Ministeriet for Fødevarer,
Landbrug og Fiskeri
Styrelsen for Fødevarer,
Landbrug og Fiskeri

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Dato: 24-04-2026

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CVR-nr.: 15230614

Godkendelse af ansøgning om forsøgsudsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (Phytophthora infestans)

Den 20. marts 2026 modtog Styrelsen for Fødevarer, Landbrug og Fiskeri en fuldstændig ansøgning fra KMC Amba, om tilladelse til forsøgsudsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel *(Phytophthora infestans)* på et areal ved Arnborg i Herning Kommune i vækstperioden frem til 30. oktober 2028, jf. bilag 1.

Styrelsen for Fødevarer, Landbrug og Fiskeri vurderer, at ansøgningens oplysninger[i] er i overensstemmelse med direktiv 2001/18/EF[ii], og at der ikke vil være uønskede miljø- og sundhedsmæssige konsekvenser forbundet med forsøgsudsætningen. Derfor vurderer Styrelsen for Fødevarer, Landbrug og Fiskeri, at udsætningen kan finde sted[iii].

Godkendelsen gives på baggrund af ansøgningens oplysninger om forsøgsudsætningens gennemførelse og oplysninger om den genetisk modificerede plante, ansøgers risikovurdering og med de i denne afgørelse fastsatte vilkår[iv].

Vilkårene i denne afgørelse kan påklages særskilt. Se nærmere herom i afsnittet ”Hvis I vil klage”.

Godkendelsen til forsøgsudsætningen gives for en periode fra dags dato indtil 30. oktober 2028. Herefter følger en periode, hvor tilladelsesarealet skal overvåges, jf. de i denne afgørelse fastsatte vilkår.

Forsøgsudsætningen er omfattet af reglerne i lov om miljø og genteknologi[v].

Denne afgørelse fremsendes i øvrigt til Herning Kommune, da kommunalbestyrelsen, ifølge lov om miljø og genteknologi[vi], er klageberettiget.

Forsøgsudsætningen

Godkendelsen omfatter forsøgsudsætning af cisgene stivelseskartofler med flere komplementære resistensgener med forventet øget resistens imod kartoffelskimmel

(*Phytophthora infestans*), for at undersøge muligheden for at reducere anvendelse af kemiske plantebeskyttelsesmidler imod kartoffelskimmel i kartoffel.

Der er ikke fundet tegn på, at uønskede dele af den anvendte plasmid-vektor er indsat i de anvendte linjer, og der er ikke tilføjet antibiotikaresistensmarkørgener.

Forsøgsudsætningen, som er registreret i Styrelsen for Grøn Arealomlægning og Vandmiljø's fællesskema (FS), skal foregå i bloknummer 500207-20 (internet markkort), som ligger ved Arnborg i Herning Kommune, jf. bilag 1. Det samlede forsøgsareal, hvor der dyrkes GM-kartofler, vil under hele markforsøget ikke overstige 400 m² brutto. Forsøgsarealet inklusive værn og stier vil udgøre ikke over 500 m² brutto m². Forsøgsudsætningen vil foregå i dyrkningssæsonen (april – oktober) i 2026-2028.

Formålet med udsætningen er at undersøge muligheden for at reducere anvendelse af kemiske plantebeskyttelsesmidler imod kartoffelskimmel (*Phytophthora infestans*) i kartoffel.

Hele den forsøgsmæssige udsætning er afsluttet, når overvågningsperioden og virksomhedens egenkontrol er afsluttet (jf. Egenkontrol og logbog, vilkår 1.9- 1.10).

Begrundelse
Styrelsen for Fødevarer, Landbrug og Fiskeri har anmodet Danmarks Tekniske Universitet (DTU) og Aarhus Universitet (AU) om at vurdere ansøgningsmaterialet i forhold til den gældende lovgivnings krav og at foretage en vurdering i forhold til påvirkninger af hhv. mennesker og dyrs sundhed, natur og miljø samt af den risikovurdering, som er fremlagt af ansøger.

AU har vurderet risikoen for, om de cisgene kartofler kan spredes til:
- Omgivelserne (dyrkningsfladen),
- Naturen,
- Vilde slægtninge (via pollen),
- Konventionelt dyrkede kartofler.

AU har endvidere vurderet risikoen for effekter på naturen og miljøet i øvrigt. Endelig har AU vurderet behovet for overvågning, samt om ansøgers risikohåndtering er fyldestgørende.

AU har vurderet, at de af ansøger foreslåede tiltag for at hindre spredning af materiale fra de cisgene kartofler sikrer en meget lille sandsynlighed for spredning af de cisgene kartofler til omgivelserne. Den genetiske modificering, som har fået indsat ekstra resistensgener mod kartoffelskimmel forårsaget af *Phytophthora infestans*, forventes ikke at ændre den modificerede kartoffels påvirkning på natur og miljø i forhold til andre konventionelt dyrkede kartofler. AU oplyser endvidere, at den af ansøger foreslåede overvågning vurderes at være tilstrækkelig.

DTU har foretaget en sundhedsmæssig risikovurdering af den genetisk modificerede kartoffel, herunder af den genetiske ændring. DTU vurderer, at et 'worst case scenario', hvor de genetisk modificerede kartofler spredes til kartoffelmarkerne via knolde

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eller pollen (f.eks. til forbrug eller formering), vil det ikke udgøre et sundhedsproblem baseret på kendskab til designet. De forventede nye egenskaber vurderes ikke at være forbundet med en sundhedsrisiko ved kartofler og forårsager ikke dannelsen af nye bestanddele, der ikke er tiltænkt.

Universiteternes vurderinger er vedhæftet denne afgørelse, jf. bilag 2, og er desuden tilgængelige på styrelsens hjemmeside.

Styrelsen har gennemført en høring blandt Europa-Kommissionen og EU-medlemsstaterne. Der er ikke kommet nogen høringssvar i denne høring. Styrelsen har endvidere gennemført en høring blandt myndigheder, organisationer og offentligheden i Danmark om forsøgsudsætningen[vii]. Et notat, der sammenfatter de indkomne høringssvar, kan findes på Høringsportalen[viii].

Det er på grundlag af oplysningerne i ansøgningen, universiteternes risikovurdering samt de modtagne høringssvar styrelsens vurdering, at der ikke vil være uønskede miljø- og sundhedsmæssige konsekvenser forbundet med forsøgsudsætningen, hvis forsøgsudsætningen gennemføres som beskrevet i ansøgningen.

Styrelsen for Fødevarer, Landbrug og Fiskeri finder dog, at det er nødvendigt, bl.a. af hensyn til styrelsens tilsyn, at fastsætte yderligere vilkår for forsøgsudsætningen. Forsøgsudsætningen skal derfor udføres i overensstemmelse med de nedenfor anførte vilkår.

Vilkår

1. Vilkår for gennemførelse af forsøgsudsætningen

I dette afsnit forstås ved
Tilladelsesarealet: Arealet, som godkendelsen omfatter (markblokken).
Forsøgsarealet: Det eller de arealer indenfor tilladelsesarealet, hvor der dyrkes genetisk modificerede kartofler og konventionelle kartofler, som indgår i forsøget (bruttoarealet).
GMO-forsøgsarealet: Det eller de arealer på forsøgsarealerne, hvor der alene dyrkes genetisk modificeret kartoffel (netto-arealet).
Sikkerhedsafstand: Afstanden til andre afgrøder, målt i meter, fra ethvert punkt i forsøgsarealets afgrænsning.

Forberedelse af forsøget

1.1 Forsøgsarealer skal markeres tydeligt, f.eks. med pinde, der afgrænser hhv. tilladelsesarealet og GMO-forsøgsarealet. En tegning eller foto af dette sendes til styrelsen på mail: planterogbiosikkerhed@fvst.dk, senest ved lægning af kartofler. Denne markering opretholdes, indtil egenkontrollen med tilladelsesområdet er ophørt, jf. egenkontrol og logbog, vilkår 1.9-1.10. Årsagen til markeringen er, at de to arealer (tilladelsesarealet og GMO-forsøgsarealet), af hensyn til tilsynet, skal kunne identificeres, mens GM-kartoflerne dyrkes samt i den efterfølgende overvågningsperiode.

Gennemførelse af forsøget

1.2 Styrelsen for Fødevarer, Landbrug og Fiskeri skal forud for lægningen underrettes om dato og tid for lægning. Denne underretning skal finde sted senest kl. 12:00

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dagen før lægningen. Årsagen er, at styrelsen skal have mulighed for at føre tilsyn med lægningen og skal kunne være til stede ved lægningens påbegyndelse.
1.3 Styrelsen for Fødevarer, Landbrug og Fiskeri skal underrettes om påbegyndt blomstring i GM-afgrøden. Denne underretning skal finde sted hurtigst muligt ved synlige blomster i GM-afgrøden. Årsagen er, at styrelsen skal have mulighed for at føre tilsyn med, at blomster afklippes for at minimere risikoen for spredning af pollen.
1.4 Styrelsen for Fødevarer, Landbrug og Fiskeri skal forud for høst underrettes om dato og tid for høst. Denne underretning skal finde sted senest kl. 12:00 dagen før høsten. Årsagen er, at Styrelsen for Fødevarer, Landbrug og Fiskeri skal have mulighed for at føre tilsyn med høsten og derfor skal kunne være til stede ved høstens påbegyndelse.

Overvågning af tilladelsesarealet efter høst
1.5 Eventuelle fremspirende kartofler skal fjernes og testes med den i ansøgningen angivne metode. Resultatet af denne test meddeles Styrelsen for Fødevarer, Landbrug og Fiskeri, jf. vilkår 1.8. Årsagen til dette vilkår er, at Styrelsen for Fødevarer, Landbrug og Fiskeri skal føre tilsyn med de i ansøgningen godkendte oplysninger om tilsyn, overvågning og efterbehandling- og affaldshåndteringsplaner, jf. pkt. 4 i ansøgningen.
1.6 De pågældende områder, hvor forsøgsudsætningen er sket, overvåges i yderligere fire vækstsæsoner for eventuelle fremspirende kartofler. Dette krav er baseret på rådgivning fra eksperter fra Aarhus Universitet, jf. bestillingen fra 2021: 'Opdatering af det faglige bidrag vedrørende dyrkning af GM-afgrøder af raps, majs, kartofler, bederoer, hvede og byg'[ix].
1.7 Hvis der i overvågningsperioden fremspirer GM-kartofler på tilladelsesarealet, vil det medføre, at overvågningsperioden forlænges med fire vækstsæsoner, medmindre det kan påvises, at det ikke er en GM-kartoffel. Forlængelsen af overvågningsperioden er baseret på rådgivning fra eksperter fra Aarhus Universitet, jf. bestillingen fra 2021: 'Opdatering af det faglige bidrag vedrørende dyrkning af GM-afgrøder af raps, majs, kartofler, bederoer, hvede og byg'.
1.8 Hvis der i overvågningsperioden findes fremspirende kartofler, skal ansøger uden ugrundet ophold underrette Styrelsen for Fødevarer, Landbrug og Fiskeri, medmindre det kan påvises, at det ikke er en GMO-kartoffel. Årsagen til dette vilkår er, at Styrelsen for Fødevarer, Landbrug og Fiskeri skal føre tilsyn med de i ansøgningen godkendte oplysninger om tilsyn, overvågning og efterbehandling- og affaldshåndteringsplaner, jf. pkt. 4 i ansøgningen.

Egenkontrol og logbog
1.9 Gennemførelse af egenkontrollen skal dokumenteres i en logbog. Det skal af logbogen klart fremgå:

- Hvilke forhold, der er ført egenkontrol med.
- Hvornår egenkontrolaktiviteten er gennemført.
- Hvem der har gennemført egenkontrolaktiviteten.
- Resultaterne af egenkontrolaktiviteten.

Årsagen er, at egenkontrollen indgår i den endelige vurdering af, hvornår overvågningen er afsluttet, jf. afsnittet "Afslutning af forsøget".
1.10 Logbogen skal føres af den for forsøgsudsætningen ansvarlige eller en eller flere af denne udpegede medarbejdere, og Styrelsen for Fødevarer, Landbrug og Fiskeri

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skal underrettes om, hvem der udpeges. Årsagen er, at styrelsen til hver en tid skal være bekendt med, hvem der er ansvarlige for forsøgsudsætningen.

Afslutning af forsøget

1.11 Forsøgsudsætningen og egenkontrollen er afsluttet, når ansøger har dokumenteret, at der i fire vækstsæsoner i træk ikke har været fremspirende GM-kartofler på forsøgsarealet blandt andet på grundlag af dokumentationen i logbogen, jf. Egenkontrol og logbog, vilkår 1.9-1.10.

2. Vilkår for rapportering

2.1 Hvis der sker personændringer i kredsen af ansvarlige for forsøgsudsætningen eller den daglige drift, skal dette uden ophold meddeles Styrelsen for Fødevarer, Landbrug og Fiskeri. Årsagen er, at Styrelsen for Fødevarer, Landbrug og Fiskeri til hver en tid skal være bekendt med, hvem der er ansvarlige for forsøgsudsætningen.
2.2 Ansøger skal en gang årligt afrapportere resultaterne af egenkontrollen (logbogen) til Styrelsen for Fødevarer, Landbrug og Fiskeri. Afrapporteringen af egenkontrollen skal være styrelsen i hænde senest med udgangen af januar hvert år, frem til afslutningen af hele forsøgsudsætningen, jf. afsnittet 'Afslutning af forsøget'. Årsagen er, at egenkontrollen indgår i den endelige vurdering af, hvornår overvågningen er afsluttet.
2.3 Ansøger indsender delrapporter efter endt overvågningsperiode for de i dyrkningsåret 2026, anvendte arealer samt en endelig rapport efter overvågningsperioden når dyrkningsåret 2028 er afsluttet. Delrapporterne udarbejdes i overensstemmelse med skabelonen i bilaget til Kommissionens beslutning (2003/701/EF) af 29. september 2003, jf. beslutningens artikel 3, stk. 1. Kommissionens beslutning med tilhørende bilag er vedlagt denne afgørelse (bilag 3). Årsagen til delrapporteringerne er, at de enkelte dyrkningsarealer kan frigives løbende. Indsendelse af rapporterne skal ske elektronisk.
2.4 Når forsøgsudsætningen og overvågningsperioden er endeligt afsluttet, skal ansøger udarbejde en endelig rapport om forsøget. Slutrapporten skal bl.a. bruges til at underrette Europa-Kommissionen og de øvrige medlemsstater i EU om, at forsøgsudsætningen er afsluttet.
2.5 Til den endelige afrapportering benyttes den rapporteringsmodel, der fremgår af bilaget til Kommissionens beslutning (2003/701/EF) af 29. september 2003 om fastlæggelse i henhold til Europa-Parlamentets og Rådets direktiv 2001/18/EF af en model for fremlæggelse af resultatet af udsætning i miljøet af genetisk modificerede højerestående planter i andet øjemed end markedsføring.
2.6 Slutrapporten skal være Styrelsen for Fødevarer, Landbrug og Fiskeri i hænde senest 30 dage efter forsøgsudsætningens endelige afslutningsdato.

Tilsyn og offentliggørelse

Styrelsen for Fødevarer, Landbrug og Fiskeri skal have mulighed for at føre tilsyn med, at forsøgsudsætningen følger de i ansøgningen anførte foranstaltninger og med, at ovenstående vilkår overholdes[x]. Tilsynet vil blive planlagt og varslet, så Styrelsen for Fødevarer, Landbrug og Fiskeri kan overvåge lægning af kartofler, høst og evt. et tilsyn i løbet af blomstringsperioden[xi]. Tilsynet koordineres med ansøger, så det foregår på et for planternes udvikling hensigtsmæssigt tidspunkt.

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Manglende overholdelse af de i denne afgørelse fastsatte vilkår kan straffes med bøde[xii].

Styrelsen for Fødevarer, Landbrug og Fiskeri har oprettet et register på styrelsens hjemmeside, www.lfst.dk, hvor følgende oplysninger om forsøgsudsætningen bliver offentliggjort[xiii]:

1) Ansøgers navn og adresse, beskrivelse af den eller de genetisk modificerede organismer, formålet med udsætningen og stedet for udsætningen.
2) Resumé af de miljø-, natur- og sundhedsmæssige risikovurderinger.
3) Styrelsen for Fødevarer, Landbrug og Fiskeris vurdering af sagen.
4) Vilkårene for gennemførelsen af forsøgsudsætningen (pkt. 1) samt vilkår om rapportering under og efter at udsætningen er fuldført (pkt. 2).

Hvis I vil klage
Hvis I er uenig i vores afgørelse med tilhørende vilkår, kan I klage over den. I skal sende klagen inden 4 uger fra den dag, hvor I fik dette brev.

I klager via klageportalen, som I finder på Nævnenes Hus' hjemmeside. Derinde kan I læse, hvordan I skal gøre, og se status på jeres sag. I logger på klageportalen med MitID.

Jeres klage bliver automatisk sendt til os i Styrelsen for Fødevarer, Landbrug og Fiskeri. Hvis vi fastholder vores afgørelse, sender vi klagen videre til Miljø- og Fødevareklagenævnet via klageportalen. I får besked, hvis vi sender jeres klage videre.

Hvis I ikke sender jeres klage via klageportalen, afviser Miljø- og Fødevareklagenævnet jeres klage, medmindre I er fritaget for brug af klageportalen. I kan læse mere om fritagelse fra klageportalen på nævnets hjemmeside.

Venlig hilsen

Morten Storgaard / morsto@fvst.dk
Chefkonsulent
Styrelsen for Fødevarer, Landbrug og Fiskeri

Bilag
Bilag 1: Ansøgning med tilhørende bilag
Bilag 2: Universiteternes miljø- og sundhedsmæssige risikovurderinger
Bilag 3: Kommissionens beslutning (2003/701/EF) af 29. september 2003 om fastlæggelse i henhold til Europa-Parlamentets og Rådets direktiv 2001/18/EF af en model for fremlæggelse af resultatet af udsætning i miljøet af genetisk modificerede højerestående planter i andet øjemed end markedsføring

[i] jf. § 4, stk. 1, i BKG nr. 37 af 19. januar 2012 om godkendelse af udsætning i miljøet af genetisk modificerede organismer.

[ii] Direktiv 2001/18/EF af 9. marts 2001 om udsætning i miljøet af genetisk modificerede organismer

[iii] § 9, stk. 1, og stk. 2 nr. 1, i LBK nr. 9 af 4. januar 2017 om godkendelse af udsætning i miljøet af genetisk modificerede organismer.

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[iv] Jf. § 16, stk. 1, i LBK nr. 9 af 4. januar 2017 om godkendelse af udsætning i miljøet af genetisk modificerede organismer.

[v] LBK nr. 9 af 4. januar 2017 om miljø og genteknologi.

[vi] Jf. § 26, stk. 2, og § 30, stk. 1, nr. 3, i LBK nr. 9 af 4. januar 2017 om miljø og genteknologi.

[vii] § 9 i BKG nr. 37 af 19. januar 2012 om godkendelse af udsætning i miljøet af genetisk modificerede organismer.

[viii] www.hoeringsportalen.dk

[ix] https://pure.au.dk/portal/files/101255558/Opdatering_af_det_faglige_bidrag_

vedr_rende_dyrkning_af_GM_afgr_der_280915.pdf.

[x] Jf. § 20, stk. 1, i LBK nr. 9 af 4, januar 2017 om miljø og genteknologi.

[xi] § 4, stk. 3, i BKG nr. 37 af 19. januar 2012 om godkendelse af udsætning i miljøet

af genetisk modificerede organismer.

[xii] Jf. § 36, stk. 1, nr. 3 og stk. 5, i LBK nr. 9 af 4. januar 2017.

[xiii] § 10, stk. 2, i BKG nr. 37 af 19. januar 2012 om godkendelse af udsætning i miljøet af genetisk modificerede organismer.

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Ansøgning om udsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (Phytophthora infestans)

Forår 2026, 2027 og 2028.

Indhold

Ansøgning om udsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (Phytophthora infestans)

A. Generelle oplysninger

A.1. Anmelderens navn og adresse

Kåre Lehmann Nielsen, Senior R&D scientist, KMC Amba, Herningvej 60, 7330 Brande
e-mail: kln@kmc.dk

Kristian Elkjær, Team Leader, Agro R&D, KMC Amba, Herningvej 60, 7330 Brande
e-mail: kel@kmc.dk

A.2. De ansvarligere forskeres navne

Kåre Lehmann Nielsen, senior R&D scientist, KMC, samt professor i Genomik på Aalborg Universitet, PhD, Gruppeleder, > 25 års erfaring i kartoffel-genomik og biologi, samt udvikling af avancerede forædlingsmetoder baseret på statistiske, bioinformatiske metoder samt nye forædlingsteknologier.

Christian Kjær Olesen, R&D scientist, KMC. Cand polyt I bioteknologi fra AAU med speciale i plantetransformation. Har 4 års erfaring med metodeudvikling og implementation af NGT i planter, herunder de sidste 3 år i kartofler.

Kristian Elkjær, Team Leader, Agro R&D, KMC. Har arbejdet med udvikling, avl og forædling af kartofler siden 2017.
Erhvervede GMO-kørekort i 2021.

Markpersonalet er uddannede jordbrugsteknologer og har erhvervet GMO - kørekort på Bygholm Landbrugsskole i december 2021 eller marts 2023.
Forsøgsarbejdet vil blive udført i samarbejde med Ytteborg Field Trials, Hjermvej 94, 7560 Hjerm.

A.3. Projektets titel

Cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (Phytophthora infestans)

A.4. Udsætningen

A.4.a. Formålet med udsætningen

Undersøge muligheden for at reducere anvendelse af kemiske plantebeskyttelsesmidler imod kartoffelskimmel (*Phytophthora infestans*) i kartoffel.
Forsøget vil bestå af ubehandlede parceller.

A.4.b. Udsætningens startdato og varighed

Udsætning sker i perioden 01. april – 15. juli 2026/2027/2028 og høst i perioden 01. september – 30. oktober 2026/2027/2028.

A.4.c. Udsætningsmetode

Kartoffelknolde håndlægges i rækker og dækkes med kartoffelkamme (hyppes), og/eller der udplantes pottedyrkede kartoffelplanter, som sættes i en færdighyppet kam.

A.4.d. Fremgangsmåde ved forberedelse og behandling af udsætningsstedet inden, under og efter udsætningen, herunder dyrknings- og høstpraksis

Forår:
Marken er pløjet og/eller harvet op inden lægning af kartoffelknolde/udplantning af pottedyrkede kartoffelplanter.

Under (udsætningen) væksten:
Normal behandling mod ukrudt, skadedyr og sygdomsbekæmpelse imod andre svampesygdomme end kartoffelskimmel (*Phytophthora infestans).* Forsøget vil bestå af ubehandlede parceller mod kartoffelskimmel, men kontrol og værneplanter kan blive sprøjtet.
Planterne vil løbende blive vandet efter behov.

Høst (optagning):
Høst af de cisgenetisk modificerede kartofler vil foregå ved en rodunderskæring og løsning af kammen, efterfulgt af håndopgravning og opsamling samt vejning i marken.

De høstede knolde pakkes og transporteres til analyse og viderebehandling på KMCs GMO godkendte laboratorium på Herningvej 60, Brande.

A.4.e. Omtrentlig antal planter per kvm.

3 - 6 planter per kvm.

A.5. Oplysninger om udsætningsstedet

A.5.a. Udsætningsstedets størrelse og beliggenhed

Udsætningsstedet er beliggende i markbloknummer 500207-20.
Området, der vil blive tilplantet med cisgenetisk modificerede kartofler, vil være 100-500 m² brutto og 50-400 m² netto.
Forskel mellem brutto og netto areal er værn og sti.

A.5.b. Beskrivelse af udsætningsstedets økosystem, herunder klima, flora og fauna

Udsætningsstedet er beliggende i et konventionelt dansk landbrugsareal.

A.5.c. Forekomsten af krydsningskompatible beslægtede vilde eller dyrkede plantearter

Kartoffel krydser ikke spontant med vilde arter af kartoffel eller andre dyrkede *Solanum* arter. Dog kan kartofler i meget sjældne tilfælde bestøve andre kartoffelsorter. (Rizov et al. 2018)

A.5.d. Afstanden til officielt anerkendte biotoper eller beskyttede områder, som vil påvirkes

Afstande

§3 Hede: 230 meter

§3 Overdrev: 470 meter

§3 Eng: 550 meter

Fredskov: min. 15 meter

B. Videnskabelige oplysninger

B.1. Oplysninger om recipientplanten eller - hvor det er relevant - forældreplanter

B.1.a. Fuldstændigt navn

Taxonomi	Latinske navn
i) Familie	*Solanaceae*
ii) Slægt	*Solanum*
iii) Art	*Solanum tuberosum*
iv) Underart	*Tuberosum*
v) Kultivar	Ydun
vi) Almindeligt navn	Kartoffel (stivelse) ”Ydun”

B.1.b. Udbredelse og dyrkning i Unionen

Kartofler dyrkes bredt i alle lande i Unionen og anvendes til almindeligt konsum, pommes frites, chips, dehydrede produkter, alkohol og stivelsesproduktion.

B.1.c. Reproduktion

i)

Kartofler opformeres (reproduceres) normalt klonalt ved udplantning af læggeknolde, som producerer nye knolde.

I forsknings- og forædlingsøjemed bruges frø til at frembringe F1 generationen, som producerer den første knold. Da bestøvning i kartofler er en ineffektiv proces, foregår det i drivhuse, hvor pollen overføres til støvdrager med pensel, populært betegnet som ”kunstig befrugtning”.

Langt de fleste kommercielle kartoffel kultivarer (sorter) er tetraploide, hvilket vil sige at der er fire kopier (kaldet alleler) af hvert gen i kartoflens genom.

ii)

I naturen sker der yderst sjældent spontant krydsning mellem kultivarer (sorter) af kartofler, hvorfor risiko for krydsbestøvning anses som værende teoretisk. Risikoen er meget lille: i) fordi kartofler generelt ikke er særligt effektive til kønnet formering, men som hovedregel forlader sig på vegetativ propagation via knolde. ii) Kartoffelbær modnes meget sjældent i Danmark inden høst eller afvisning af planten, hvorfor viable frø sjældent dannes.

Ydermere er risikoen meget afstandsafhængig og selv en lille afstand på 10 m til andre sorter har vist at være en effektiv nedsættelse af krydsningsrisiko (Rizov et al. 2018).

For at eliminere selv den mindste risiko vil vi, som ekstra sikkerhed, klippe blomster af de cisgenetisk modificerede kartoffelplanter ved blomstring. Dette vil elimere enhver teoretisk mulighed for krydsbestøvning.

iii)
Kartofler er 1. årige.

B.1.d. Krydsningskompatibilitet med andre dyrkede eller vilde plantearter, herunder udbredelsen i Europa af de kompatible arter
Der kendes ikke til krydsninger mellem kartofler og andre dyrkede eller vilde arter i Europa. Krydsningskompatibiliteten må derfor anses for at være ikke eksisterende.

B.1.e. Overlevelsesevne
i)
Evne til at danne strukturer, der fremmer overlevelse eller vækstdvale:
Ikke høstede knolde kan overleve i jorden hen over en mild vinter uden betydende frost. Almindeligt vintervejr med gentagen nat og dagsfrost vil slå eventuelle overskydende knolde i jorden ihjel, de fryser væk.
Alle kartoffelknolde i forsøget på udsætningsstedet vil blive rodunderskåret og jordløsnet, efterfulgt af håndopgravning og opsamling, hvorfor sandsynligheden for at der skal være knolde i jorden efter høst er ubetydelig.

ii)
Ingen særlige faktorer.

B.1.f. Spredning
i)
Maskinoptagning vil i nogle tilfælde spilde små knolde, som kan give ny vækst året efter. Derfor vælges den manuelle håndopgravning og opsamling, som er et effektivt værn imod knolde, der ikke bliver høstet.

ii)
Generel betragtning vedr. risiko for spredning

Det må overordnes vurderes med de forslåede tiltag, at der ikke er nogen risiko for spredning af genetisk materiale til vildarter i natur og kun en ekstrem lille risiko for spredning til andre kartoffelsorter. Da der ikke dyrkes læggekartofler i umiddelbar nærhed af forsøgsmarken, så er der reelt ingen risiko for at genetisk materiale skulle finde vej ind i den etablerede kartoffelproduktion.

Det er velkendt, at spildkartoffelplanter fra den almindelige kartoffelproduktion ikke kan klare sig i flere vækstsæsoner i naturen, heller ikke sorter med betydelig genetisk resistens mod kartoffelskimmel. Derfor er risiko for etablering af kartofler (resistente eller ej) som invasiv art i naturen allerede kendt som ikke-eksisterende.

B.1.g.

Ikke relevant

B.1.h.

Kartoflen vekselvirker ikke med andre planter eller organismer, hvor den dyrkes konventionelt, og der er ikke nogen kendt toksisk virkning på mennesker, dyr eller andre organismer.

B.2. Molekylær karakterisering

a) Oplysninger om den genetiske modifikation

Tre gener fra 2 *Solanum* arter som er krydsningkompatible med *Solanum tuberosum*, vnt1.1 fra *Solanum venturii*; blb1 og blb2 fra *Solanum bulbocastanum*, samt deres flankerende region (~1 kb upstream) og 400-600 bp downstream) er forsøgt indsat som een kontinuær insertion i kartoffelsorten Ydun.

Tre linjer i denne ansøgning indeholder dog kun de hele gener for blb1 og blb2, hvorimod der er sket en trunkering af genet for vnt1.1, hvor hele promotersekvensen for alle linjer og 1781 bp, 367 bp og 218 bp for hhv. YSF5, YSF12 og YSF13 mangler. Derfor har alle tre linjer kun indsat forventede funktionelle gener for blb1 og blb2. Disse linjer er identiske med linjerne fra forsøg i 2025.

YA10_51 indeholder alle tre gener i fuld længde.

Yderligere 4 linjer indeholder alle 3 gener i deres fulde længde med flankerede regioner. Herudover indeholder de enten hele potato spacer sekvensen (YA_mfp_1) eller dele af potato spacer sekvensen, hvor hhv. 611 bp (YA_mfp_2); 89 bp (YA_mfp_3) og 150 bp (YA_mfp5) mangler. Herudover er der i (YA_mfp_1 og YA_mfp_5) indsat to tandem kopier af genkassetten, så YA_mfp_1 og YA_mfp_5 indeholder 2x af alle gener samt 1 kopi af Right-border sekvensen: TGACAGGATATATTGGCGGGTAAACCT imellem de to genkassetter. Potato spacer sekvensen er en kontinuær DNA sekvens fra kartoffel reference genomet fra kromosom 1. Sekvenser er en kontinuær sekvens fra kromosom 1 og indeholder ingen fuld-længde gener, men indeholder en del af intron 2, exon 2 og en del af intron 3 af genet Soltu.DM.01G000200 - Protein of unknown function (DUF1423) - homologous to protein OBERON 3-like. Da promoter region og den første exon ikke er inkluderet, vil dette genfragment ikke blive transkriberet (og translateret). Derfor er det højst usandsynlygt, at det skulle bidrage med nogen biologisk funktion. Formålet med fragmentet er at mitigere effekt af hyppige trunkeringer ved Left Border under indsættelse i genomet. Sådanne trunkeringer ville inaktivere det første R-gen (vnt1) i kassetten (se YSF5, YSF12 og YSF13). De 3 R-gener er kendte racespecifikke resistensgener mod kartoffelskimmel forårsaget af *Phytophthora infestans* af typen NB-LRR gener, som populært kaldes R-gener i kartoffelforskningen. R-gener fungerer som immunreceptorer, som kan registrere infektion og forårsage et passende immunrespons som forhindrer videre infektion. De tre gener genkender distinkte molekylære strukturer fra *P. infestans,* og vil derfor i kombination med hinanden udgøre en meget effektivt værn mod infektion her og nu, men også være en meget betydelig forhindring for genetisk udvikling af *P. infestans* stammer, som kan undgå detektion af hvert af disse gener og dermed bryde resistensen. Det er derfor også forventningen, at denne *stacking* af komplementære R-gener giver en betydeligt længerevarende effektiv resistens end hos sorter, som kun indeholder et enkelt R-gen.

i) Beskrivelse af de metoder der er anvendt

Konstruktion af DNA sekvens.

DNA sekvenserne af vnt1.1; blb1 og blb2, samt plasmidvektor PCambia2300, herefter benævnt som pCambia2300_3Rgenes (se appendix 1 for præcis sekvenser og plasmid maps) er kemisk synteseret. Plasmiderne blev propagaderet ved introduktion i *Escherichia coli* Top10, vækst og efterfølgende plasmid oprensning. DNA sekvensen af det resulterende plasmid blev verificeret ved Long-Read DNA sekventering (Oxford Nanopore MINIon).

Transformation og regeneration af Kartoffelplanter.

Herefter blev plasmidet transformeret ind i *Agrobacterium tumefaciens* AGL-1. En koloni blev opdyrket i flydende kultur og denne kultur blev efterfølgende brugt til at transformere plantemateriale som beskrevet nedenfor.

Sterile *In vitro* kartoffelplanter af sorten Ydun blev skåret i stykker af ca. 0,5 cm^2 (bladmateriale) og ca. 1 cm nodale stængelstykker, og co-kultiveret med *Agrobacterium*-kulturen. Herefter blev plantemateriale overført til callus-inducing media (CIM) agarplader indeholdende Timentin, for at fjerne Agrobakterier fra den videre vækst. CIM-plader indeholder hormoner som fremmer plantecelledeling, men hæmmer vævsdifferentiering. Efter 7-9 dage blev plantestykker med synlige calli overført til shoot-inducing media (SIM), som indeholder plantehormoner som stimuleret skuddannelse og vækst. Efter 4-12 uger blev synlige skud skåret fra enkeltvis og overført til root-inducing media. Efter 4 uger blev de planter, som havde dannet rødder overført til almindeligt in vitro plante vækstmedie uden hormoner, og propageret som individuelle *in vitro* kultur linjer og karakteriseret som beskrevet nedenfor.

For at verificere at Agrobacterium er elimineret, testes plantematerialet 40-60 dage efter transformationen via PCR.

Karakterisering af udvalgte kartoffellinjer

Formodede transformante *in-vitro* linjer blev screenet med PCR. En lille plantedel, typisk 0,5 mm3, blev udtaget fra hver linje samt fra den negativ kontrol (baggrundssorten Ydun) og brugt som template i en PCR reaktion med kemisk syntetiserede DNA primere, som er komplementære til deel af det indsatte DNA. Fragmenterne blev detekteret ved standard TAE-agarose gel-elektroforese. Tilstedeværelse af fragmenter med den forventede størrelse i de rekombinante linjer (og fravær i den negative kontrol, Ydun) blev taget som indikation for mindst delvis tilstedeværelsen af gen-kassetten.

For YA10_51's vedkommende blev der efterfølgende generet protoplaster plantevæv af den primære postitive transformant (YA10). Protoplasterne blev behandlet med CrispR/Cas9 nucleoprotein kompleks ladet med gRNAs flankerende KanR-gen (se appendix 1b) med det formål at deletere hele KanR genet. Herefter blev planter blev regenereret fra enkelt-protoplaster og den ønskede deletion detekteret med PCR.

Fuld genom DNA sekventering af primære linjer.

Genomisk DNA fra rekombinante linjer samt baggrundsorten, Ydun, blev isoleret med standard CTAB metode efterfulgt af Genomic Tip oprensning (Qiagen). Efterfølgende blev det genomiske DNA forberedt til Long-read DNA sekventering med Ligation Sequencing protokol (Oxford Nanopore Technologies). Der blev frembragt mindst 80 Gigabp DNA sekvens, svarende til min. 80x dækning af det haploide genom.

Bioinformatisk analyse.

De rå sekvensreads blev importeret ind i CLC Genomics workbench v25, og mappet til et sekvensfragment indeholdende 100 bp i hhv. overgangen mellem henholdsvis vnt1.1 og blb1 og blb2 og/eller et 100 bp fragment fra den forventede overgang mellem upstream sekvens og vnt1.1. (YA_10_51) Formålet er at isolere sekvensreads som er specifikke for den indsatte sekvens og ikke kontamineres af reads, som kommer fra homologer gener fra andre dele af kartoffelgenomet. Reads som mapper til disse områder blev isoleret og mappet til hele den oprindelige vektor sekvens. Herfra kan insert-enderne bestemmes (se appendix 3).

5'-Insert-flankerende sekvenser blev identificeret og isoleret og sammenlignet med reference genomsekvensen for *S. tuberosum* (DMv6.1) for at identificere insertionssite. Bedste signifikante BLAST hit blev antaget som insertionssite. Kun linjer som havde et unikt insertionssted blev udvalgt. Det blev fundet at YSF5-insert er indsat på chr7 nt 20034368; YSF12-insert er indsat på kromosom 1 nt 81232018 og YSF13-insert er indsat på kromosom 11 nt 35056228. YSF5 og YSF12 er indsat i områder som ikke koder for andre kendte gener, men for YSF-13 er kassetten indsat i en intron af genet of Soltu.DM.11G017710.1, som koder for en *alpha/beta-Hydrolases superfamily protein*. Det er sandsynligt, at denne allel af dette gen er inaktiveret i YSF-13. Det vides ikke om funktionen af dette gen er vigtigt for kartoffel, men givet at kartofler er tetraploide og vores insertion kun er sket i en enkelt genomisk fase, er det sandsynligt, at den biologiske funktion af dette gen locus er uændret. Vi har ikke i øvrigt ikke observeret nogen umiddelbar fænotypisk forskel mellem de tre plantelinjer.

YA10_51 er indsat på kromosom 6 26735530, YA_mfp_1 kromosom 6 1394410, YA_mfp_2 kromosom 4 6084710, YA_mfp_3 kromosom 2 29646471, og YA_mfp_5 kromosom 6 34958838. Alle disse linjer, undtaget YA_mfp_5, er indsat i områder som ikke koder for andre kendte gener, men YA_mfp_5 er indsat i en intro af genet Soltu.DM.06G011580 som koder for et *hypothetical protein.* Det vides altså ikke med sikkerhed, om det er et aktivt gen eller hvilken funktion det gen måtte have. Det er sandsynligt, at denne allel af dette gen er inaktiveret i YA_mfp_5. Det vides ikke om funktionen af dette gen er vigtigt for kartoffel, men givet at kartofler er tetraploide og vores insertion kun er sket i en enkelt genomisk fase, er det sandsynligt, at den biologiske funktion af dette gen locus er uændret.

Vi har ikke observeret nogen umiddelbar fænotypisk forskel mellem nogle af de 8 plantelinjer.

For at analysere om uønskede dele af vektoren (herunder et Kanamycin resistens gen, som bruges til selektion af Agrobacterier inden transformation af planter) skulle være indsat andre steder i genomet, så blev reads mappet vektorsekvens uden insert. Der blev for alle linjer ikke fundet reads som mapper til uønskede vektordele, ej heller reads som mapper til gener som koder for antibiotikaresistens. Vi konkluderer, at kun insertsekvenserne som beskrevet er indsat i genomet.

ii) Den anvendte vektors art og oprindelse

Vektordesign for cisgenetisk modifikation er baseret på plasmid pCambia2300 modificeret i forhold til Appendix 1. Selve DNAet er kemisk syntetiseret og efterfølgende propageret og oprenset fra *E. coli*. *Agrobacterium tumefaciens* AGL-1 er brugt til at levere DNA ind i cellerne og translokation ind i genomisk DNA.

iii) Kilden til den/de til transformationen anvendte nukleinsyre(r) samt størrelse og tilsigtet funktion af hver bestanddel af den region, der skal indsættes

Design af de tre R-gener vnt1, blb1 og blb3 er baseret på sekvenser i Genbank databasen med Accession numre FJ423044.1, AY426259, DQ122125.1. De præcise sekvenser af angivet i appendix 2 og de tre gener har henholdsvis størrelserne 3785 bp, 4992 bp og 5491 bp inkl. promoter og 3'-UTR. Potato spacer har størrelsen 2081 bp.

b) Oplysning om GMHP'erne

i) Overordnet beskrivelse af de egenskaber og karakteristika, der er indført eller ændret

Præcis nukleotid information for alle tre gener, samt potato spacer er angivet i appendix 2. Bemærk at for YSF5, YSF12 ogYSF13 er det kun er blb1 og blb2, som forventes at være aktive, da kun disse gener er indsat i fuld længde. De øvrige linjer indeholder alle 3 R-gener i fuld længde. De tre R-gener er kendte og velkarakteriserede racespecifikke resistensgener mod *P. infestans* (van der Vossen et al. 2003; van der Vossen et a. 2005 and Pel et al. 2009). Derfor forventes kartoffellinjer, som indeholder og udtrykker disse gener, at være mere modstandsdygtige overfor kartoffelskimmel end deres baggrundsort (Ydun). I alle andre aspekter forventes de rekombinante linjer at være fænotypisk identiske med baggrundssorten. Potato spacer sekvensen har ingen biologisk funktion.

De tre R-gener genkender forskellige molekyler fra *P. infestans* og derfor er derfor forventes deres effekt at være additiv og samlet set meget stor. Men mindst lige så vigtigt er det, at en stor udfordring for brug af racespecifikke R-gener i landbrugets monokultur, er at den genetiske tilpasningsevne i den meget store *P. infestans* population er stor, og der opstår mutationer som kan overkomme et enkelt R-gen med jævne mellemrum som en naturlig konsekvens af udviklingen af populationen. Varianter som opstår spontant, der kan overkomme enkeltresistensgener bliver selekteret med høj effektivitet pga. de store arealer, som dyrkes netop dette R-gen, hvor de jo har en stor fordel i forhold til andre *P. infestans* varianter. Ved brug af komplementære R-gener, skal der ske flere specifikke genetiske ændringer samtidigt i en enkelt *P. infestans* variant for at opnå en selektiv fordel. Dette anses som meget usandsynligt og derfor forventes resistensen af være langvarig i forhold til sorter med et enkelt R-gen eller sorter med R-gener som genkender samme dele af *P. infestans*.

Derfor er det vores forventning, at de frembragte sorter har brug for betydeligt færre behandlinger med svampebekæmpelsesmidler og med mindre mængde aktivt stof. Både på kort og lang sigt.

ii) Oplysninger om faktisk indsatte/deleterede sekvenser

YSF5, YSF12 og YSF13: Der er indsat en enkelt kopi i cellekerne DNA i en enkelt fase (dvs. 1 ud af 4 kromosomsæt). Størrelsen af inserts er YSF5: 11765 bp (nt 8695-20459 i appendix 1), YSF12: 13156 bp (nt 7289-20444 i appendix 1) og YSF13: 13328 bp (nt7132-20459 i appendix 1). Disse sekvenser består af de tre gener (sekvenser angivet i appendix 2), sammensat præcist ende mod ende i rækkefølgen vnt1, blb1 og blb2, dog med de omtalte trunkeringer af vnt1. Der er anvendt de native promoter sekvenser, så det er vores forventning at generne overordnet er udtrykt på samme måde som i S. bulbocastanum (blb1 og blb2). Det er kendt at det ikke er muligt at måle genekspressionen af R-gener pålideligt direkte vha. RNASeq eller RT-PCR, pga. den meget store mængde (> 300) homologe og næsten identiske R-gener, som findes naturligt i kartoflens genom som forstyrrer analysen.

YA_10_51: Der er indsat en enkelt kopi i cellekerne DNA af området mellem LeftBorder og RightBorder ideholdende de 3 R gener, samt KanR gen (se appendix 1b). Efterfølgende er området mellem upstream og downstream CrispR excission site og dermed KanR genet fjernet ved CrisPR/Cas9 mediated genome editing og en del af gRNA sekvensen (5'-GACCATTACCAGACTC).

YA_mfp1, YA_mfp2 og YA_mfp_3: Der er indsat en enkelt kopi i cellekerne DNA af alle 3 R-gener i deres fulde længde med flankerede regioner og enten hele potato spacer sekvensen (YA_mfp_1) eller dele af

potato spacer sekvensen, hvor hhv. 611 bp (YA_mfp_2); 89 bp (YA_mfp_3) og 150 bp (YA_mfp5) mangler fra 5-enden. Herudover er der i YA_mfp_1 og YA_mfp_5 indsat to tandem kopier af genkassetten, så YA_mfp_1 og 5 indeholder 2x af alle gener samt 1 kopi af Right-border sekvensen: TGACAGGATATATTGGCGGGTAAACCT imellem de to genkassetter.

iii) Dele af Planten, hvori insertet udtrykkes

Det forventes at resistensgenerne udtrykkes i hele planten.

iv) Insertets genetiske stabilitet og GMHP'ernes fænotypiske stabilitet

Kartofler propageres normalt via klonal formering via knolde, og kartoffel er kendt for at bibeholde deres genetiske setup ved denne metode. Rekombination sker i langt overvejende grad ved kønnet formering, som ikke er relevant for denne ansøgning. Opformering af plantemateriale sker udelukkende ved klonal propagadering i form af enten in-vitro kulturer (via stem-cuttings) eller via knoldopformering som andre kartoffelsorter. Hyppigt tab af gener eller rekombination af genomet under knoldopformering er ikke beskrevet i kartofler. Vi verificerer løbende vha. PCR, at de planter vi multiplicerer, er som forventede og under frembringelsen af disse linjer er insertion-sites karakteriseret flere gange over multiple klonale generationer (stem-cuttings). Der blev ikke observeret rekombination af det indsatte område på noget tidspunkt. Ydermere, har vi aldrig i nogle af de mere end 100 linjer vi har re-genereret observeret "mosaik-kartoffelplanter", hvor kun en del af planten er blevet transformeret. Derfor er det usandsynligt, at disse tre linjer skulle indeholde utransformeret væv, som kunne give ophav til ikke transformerede knolde eller stem-cuttings. Samlet set har vi ikke data som typer på, at disse linjer ikke er permanent og stabilt transformerede.

Med hensyn til fænotypisk stabilitet så er en væsentlig del af formålet med disse varianter at forøge den fænotypiske stabilitet (resistens mod kartoffelskimmel). Den kendte udfordring med fænotypiske ustabilitet for racespecifikke R-gener udgøres af den enorme genetiske tilpasningsevne af *P. infestans* populationen. Ved at indsætte flere komplementære R-gener samtidig forventes den fænotypiske stabilitet at forøges dramatisk, da det således kræves at et enkelt *P. infestans* individ opnår flere genetiske ændringer samtidig, for at opnå en selektionsfordel på disse sorter og det anses som meget usandsynligt.

c) Konklusioner af den molekylære karakterisering

3 linjer, YSF5, YSF12 og YSF13 indeholder to R-gener, blb1 og blb2 i fuld længde og tre forskellige fragmenter af vnt1 er indsat, en variant i hver linje. Det forventes derfor, at linjerne får tilført resistens forårsaget af blb1 og blb2, men ikke vnt1.

1 linje YA10_51 indeholder alle tre R-gener i fuld længde.

4 linjer indeholder alle 3 R-gener i fuld længde, samt 4 forskellige fragmenter af potato spacer sekvensen.

1 Linje indeholder 2x 3R-gener+potato spacer sekvensen i fuld længde.

Der er kun fundet evidens for indsættelse af en kopi af insert pr. linje, og derfor er hver linje heterozygot for insert med alleldosis en.

Der er ikke fundet tegn på at uønskede dele af den anvendte plasmid-vektor er indsat andre steder i genomet.

Litteratur med direkte relevans for linjerne

van Der Vossen, E., Sikkema, A., Hekkert, B.t.L., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W. and Allefs, S. (2003), An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. The Plant Journal, 36: 867-882.

van der Vossen, E.A.G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A. and Allefs, S. (2005), The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. The Plant Journal, 44: 208-222.

Pel M., A., Foster, S., J., Park, T., H., Rietman, H., van Arkel, G., Jones, J.D.G., van Eck, H., Jacobsen, E. Visser, R., G., F. and van der Vossen, E., A., G., Mapping and Cloning of Late Blight Resistance Genes from *Solanum venturii* Using an Interspecific Candidate Gene Approach. Molecular Plant-Microbe Interactions 2009 22:5, 601-615.

Rizov I, Rühl G, Langhof, M, Kathage, and Rodríguez-Cerezo, E. (JRC SCIENCE FOR POLICY REPORT: Best practice document for the coexistence of genetically modified potato with conventional and organic farming. 2018. https://dx.doi.org/10.2760/055172

B.3. Oplysninger om specifikke risikoområder

(se desuden uddybende beskrivelse i medsendte bilag 1, Miljørisikovurdering M5-D2)

a) Eventuelle ændringer i GMHP'ernes persistens...

Der forventes ingen ændringer i hverken persistens eller invasionsevne, ej heller i evnen til at overføre genetisk materiale til beslægtede plantearter.
(se desuden afsnittet '*Generel betragtning vedr. risiko for spredning'*)

b) Eventuelle ændringer i GMHP'ernes evne…

Der forventes ingen ændringer i evnen til at overføre genetisk materiale til mikroorganismer.
(se desuden afsnittet '*Generel betragtning vedr. risiko for spredning'*)

c) Vekselvirkningsmekanisme mellem GMHP'erne og målorganismerne…

Ikke relevant. Planternes forsvarsmekanismer imod kartoffelskimmel *(P. infestans)* forbedres.

d) Potentielle ændringer i GMHP'ernes vekselvirkninger…

Der forventes ingen ændringer.

e) Potentielle ændringer i landbrugspraksis…

Den potentielle ændring i landbrugspraksis vil være, at der skal sprøjtes færre gange med svampemidler i kartoflerne. Det betragtes som en positiv ændring, både i relation til landbrugspraksis og i relation til miljøet bredt set (inkl. fx forekomst i drikkevandsboringer, CO2 regnskab i forbindelse med svampemiddels fremstilling og udbringning etc.).

f) Potentielle vekselvirkninger med det abiotiske miljø…

Der forventes ingen påvirkninger på de abiotiske miljøer.

g) Oplysninger om enhver toksisk, allergenisk…

Der er ingen forventning om, at der er sket ændringer i stivelsessyntesen eller den øvrige måde planten vokser på.
Det udsatte/reducerede skimmelangreb vil forventeligt give en mere jævn vækstrytme for planten, da angreb stresser planten og presser dens vækst. Dette antages at have en positiv effekt på plantens generelle vækst, hvilket bl.a. er kendt fra den praktiske avl.
Kartofler er generelt ikke toksiske eller kendt for at udvikle allergier.

Der forventes ingen toksisk, allergisk eller anden skadelig påvirkning på menneskers eller dyrs sundhed.

h) Konklusioner vedrørende de specifikke risikoområder

Der forventes ingen øget risiko for miljøpåvirkning, hverken på mennesker, dyr eller omkringliggende natur. Den forventede reducerede mængde svampemiddel forventes derimod at mindske risikoen for skadelige påvirkninger på alle omgivelser.
De cisgenetisk modificerede kartoffelplanter transporteres fra Aalborg Universitet, Fredrik Bajers Vej 7H,

9220 Aalborg Ø eller Københavns Universitet, Thorvaldsensvej 40, 1871 Frederiksberg til KMC som knolde pakket i dobbelt sække og placeret i sikrede kasser (f.eks. med låg) mærket GMO, eller som planter i sikrede kasser (f.eks. med netlåg) mærket GMO. Transport mellem landsdele ske i bil, ledsaget af person med GMO – kørekort.

Transport mellem mark og KMC sker via KMC ejede køretøj (se desuden beskrivelser ovenfor og følgende for håndtering).

B.4. Oplysninger om kontrol, overvågning og efterbehandling- og affaldshåndteringsplaner

4.a. Trufne forholdsregler

i) Afstand fra krydsningskompatible plantearter, både beslægtede vilde plantearter og afgrøder.
Der vil være mindst 10 m til nærmeste kartoffelmark, eller mindst 20 m til konventionelle eller økologiske læggekartofler.
Udsætningsstedet for de cisgenetisk modificerede kartofler vil desuden blive omgivet af et værn af ikke-GMO-kartofler.
Kartofler krydser ikke spontant med vilde arter af kartofler eller andre *Solanum* arter.
Som ekstra sikkerhed afklippes blomster i de cisgenetisk modificerede kartofler i blomstringsperioden, typisk fra primo juli til afsluttende blomstring primo august.

ii) Forholdsregler for at mindske/undgå spredning af de modificerede planters reproduktionsorganer (F.eks. Pollen, frø, knolde).
Udsætningsstedet for de cisgenetisk modificerede kartofler vil blive omgivet af værn af ikke-GMO-kartofler, der vil fungere som pollenfanger, og derved reducere pollenspredning.
Dette bælte vil blive høstet og alt plantemateriale vil blive destrueret ved høst, som beskrevet nedenfor.
Som ekstra sikkerhed afklippes blomster i de cisgenetisk modificerede kartofler i blomstringsperioden, typisk fra primo juli til afsluttende blomstring primo august.

Spande, kurve og øvrige redskaber anvendt ved udplantningen vil blive grundigt rengjorte og efterset for lægge knolde.
3 – 5 dage før forventet høst/optagning vil toppen bliver knust med en top-knuser. Derved knuses alt top, og kartoffeltoppen bliver efterladt på GMO-forsøgsarealet, hvor det indtørre og indarbejdes i jorden.
Efter topknusning og forud for høst af kartoffelknolde, vil kammene blive rodunderskåret og løsnet, efterfulgt med håndopgravning og opsamling, for at sikre der ikke efterlades knolde i jorden.

Høstede cisgenetisk modificerede knolde vil blive opsamlet i dobbelt lukkede plastposer eller lukkede plastkasser, mærket med GMO, og transporteres til videre analyse på KMCs GMO godkendte laboratorium på Herningvej 60, Brande.
Restmaterialer efter analyse destrueres efter standardprocedurer for GMO godkendte laboratorier.
Efter brug vil transportkasser blive rengjort og desinficeret og overskydende knolde fra forsøg og værn vil blive destrueret (f.eks. forbrænding/deponi).

4.b. Metoder til efterbehandling af stedet efter udsætning
Efter høst vil jorden bliver harvet, for at fritlægge eventuelle knolde, som ikke er taget op.
1. harvning vil ske efter høst, så evt. knolde kan frilægges og fjernes. Hen over vinteren vil marken blive harvet efter frostperioder eller mindst 2 gange.
Efter hver harvning vil marken blive kontrolleret for evt. fritlagte knolde, som vil blive destrueret.
Året efter udsætningen, vil arealet ligge som sort jord med månedlige harvninger (april til september) og overvågning.
Arealet vil blive overvåget i min. 4 år eller til der ikke findes spildplanter mere (jf. vejledning fra Landbrugsstyrelsen ”Dyrkningsbestemmelser for GM kartofler” juni 2024).

Arealet forventes udlagt med slåningsbrak fra 2028, som kan slås og overvåges.

Nedenfor er vist en forventet placering af forsøgsarealet i 2026 i markblok 500207-20, hvor der ikke er overlap til tidligere GMO-forsøgsarealer.

Det skal bemærkes, at erfaringen med håndoptagning og opsamling af kartofler er at der meget sjældent efterlades knolde i jorden.

4.c. Behandlingsmetoder, efter udsætning, herunder affald

Under dyrkningen: normal plantebeskyttelse imod ukrudt, skadedyr og andre sygdomme end kartoffelskimmel.

Høst: Ved håndopgravning og opsamling er risikoen for spild meget lille. Alt overjordisk plantemateriale vil blive knust forud for høst/opsamling, og kartoffeltoppen bliver efterladt på GMO-forsøgsarealet, hvor det indtørre og indarbejdes i jorden.
Plantemateriale fra værnet udenfor de cisgenetisk modificerede kartofler vil også blive knust forud for høst/optagning.
De høstede knolde vil blive transporteret i dobbelt lukkede plastposer eller lukkede plastikkasser, mærket med GMO, og transporteres til videre analyse på KMCs GMO godkendte laboratorium på Herningvej 60, Brande.
Efter brug vil vægten og kasser blive rengjort og desinficeret, og knolde og sække vil blive kørt til forbrænding/deponeret.

4.d. Overvågningsplaner og teknikker

Udsætningsmarken vil blive observeret hver uge i vækstperioden, og væksten vil blive noteret og beskrevet. Efter høst og i årene efter (jf. pkt.4b) vil udsætningsmarken blive nøje overvåget for knolde og eventuelle planter.
Eventuelle planterester og knolde vil blive destrueret.

4.e. Beredskabsplaner

Der forventes ikke krisesituationer med mulig undtagelse af potentielle hærværksaktioner, hvilket der ikke er tradition for i Danmark.
Lokaliteten vil blive overvåget med jævne mellemrum. Der vil blive opsat skilte forskellige steder ved marken, der beskriver forsøget samt navne og telefonnumre på de ansvarlige for forsøget: Kåre Lehmann Nielsen, Senior R&D Researcher, KMC og Kristian Elkjær, Teamleder R&D, KMC.

4.f. Metoder og procedurer til beskyttelse af stedet

i)
Alt arbejde med de cisgenetisk modificerede kartofler vil ske som håndarbejde, hvorfor den mekaniske spredningsrisiko betragtes som minimal.
Al transport til og fra mark vil ske i lukkede enheder/kasser, hvorfor risiko for spredning under transport også betragtes som minimal.

ii)
Der forventes ikke at skulle gøres noget ekstra til beskyttelse af stedet mod uvedkommende personers indtrængen.

iii)
Der forventes ikke at skulle gøres noget ekstra til beskyttelse af stedet mod andre organismers indtrængen.

B.5. Beskrivelse af teknikker til påvisning og identifikation af GMHP´erne

Vi har udviklet flg. metode til detektion af GMHPerne baseret på overgangen mellem de indsatte gener blb1 og blb2: Ved brug af primerne: Blb1_F1 (ACTGATCAAGCGGTGTGAGA) og Blb2_R1 (TCACTCCACACTCTCCAACC) med en annealingstemperatur på 64,2 C og en elongeringstid på 30 sec genererer et produkt på 1157 bp ved brug af Phire Plant Direct PCR kit (Thermo Scientific). Produktet af fraværende i den genetiske baggrund (Ydun). Vi har heller ikke fået et baggrundprodukt i 6 andre sorter vi har testet og da de to gener ikke er lokaliseret ved siden af hinanden i *Solanum bulbocastanum*, hvor generne oprindeligt kommer fra, betragter vi det som grænsende til umuligt at opnå et PCR produkt med disse primere af den størrelse i nogen kartoffelsort. Skulle der, mod vores forventning, opstå tvivlstilfælde, så kan DNA sekventering af det opnåede PCR produkt præcist fastslå transformationsstatus.

B.6. Oplysninger om tidligere udsætninger af GMPH´erne

I 2025 har det været GMO forsøgsudsætning af linjerne YSF5, YSF12 og YSF13 som udsætning af in-vitro planter, og med undtagelse af kraftigt forøget resistens, opførte de tre NGT linjer sig som baggrundssorten Ydun.

Underskrift

Dato: 20. marts 2026

Kåre Lehmann Nielsen
KMC

Kristian Elkjær
KMC

Bilag:

1. **Miljørisikovurdering**
2. **KMCs GMO-godkendelse af laboratorie.**
3. **Foreløbig skitse til forsøgsplan i marken**
4. **Rodunderskæring og jordløsning**

Appendix:

1. **Nukleotidsekvens af anvendte plasmider**
2. **Gensekvenser**
3. **Sekvensdokumentation for inserts**

Bilag 1

Miljørisikovurdering

Ansøgning om udsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (Phytophthora infestans)

M5 –D2: I tilfælde af genetisk modificerede højerestående planter (GMHPer)

1. **Persistens og invasionsevne hos GMHPerne, herunder genoverførsel fra plante til plante.**
   A)
   Afklipning af blomster vil effektivt forhindre en risiko for pollenspredning. Risikoen for pollenspredning vurderes som ubetydelig, men afklipningen vil eliminere den teoretiske risiko for spredning.
   Der dannes derfor heller ingen frø, hvorfor både persistens og invasionsevne betragtes som ubetydelig.

   B)
   Håndopgravning og opsamling af knolde vil sikre, at risikoen for overlevende knolde i jorden er ubetydelig.
   Efterfølgende frost i vinterperioden og sort jord i året efter avl vil effektivt sikre at evt. overlevende knolde fra høst ikke vil overleve og spire året efter.
   Efter høst vil jorden blive harvet for at frilægge eventuelle knolde, som ikke er taget op.
   1. harvning vil ske efter høst, så evt. knolde kan frilægges og fjernes. Hen over vinteren vil marken blive harvet efter frostperioder eller mindst 2 gange.
   Efter hver harvning vil marken blive kontrolleret for evt. fritlagte knolde, som vil blive destrueret.
   Året efter udsætningen vil arealet ligge som sort jord med månedlige harvninger (april til september) og overvågning. Arealet vil blive overvåget i min. 4 år eller til der ikke findes spildplanter mere (jf. vejledning fra Landbrugsstyrelsen ”Dyrkningsbestemmelser for GM kartofler” juni 2024).
   Arealet forventes udlagt med slåningsbrak fra 2028, som kan slås og overvåges. Det skal bemærkes, at erfaringer med håndopgravning og opsamling af kartofler er at der meget sjældent efterlades knolde i jorden.

2. **Genoverførsel fra plante til mikroorganismer**
   Vurderes som værende uden betydning og er ikke kendt i kartoffel.

3. **GMPHernes vekselvirkning med målorganismer**
   Forbedret resistens overfor kartoffelskimmel vurderes at være positivt, da bedre resistens overfor skimmel vil styrke planten.
   Vi forventer ikke at der vil ske ændringer i populationen af kartoffelskimmel *(Phytophthora infestans)* eller ændringer i dennes aggressivitet over en kortere tidshorisont.
   Vi forventer nærmest 100 % resistens i en længere del af dyrkningssæsonen i ubehandlede led.

4. **GMPHernes vekselvirkning med ikke målorganismer**
   Det vurderes ikke at de udsatte planter vil have påvirkning på ikke - målorganismer

5. **Virkningerne af de specifikke dyrknings-, håndterings- og høstteknikker.**
   Det vurderes, at arbejdet i forbindelse med håndlægning af knolde og/eller udplantning af pottedyrkede kartoffelplanter, med efterfølgende maskinhypning og jordløsning med maskine forud for håndopgravning og opsamling af knolde, sikrer en meget høj grad af sikkerhed for at der ikke efterlades knolde i jorden.

   Afklipning af blomster i forbindelse med blomstringen i begyndelsen af juli vil garantere, at selv den teoretiske risiko for pollen overførsel er elimineret.
   Vi ved fra svenske kollegaer på Sveriges Lantbruks Universitet (SLU) at dette er praktiseret de seneste år ved udsætninger i Skåne.

   Transport til og fra mark vil foregå i dobbelt lukkede enheder. Al transport og håndtering vil foregå med de relevante personer, altså ingen eksterne transportører.

   De personer, som skal foretage de kritiske arbejdsopgaver, transport, lægning, høst og efterkontrol er alle uddannet med GMO - kørekort i 2021 eller 2023, hvorfor alle er opdateret med nyeste viden om emnet.

6. **Virkninger på biogeokemiske processer**
   Det forventes ikke at kartoffelskimmel populationerne vil ændre sig over en kortere tidshorisont, men kunne blive forsinket i deres udbredelse. Denne forsinkelse forventes at kunne reducere anvendelsen af fungicider væsentligt.

7. **Virkninger på menneskers og dyrs sundhed**
   Det vurderes ikke at de udsatte planter vil have virkninger på hverken menneskers eller dyrs sundhed.
   Bedre resistens imod skimmel vil principielt forventes at virke positivt på både mennesker og dyrs sundhed, da det forventes at der skal anvendes en betydelig mindre mængde plantebeskyttelsesmidler (svampemidler) end i den oprindelige cultivar (sort). De ændrede egenskaber med flere resistensgener imod kartoffelskimmel, vil kunne forekomme under naturlige forhold, hvorfor virkningen ikke vurderes som væsentlig.
   Erfaringerne fra den traditionelle forædling er, at når der selekteres på sorter med flere resistensgener, har det ikke haft betydning på hverken mennesker eller dyrs sundhed.
   I forbindelse med almindelig resistensforædling har det heller ikke haft nogen kendt betydning for hverken mennesker eller dyrs sundhed

Bilag 2

KMCs GMO-godkendelse af laboratorie

Arbejdstilsynet

KMC, KARTOFFELMELCENTRALEN, AMBA
Herningvej 60
7330 Brande

Arbejdstilsynet

Landskronagade 33
2100 København Ø

T 70 12 12 88
at@at.dk
www.at.dk

CVR 21481815

23. september 2024

Sag
2024 - 52023
Ansvarlig: Rikke Kolding Hansen

CVR 15230614

Afgørelse om klassifikation til genteknologisk arbejde klasse planter (kartofler)

Arbejdstilsynet har den 6. august 2024 modtaget anmeldelse fra KMC, KARTOFFELMELCENTRALEN, AMBA ved Line Bach Christensen (lbc@kmc.dk) vedrørende klassifikation til genteknologisk arbejde klasse planter (kartofler) i lokale 1 (Pilot Plant) beliggende Herningvej 60, 7330 Brande.

Ansøgningen er fremsendt i henhold til Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Beskrivelse
Virksomheden har fremsendt udfyldt skema ´Anmeldelse til klassifikation af genteknologiske laboratorier og laboratorieområder samt anlæg til genteknologisk storskalaforsøg eller produktion´.

Yderligere bilag fremsendt:

- Plantegning.
- KMC´s + arbejdsmiljøorganisation.
- Beredskabsplan.

Ansøgningen har været forelagt Miljøstyrelsen (MST sagsnr 2024 – 61856) der har sendt følgende udtalelse til Arbejdstilsynet den 20. september 2024.

Miljøstyrelsen har den 12. september 2024 foretaget en besigtigelse af lokalerne.

Bemærkninger til klassifikation
Miljøstyrelsen har lagt følgende forhold, som fremgår af anmeldelsen eller besigtigelsen, til grund for vurdering af den fysiske indeslutning:

- *Der er tilladelse til op/nedklassificering af lokalet.*
- *Der er skiltning på indgang til område om genteknologisk område (planter), som sættes op og tages ned ifm. opstart og afslutning af arbejde med GMO kartofler.*

- *Der er udarbejdet sikkerhedsforskrifter og rengøringsforskrifter, der forebygger spredning af genmodificerede planter og spirer til omgivende miljø.*
- *Vinduer og døre holdes lukket*
- *Gulvafløb afdækkes med rist med min 5mm huller.*
- *Der er prop til vaske.*
- *Genanvendte materialer desinficeres eller autoklaveres inden rengøring*
- *Der er ikke revner og huller i vægge*
- *Der er desinfektionsmiddel i alle lokaler*
- *Lokalet må ikke anvendes til andre GMO planter/frø.*

På dette grundlag har styrelsen ingen indvendinger imod den søgte klassifikation af lokalerne til arbejde med genetisk modificerede ***planter (Kartofler).***

Virksomheden havde oprindeligt ansøgt om storskala kl. 1+ planter, men efter aftale med Miljøstyrelsen ændres klassifikationen til en alm. GMO kl. planter (kartofler).

Vurdering
Arbejdstilsynet finder på det foreliggende grundlag, at de omhandlede lokaler, sikkerhedsforskrifter m.m. lever op til de krav, der er gældende for genteknologisk arbejde klasse planter (kartofler).

Afgørelse
På baggrund af ovenstående meddeler Arbejdstilsynet hermed klassifikation til genteknologisk arbejde klasse planter (kartofler) i 1 (Pilot Plant) beliggende KMC, Hemingvej 60, 7330 Brande, jf. § 7, stk. 1, til Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Lokalerne har fået tildelt LAB-id nr. 240 042

Vejledning
Opmærksomheden henledes på, at det af hensyn til klassifikationen er vigtigt at sikre, at forskrifter, procedurer m.v. fortsat afspejler de faktiske sikkerhedsmæssige forhold for arbejdet med GMO, herunder arbejdsmetoder og arbejdsgange. Ved at gennemgå dem med jævne mellemrum, fx i forbindelse med revideringen af virksomhedens APV kan dette sikres.

Opmærksomheden henledes endvidere på § 30 jfr. § 11 i bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008, hvorefter enhver væsentlig ændring af de oplysninger, der ligger til grund for denne klassifikation, skal anmeldes til Arbejdstilsynet.

Ligeledes henledes opmærksomheden på § 12 i samme bekendtgørelse, hvorefter det forinden skal anmeldes til Arbejdstilsynet, hvis klassifikationen ikke længere ønskes opretholdt.
Med henblik på en senere evt. afmelding af klassifikationen kan Arbejdstilsynet anbefale, at virksomheden allerede nu, udarbejder en skriftlig nedklassificeringsprocedure.

Klage
I kan klage over afgørelsen til Arbejdsmiljøklagenævnet. Klagen skal indsendes til Arbejdstilsynet og være modtaget inden fire uger efter, at I har modtaget afgørelsen.

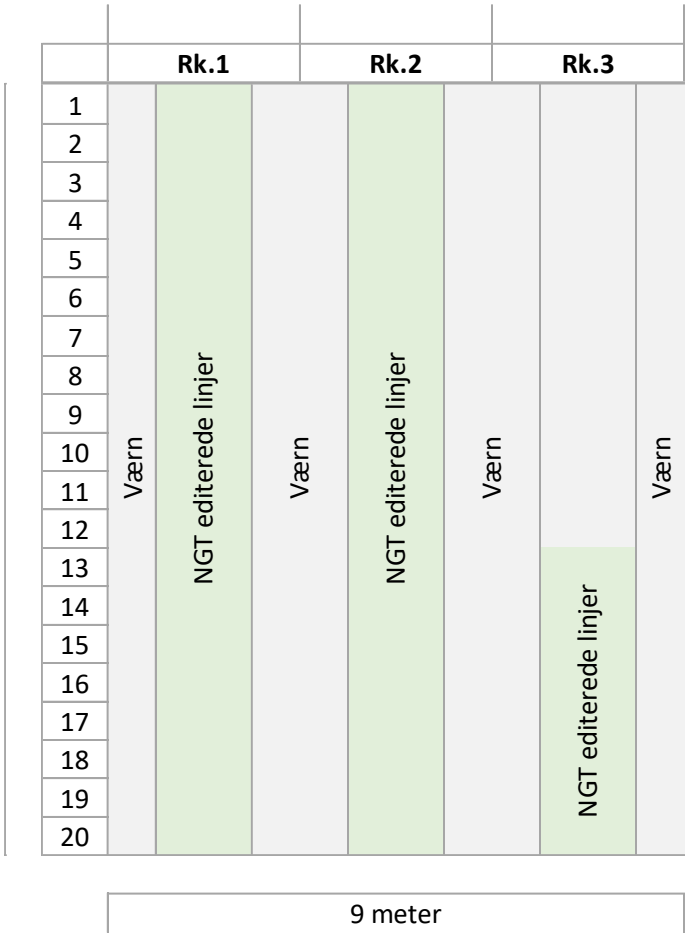
Kopi af afgørelsen sendes til Miljøstyrelsen.

Venlig hilsen

Rikke Kolding Hansen

Bilag 3

Foreløbig skitse til forsøgsplan i marken



Bilag 4

Rodunderskæring og jordløsning

De NGT editerede kartoffelplanter rodunderskæres og jordløsnes forud for håndopgravning og opsamling, for at sikre kartoffelplanterne slipper rødder, og jorden er løs. Det sikrer en mere ens håndopgravning.

Der laves en ramme, som kan påmonteres en traktors trepunktsophæng. På rammen monteres en arm, der holder et vingeskær, som kan rodunderskærer på tværs at 2 rækker. Foran armen, monteres et rulleskær – hvis nødvendigt – for at undgå at toprester vil slæbe omkring armen.

Appendix 1

a) Nukleotidsekvens af anvendte plasmider

Plasmid_RA anvendt til YSF5, YSF12 og YSF13

CATGCCAACCACAGGGTTCCCCTCGGGATCAAAGTACTTTGATCCAACCCCTCCGCTGCTATAGTGCAGTCGG
CTTCTGACGTTCAGTGCAGCCGTCTTCTGAAAACGACATGTCGCACAAGTCCTAAGTTACGCGACAGGCTGCC
GCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTTAGTCGCATAAAGTAGAATACTTGCGACTAGAACC
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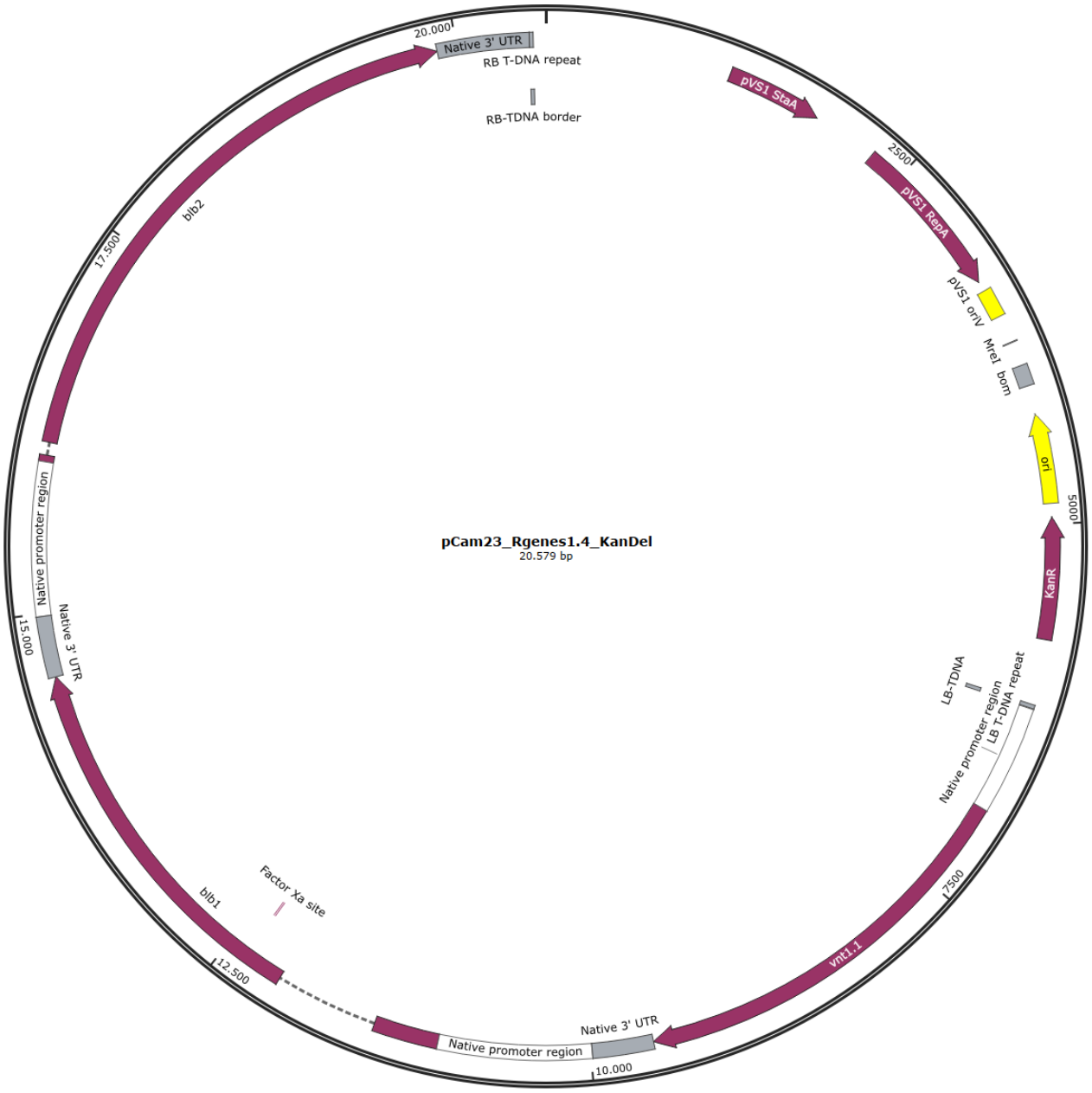

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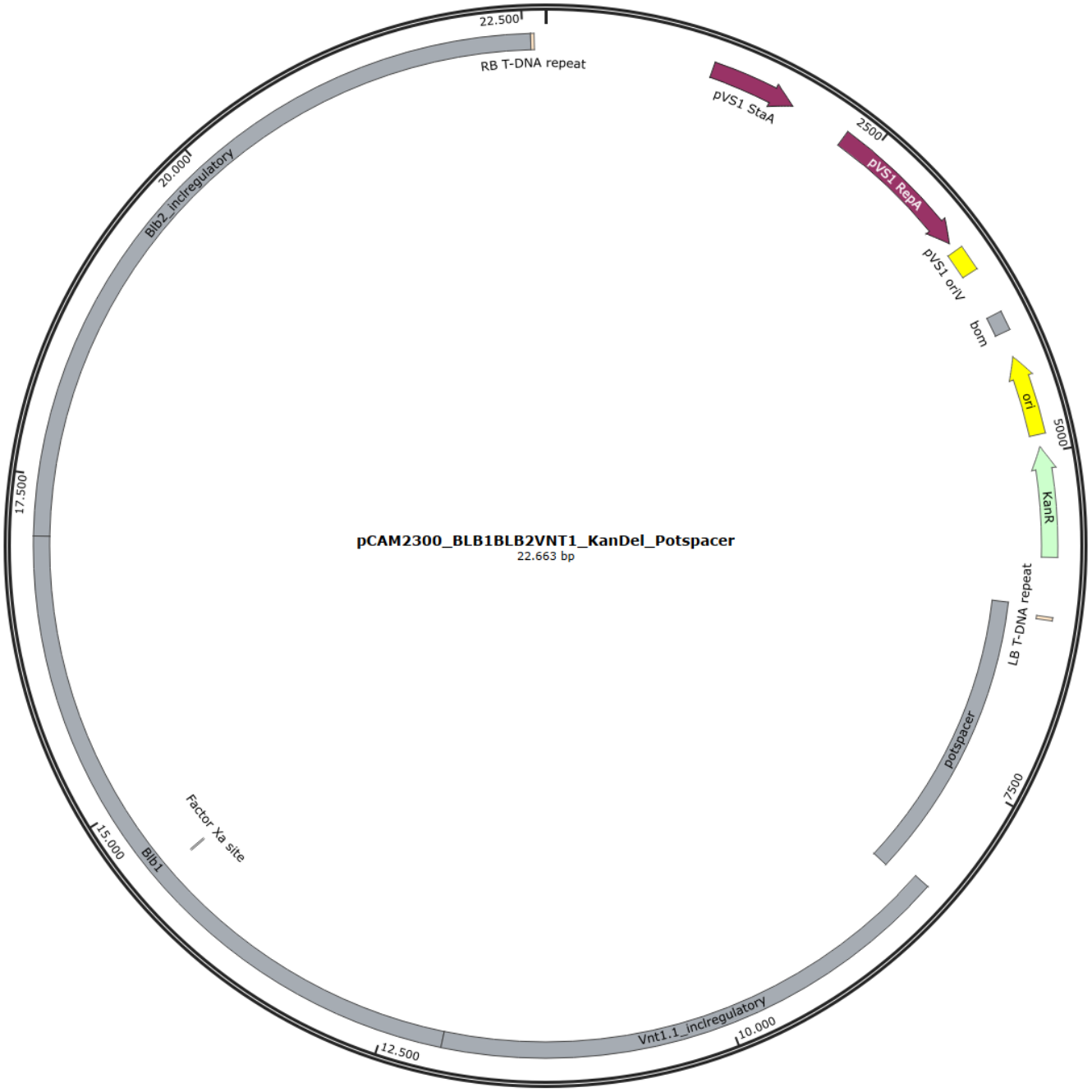
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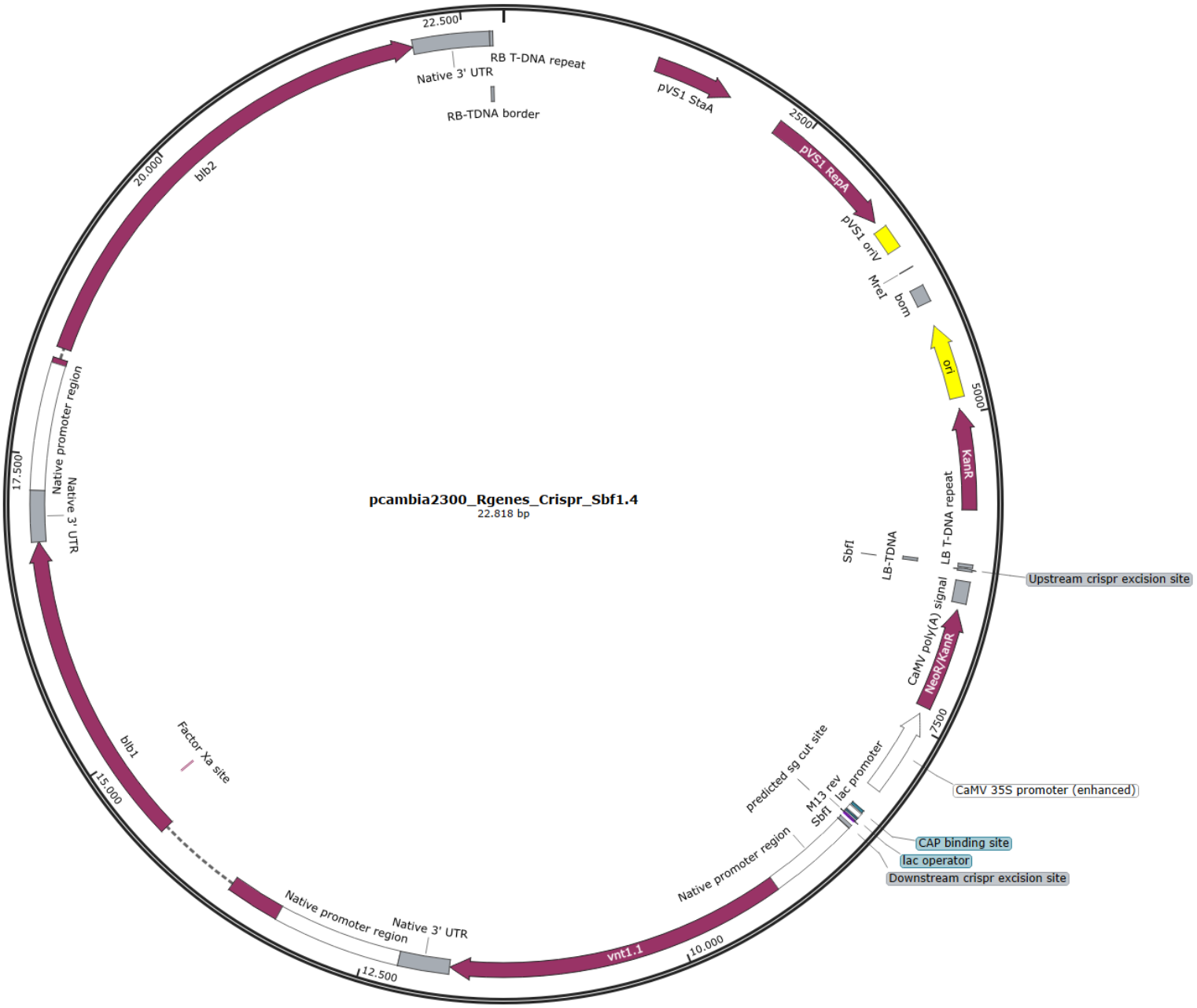
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KanR
LB T-DNA repeat
SbfI
LB-TDNA
Upstream crispr excision site
CaMV poly(A) signal
NeoR/KanR
7500
CaMV 35S promoter (enhanced)
predicted sg cut site
M13 rev
SbfI
lac promoter
CAP binding site
lac operator
Downstream crispr excision site
Native promoter region
vnt1.1
10.000
Native promoter region
Native 3' UTR
12.500
Factor Xa site
blb1
15.000
17.500
Native promoter region
Native 3' UTR
blb2
20.000

Appendix 2
Gensekvenser

>Vnt1.1 inklusiv Promoter og 3′-UTR

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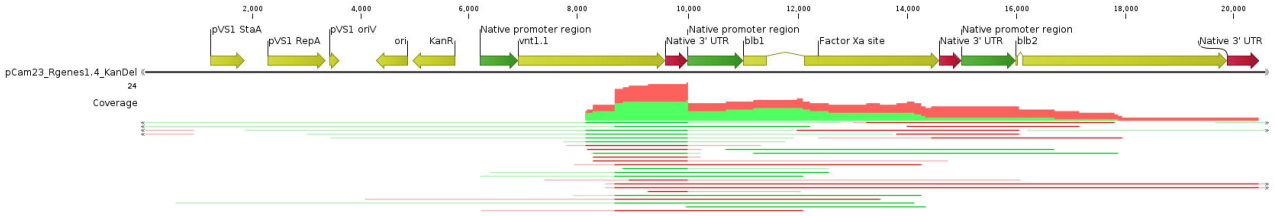
Appendix 3

Sekvensdokumentation for inserts.

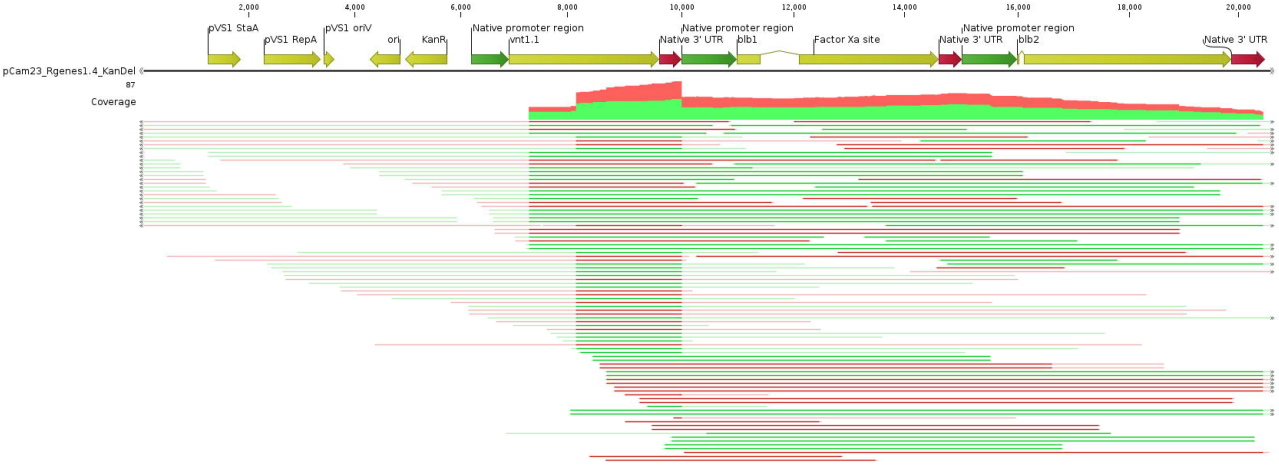
Figurforklaring: Øverst ses en lineær angivelse af den cirkulære vektor (appendix 1) med funktionelle elementer angivet. Nedenunder findes linjer/reads som repræsenterer DNA sekvens read i hhv forward (grøn) og reverse (rød) orientering. Når linjen/read er skraveret, så betyder det at dette område af sekvens-readet ikke aligner til vektoren, hvorimod uskraveret linje/read angiver den del af reads som aligner til vektor sekvens. Bemærk at det er umuligt ikke at lave sjældne sekvensfejl under sekventering. Derfor inoreres sekvensvarianter, der kun er set få gange fra analysen). Note: for YSF5, YSF12 og YSF13 er dokumentationen identisk med den godkendte ansøgning i 2025.

A: Oversigt over mapping af insert-specifikke reads fra rekombinante linjer til hele vektor. Der ses kun reads som mapper til det forventede insert og ikke til de dele af plasmidet som ikke skal indsættes. Den genetiske baggrund, Ydun, er medtaget for sammenligning. Bemærk, at der er en ikke-relevant baggrundsmapping fra Ydun, som ikke stammer fra vores behandling og derfor ikke relevant for insert-karakteriseringen.

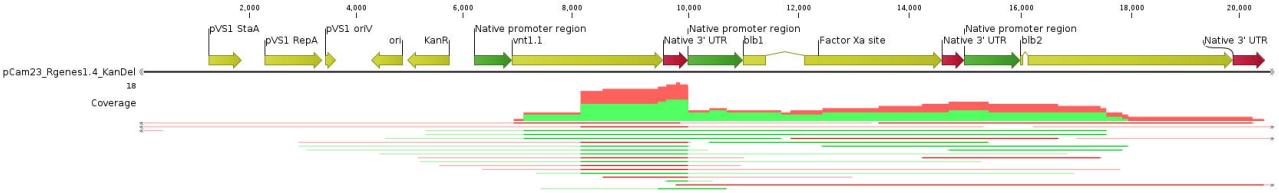
YSF5: (gener er markeret med gule pile, promoter med grønne pile, 3'-UTR regioner med røde pile)



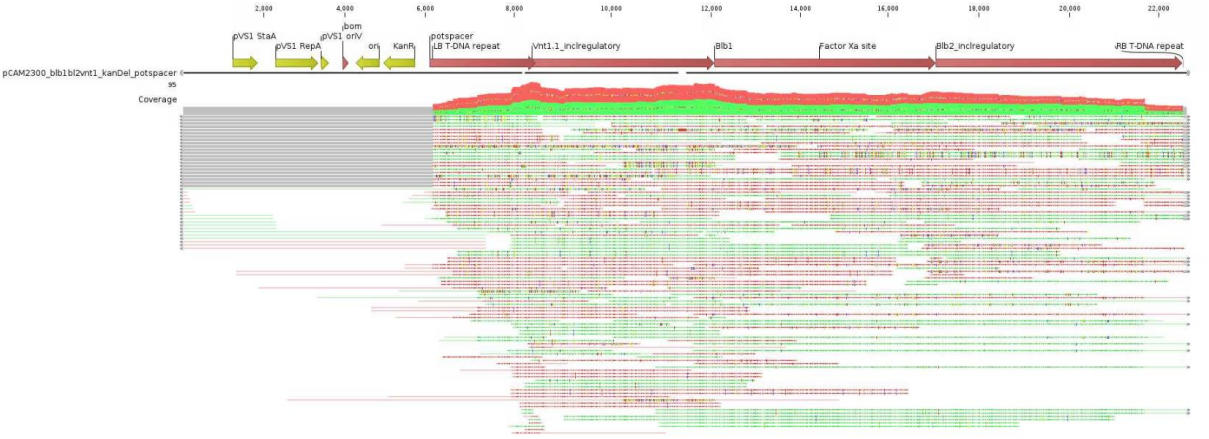
YSF12: (gener er markeret med gule pile, promoter med grønne pile, 3'-UTR regioner med røde pile)



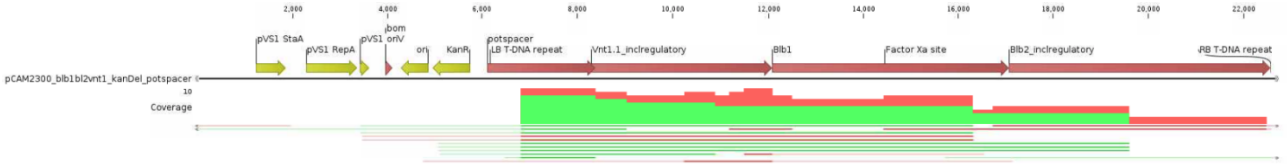
YSF13: (gener er markeret med gule pile, promoter med grønne pile, 3'-UTR regioner med røde pile)



YA_mfp_1: (gener tilhørende vektor er markeret med gule pile, ønskede inserts (inkl. evt. native promoter og 3'-UTR) er markeret med røde pile. Bemærk at i denne figur er enkelte sekvensfejl på individuelle reads angivet. Det grå område upstream opstår fordi der er to kopier af de indsatte gener som mapper til den cirkulære vektor, men som ikke indeholder de områder af sekvensen som markeres med "." som fremstår grå i denne figur. I figur B ses detaljer for mfp-1, hvor det tydeligt fremgår, at der er tale om ".", og dermed at reads ikke indeholder denne del af vektor-sekvensen)

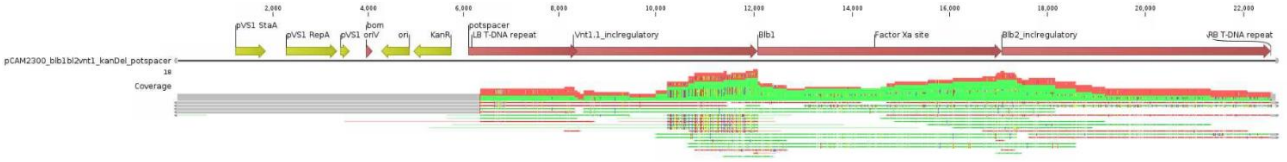


YA_mfp_2: (gener tilhørende vektor er markeret med gule pile, ønskede inserts (inkl. evt. native promoter og 3'-UTR) er markeret med røde pile.

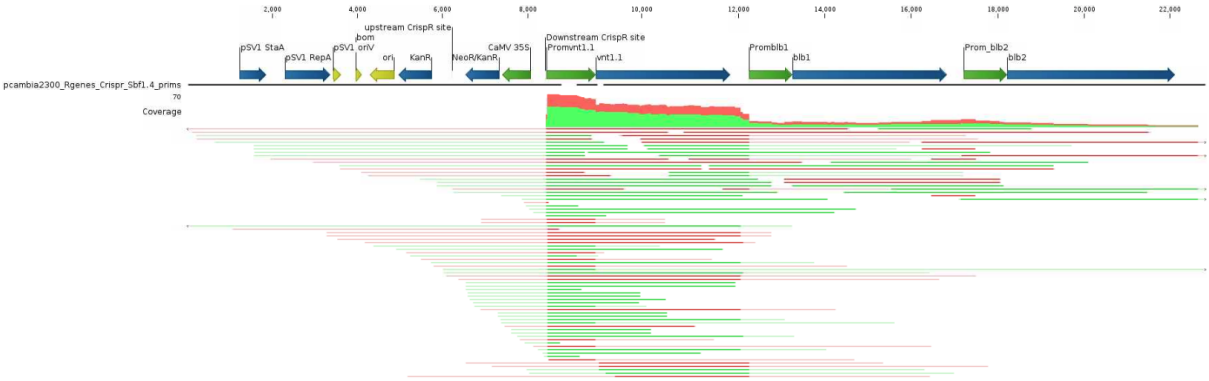


YA_mfp_3: (gener tilhørende vektor er markeret med gule pile, ønskede inserts (inkl. evt. native promoter og 3'-UTR) er markeret med røde pile.

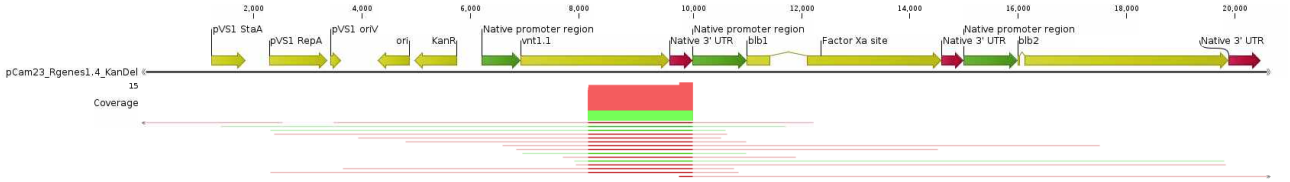
YA_mfp_5: (gener tilhørende vektor er markeret med gule pile, ønskede inserts (inkl. evt. native promoter og 3'-UTR) er markeret med røde pile. Bemærk at i denne figur er enkelte sekvensfejl på individuelle reads angivet. Det grå område upstream opstår fordi der er to kopier af de indsatte gener som mapper til den cirkulære vektor, men som ikke indeholder de områder af sekvensen som markeres med "." som fremstår grå i denne figur. I figur B ses detaljer for mfp-1, hvor det tydeligt fremgår, at der er tale om ".", og dermed at reads ikke indeholder denne del af vektorsekvensen)



YA10_51: (gener er markeret med blå pile, regulatoriske elementer fra vektor med gule pile og promoter elementer med grønne pile). Bemærk at der er betydelig cross-mapping af reads fra baggrundsloci for vnt1-genet (derfor den høje coverage i netop det område) – det er kun de reads som spanner vnt1-Pblb1 junction, som vi med sikkerhed ved stammer fra insert. Det er et resultat af at vi for denne CrispR editerede linje ikke har samme mulighed for at effektivt at filtrere baggrundsreads fra, som med de andre linjer, hvor vi har kendt sekvens på begge sider af DNA-element overgangene, da vi i dette tilfælde kun har CrispR downstream information.

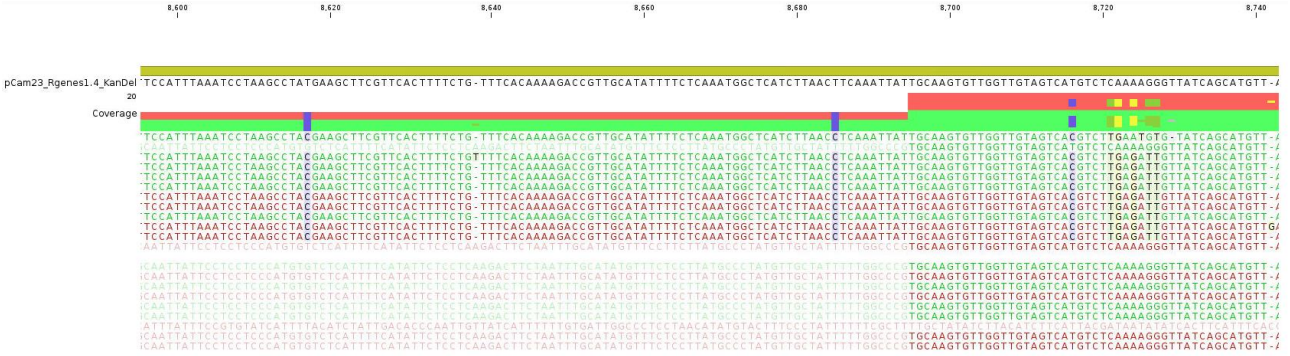


Ydun: (gener er markeret med gule pile, promoter med grønne pile, 3'-UTR regioner med røde pile)



B: Udsnit fra A som fokuserer på 5'-enden af insert. Bemærk at den insert-flankerede sekvens (når man ser bort fra Ydun baggrundssekvenser) er entydige, hvilket betyder, at der kun er et enkelt insertionsite.

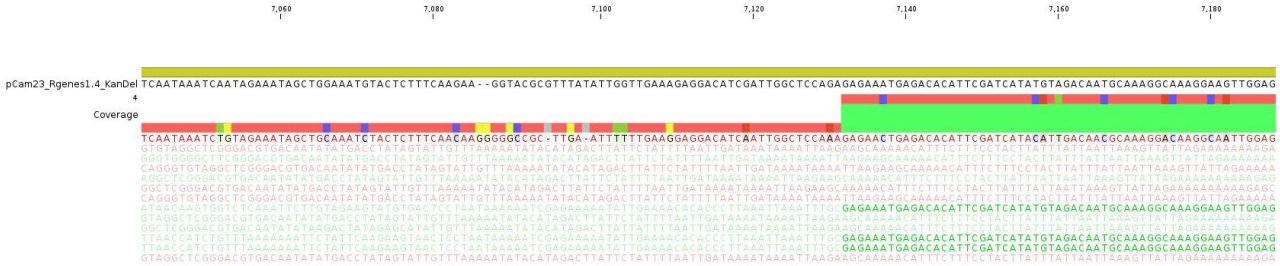
YSF5: (bemærk at det er de reads som aligner til vektor nt 8665- tilhører vores insert – de andre er Ydun baggrundsreads)



YSF12:

YSF13:

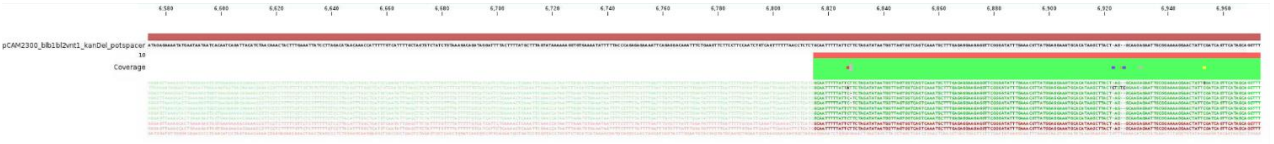
(bemærk at det er reads som aligner til vektor nt 7132- som tilhører vores insert)



YA_mfp_1:

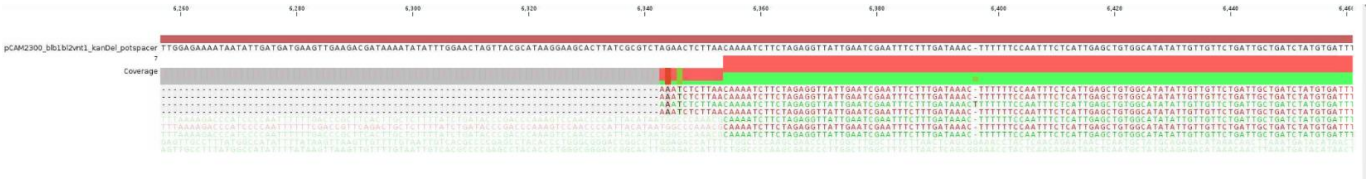
(bemærk at dobbeltkopien af R-generne forårsager et ”gap” i mappingen som markeres med ”.” for de reads som stammer fra downstream kopi af insert). Det er disse som fremstår ”grå” i figur A.

YA_mfp_2: Bemærk at det nederste ”skraverede” read er flankerende sekvens fra reads som krydsmapper fra vnt1-homologe gener i Ydun baggrund – se figur A hhv Ydun og YA_mfp_2).



YA_mfp_3: Bemærk at de to øverste og det nederste skraverede reads stammer fra reads som krydsmaper fra vnt1-homologe gener i Ydun baggrund – figur A hhv. Ydun og YA_mfp_3.

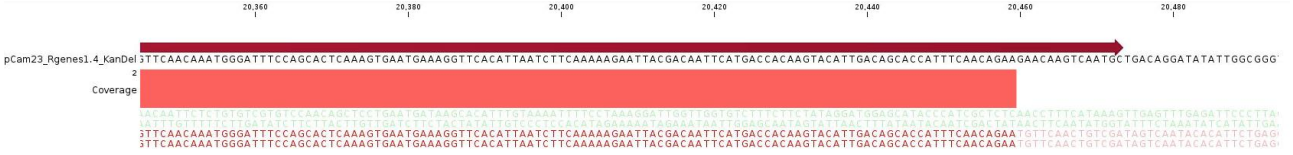
YA_mfp_5: (Bemærk at dobbeltkopien af R-generne forårsager et ”gap” i mappingen som markeres med ”.” for de reads som stammer fra downstream kopi af insert. Det er disse som fremstår ”grå” i figur A. De to nederste ”skraverede” reads er flankerende sekvens fra reads som krydsmapper fra vnt1-homologe gener i Ydun baggrund – se figur A).



YA10_51: Bemærk at der er betydelig cross-mapping af reads fra baggrundsloci for vnt1-genet (derfor den høje coverage i netop det område). Det er et resultat af at vi for denne CrispR editerede linje ikke har samme mulighed for effektivt at filtrere dem fra, som med de andre linjer, hvor vi har kendt sekvens på begge sider af DNA-element overgangene, hvor vi i dette tilfælde kun har CrispR downstream information. Disse reads er de skraverede 36 nederste reads)

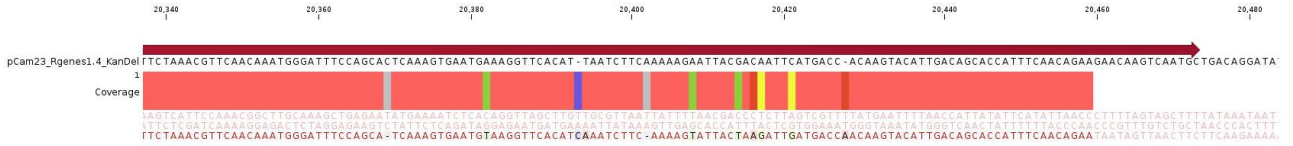
C: Udsnit af A som fokuserer på 3'-enden af insert.

YSF5:



YSF12:

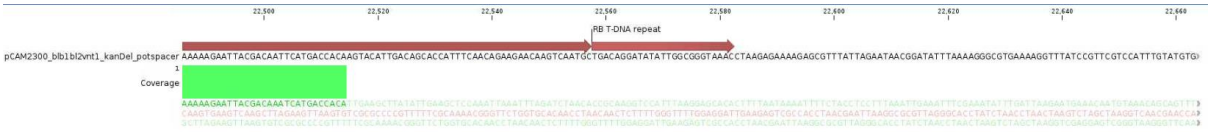
YSF13:



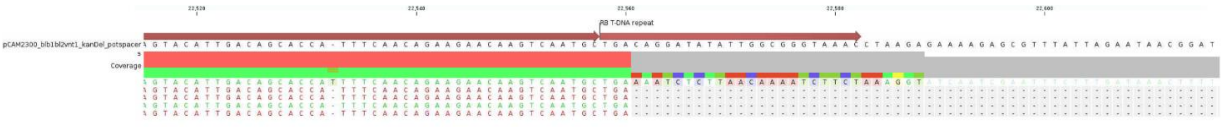
YA_mfp_1: (bemærk at YA_mfp1 har tandem insertion og dermed er der sekvenssupport for både overgangen fra den ene tandem kopi til næste (den længste match) og overgangen fra tandem kopi nr. 2 og downstream for insertion site kartoffel flankerede gDNA)

YA_mfp_2:

YA_mfp_3: (kun et enkelt read)



YA_mfp_5: (bemærk at YA_mfp1 har tandem insertion og dermed er der sekvenssupport for både overgangen fra den ene tandem kopi til næste (den længste match) og overgangen fra tandem kopi nr. 2 og downstream for insertion site kartoffel flankerede gDNA – dog kun med et enkelt read).



YA10_51:


22,540
22,560
22,580
22,600
22,620
22,640
22,660
22,680
22,700
22,720
LB-T-DNA repeat
pcambia2300_Rgenes_Crispr_Sbf1.4_prims
4
Coverage

Appendix 3

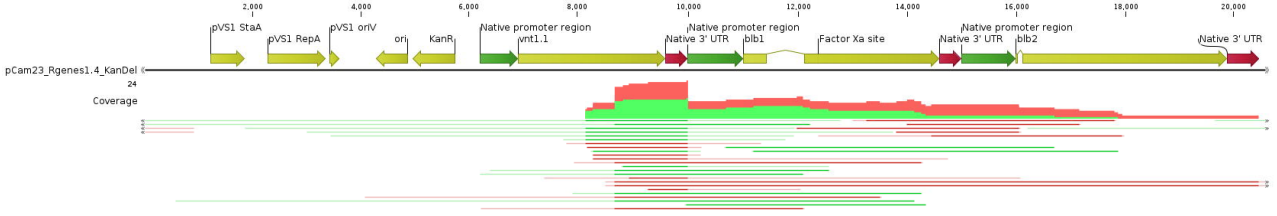
Sekvensdokumentation for inserts.

Figurforklaring: Øverst ses en lineær angivelse af den cirkulære vektor (appendix 1) med funktionelle elementer angivet (gule piler er gener; grønne pile er promotorer og røde pile er 3-UTR regioner). Nedenunder findes linjer/reads som repræsenterer DNA sekvens read i hhv forward (grøn) og reverse (rød) orientering. Når linjen/read er skraveret, så betyder det at dette område af sekvens-readet ikke aligner til vektoren, hvorimod uskraveret linje/read angiver den del af reads som aligner til vektor sekvens. Bemærk at det er umuligt ikke at lave sjældne sekvensfejl under sekventering. Derfor udelukkes sekvensvarianter, der kun er set en enkelt gang fra analysen).

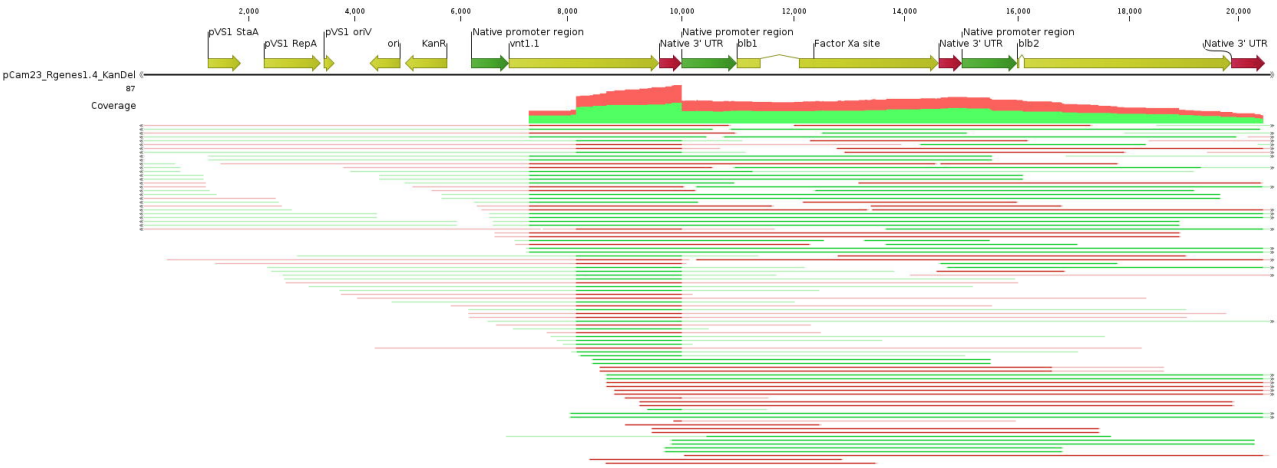
Note: for YSF5, YSF12 og YSF13 er dokumentationen identisk med den godkendte ansøgning i 2025.

A: Oversigt over mapping af insert-specifikke reads fra rekombinante linjer til hele vektor. Der ses kun reads som mapper til det forventede insert og ikke til de dele af plasmidet som ikke skal indsættes. Den genetiske baggrund, Ydun, er medtaget for sammenligning. Bemærk, at der er en ikke-relevant baggrundsmapping fra Ydun, som ikke stammer fra vores behandling og derfor ikke relevant for insert-karakteriseringen.

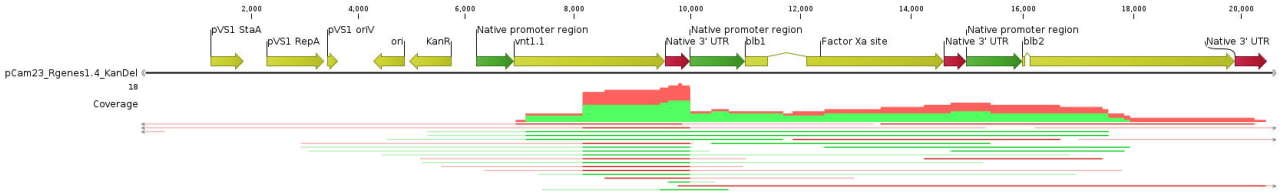
YSF5:



YSF12:



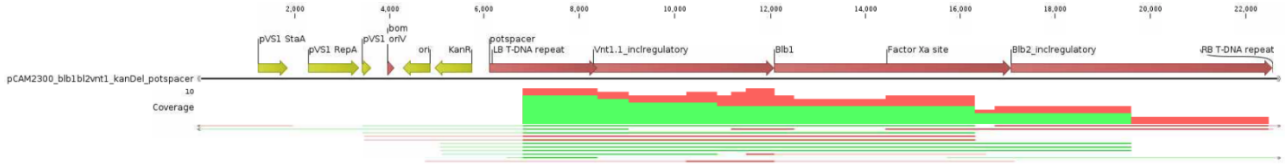
YSF13:



YA_mfp_1:

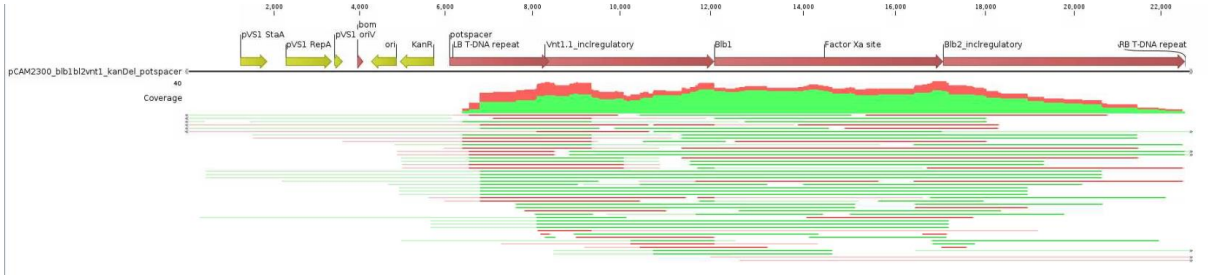
(bemærk at i denne figur er enkelte sekvensfejl på individuelle reads angivet)

YA_mfp_2:



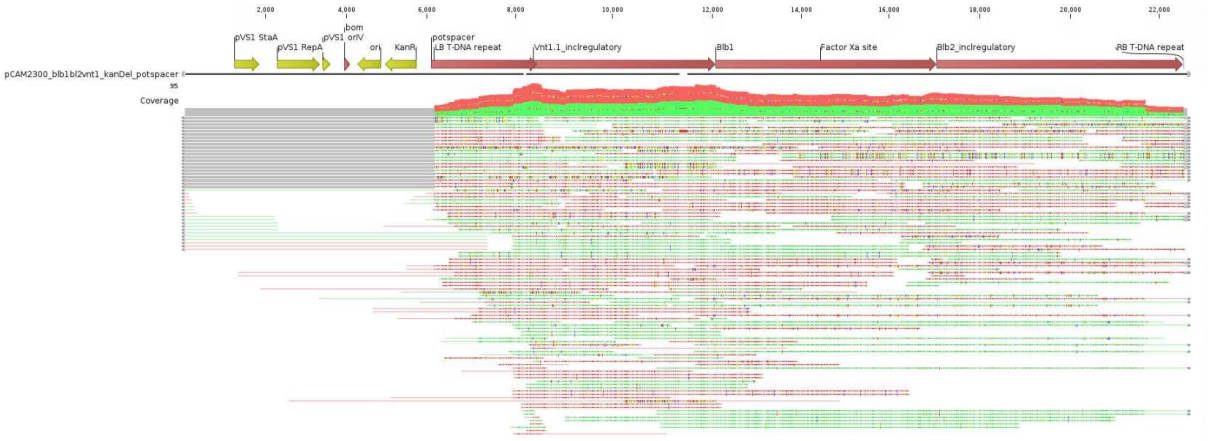
YA_mfp_3:

YA_mfp_4:

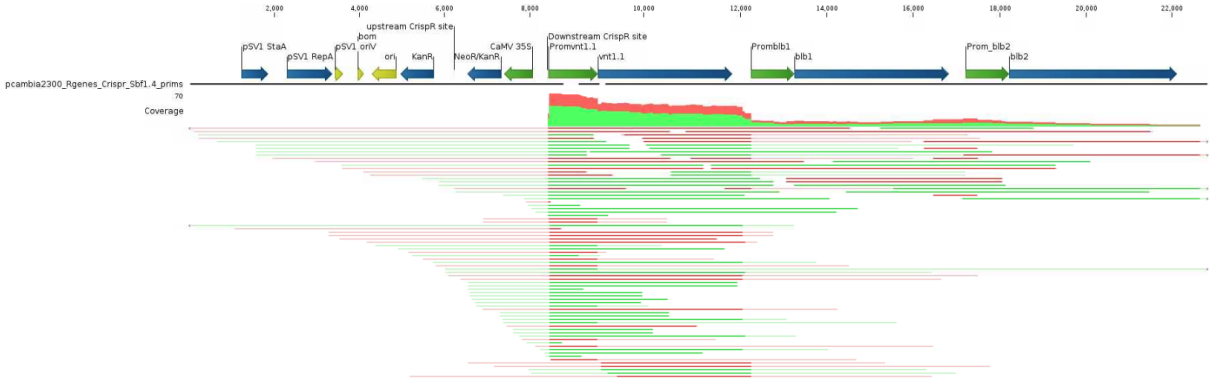


YA_mfp_5:

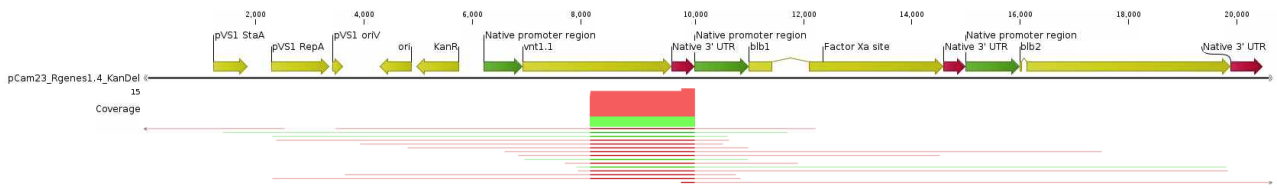
(bemærk at i denne figur er enkelte sekvensfejl på individuelle reads angivet)



YA10_51:

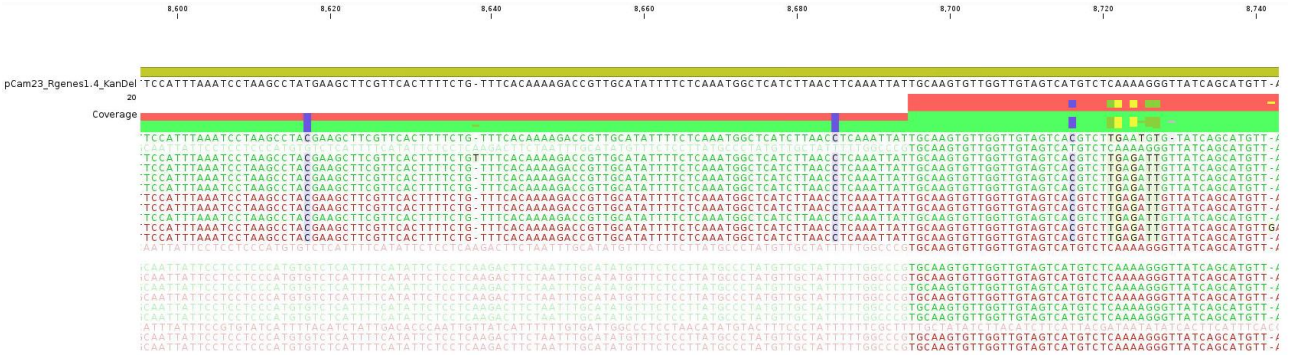


Ydun:

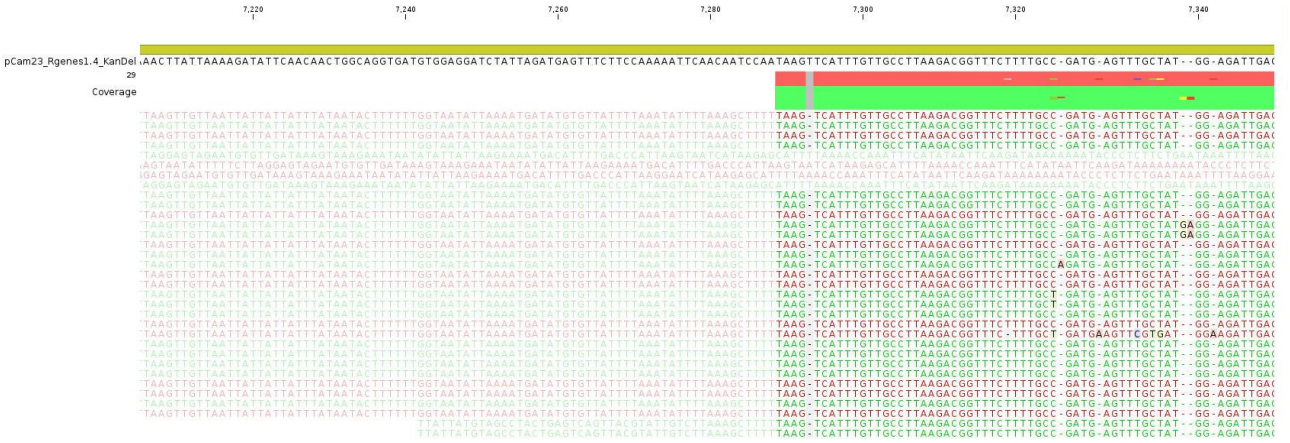


B: Udsnit fra A som fokuserer på 5'-enden af insert. Bemærk at den insert-flankerede sekvens (når man ser bort fra Ydun baggrundssekvenser) er entydige, hvilket betyder, at der kun er et enkelt insertionsite.

YSF5: (bemærk at det er de reads som aligner til vektor nt 8665- tilhører vores insert – de andre er Ydun baggrundsreads)

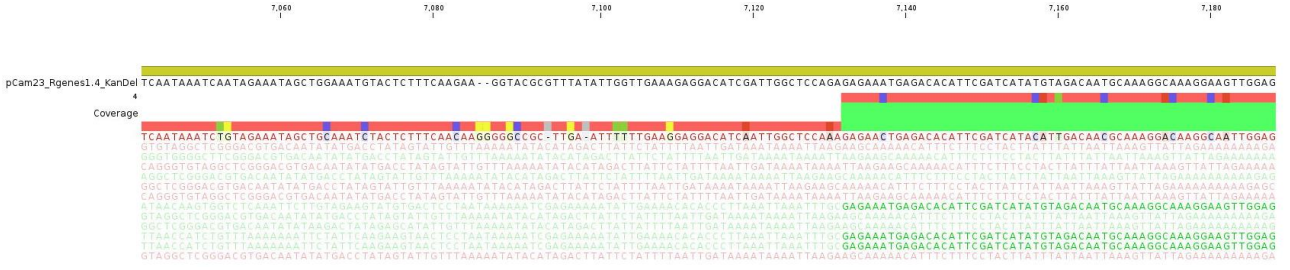


YSF12:



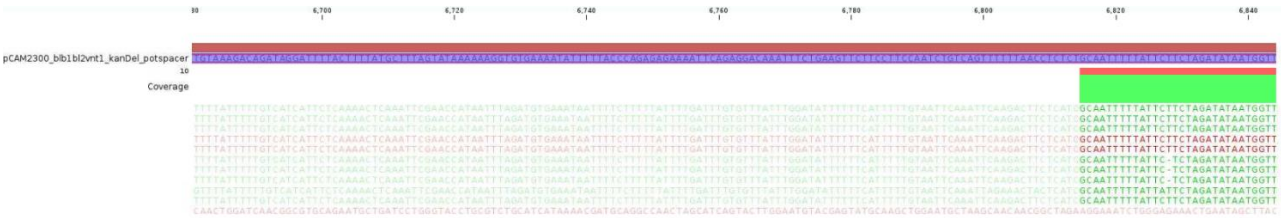
YSF13:

(bemærk at det er reads som aligner til vektor nt 7132- som tilhører vores insert)

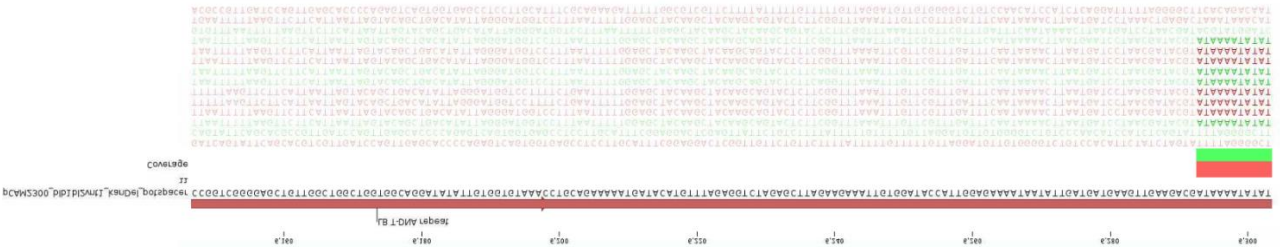


YA_mfp_1:

YA_mfp_2:



YA_mfp_3:



YA_mfp_4:

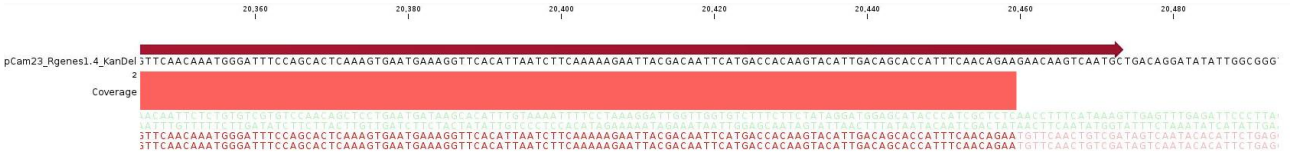
YA_mfp_5:

YA10_51:


Downstream CrispR site
Promvnt1.1
pcambia2300_Rgenes_Crispr_Sbf1.4_prims
Coverage

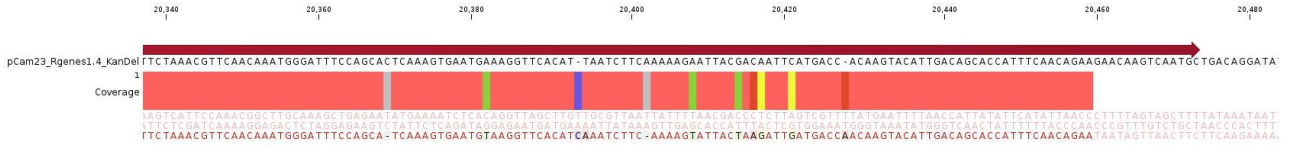
C: Udsnit af A som fokuserer på 3'-enden af insert.

YSF5:



YSF12:

YSF13:

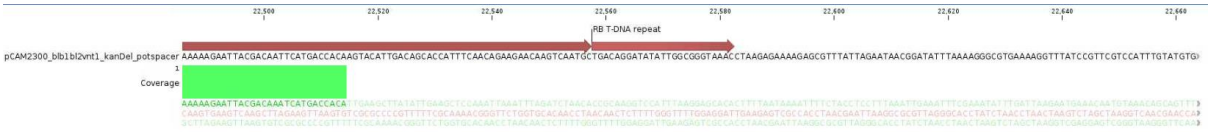


YA_mfp_1:

(bemærk at YA_mfp1 har tandem insertion og dermed er der sekvenssupport for både overgangen fra den ene tandem kopi til næste (den længste match) og overgangen fra tandem kopi nr. 2 og downstream for insertion site kartoffel flankerede gDNA)

YA_mfp_2:

YA_mfp_3:



YA_mfp_4:

YA_mfp_5:

(bemærk at YA_mfp1 har tandem insertion og dermed er der sekvenssupport for både overgangen fra den ene tandem kopi til næste (den længste match) og overgangen fra tandem kopi nr. 2 og downstream for insertion site kartoffel flankerede gDNA)

YA10_51:

The Plant Journal (2003) **36**, 867–882 doi: 10.1046/j.1365-313X.2003.01934.x

An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato

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Summary

Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease for potato cultivation. Here, we describe the positional cloning of the *Rpi-blb1* gene from the wild potato species *Solanum bulbocastanum* known for its high levels of resistance to late blight. The *Rpi-blb1* locus, which confers full resistance to complex isolates of *P. infestans* and for which race specificity has not yet been demonstrated, was mapped in an intraspecific *S. bulbocastanum* population on chromosome 8, 0.3 cM from marker CT88. Molecular analysis of a bacterial artificial chromosome (BAC) clone spanning the *Rpi-blb1* locus identified a cluster of four candidate resistance gene analogues of the coiled coil, nucleotide-binding site, leucine-rich repeat (CC-NBS-LRR) class of plant resistance (*R*) genes. One of these candidate genes, designated the *Rpi-blb1* gene, was able to complement the susceptible phenotype in a *S. tuberosum* and tomato background, demonstrating the potential of interspecific transfer of broad-spectrum late blight resistance to cultivated *Solanaceae* from sexually incompatible host species. Paired comparisons of synonymous and non-synonymous nucleotide substitutions between different regions of *Rpi-blb1* paralogues revealed high levels of synonymous divergence, also in the LRR region. Although amino acid diversity between Rpi-blb1 homologues is centred on the putative solvent exposed residues of the LRRs, the majority of nucleotide differences in this region have not resulted in an amino acid change, suggesting conservation of function. These data suggest that *Rpi-blb1* is relatively old and may be subject to balancing selection.

Keywords: *Rpi-blb1*, disease resistance, *Phytophthora infestans*, *Solanum bulbocastanum*, potato, tomato.

Introduction

Despite the notorious Irish potato famine of the mid-19th century, late blight, caused by the oomycete pathogen *Phytophthora infestans*, still continues to be one of the most devastating of all plant diseases (Duncan, 1999). Although chemicals targeted against *P. infestans* provide reasonable levels of disease control, world-wide losses because of late blight and costs of control measures are estimated to still exceed $3 billion annually. *P. infestans* is a specialised pathogen, primarily causing disease on the foliage and fruits of a range of Solanaceae species (Erwin and Ribeiro, 1996), especially potato and tomato (Fry and Goodwin, 1997). The necessity of developing crops that possess durable resistance increases as more virulent, crop-specialised and pesticide-resistant strains of the pathogen are rapidly emerging. The relatively recent introduction of both mating types to major potato-growing areas of the world has enabled the pathogen to reproduce sexually (Goodwin *et al.*, 1995; Smart and Fry, 2001; Spielman *et al.*, 1991). This allows the recombination of traits in the sexual cycle, thereby generating new possibilities for quick adaptation.

To obtain late blight resistance, breeders have in the past focussed on the introgression of dominant resistance (*R*) genes from *Solanum demissum*, a wild potato species indigenous to Mexico (Malcomson and Black, 1966; Wastie, 1991). Eleven such *R* genes have been identified, several of which have been mapped to specific loci on the genetic map of potato (reviewed by Gebhardt and Valkonen, 2001).

Unfortunately, the resistance caused by these *R* genes, although nearly complete to specific races of the pathogen, appeared not to be durable. Once newly bred potato cultivars are grown on larger scale in commercial fields, new virulences emerge in *P. infestans*, which render the pathogen able to overcome the introgressed resistance (Wastie, 1991). More durable field resistance to late blight, often quantitative in nature and presumed to be race non-specific, can be found in several Mexican and Middle and South American *Solanum* species (Ross, 1986). However, this type of resistance is difficult to transfer into potato cultivars through crossing and phenotypic selection. The ultimate goal of breeders, therefore, is to introduce into potato varieties major *R* genes that confer broad-spectrum resistance to late blight through recognition of pathogen factors that are essential for pathogen fitness.

Diploid *S. bulbocastanum* from Mexico and Guatemala is one of the tuber-bearing species that is known for its high levels of resistance to late blight (Niederhauser and Mills, 1953). Unfortunately, classic transfer of resistance from wild *Solanum* species to cultivated potato is not easy because of differences in ploidy and Endosperm Balance Number (Johnston *et al.*, 1980). Despite these problems, introgression of the *S. bulbocastanum* resistance trait has been successful. Ploidy manipulations and a series of tedious bridge crosses resulted in *S. bulbocastanum*-derived, *P. infestans*-resistant germplasm (Hermsen and De Boer, 1971; Hermsen and Ramanna, 1969, 1973; Ramanna and Hermsen, 1971). However, 40 years of intense and continuous breeding efforts with this germplasm has still not resulted in market introduction of resistant cultivars. More recently, somatic hybrids of *S. bulbocastanum* and *S. tuberosum* and backcrossed germplasm were found to be highly resistant to late blight, even under extreme disease pressure (Helgeson *et al.*, 1998). However, suppression of recombination within this material could form a potential obstacle for reconstitution of the cultivated potato germplasm by recurrent crosses, to a level that could meet the standards for newly bred potato cultivars (Helgeson *et al.*, 1998). Isolation of the genes that code for the resistance traits found in wild sources and subsequent transformation of existing potato cultivars with these genes could be a much quicker means of exploiting potentially durable late blight resistance in wild *Solanum* species.

According to the gene-for-gene hypothesis (Flor, 1971), disease resistance starts with recognition of pathogen avirulence (Avr) factors by plant R proteins, followed by signal transduction leading to a hypersensitive response (HR) and resistance (Dangl *et al.*, 1996). The cloning and molecular characterisation of over 30 plant *R* genes conferring resistance to bacteria, fungi, oomycetes, viruses, nematodes or insects has allowed their classification in structural classes regardless of pathogen specificity (reviewed by Dangl and Jones, 2001; Hulbert *et al.*, 2001). The most abundant class of characterised *R* genes, comprising approximately 0.5% of the genes predicted in the *Arabidopsis* genome (Meyers *et al.*, 2002), is predicted to encode intracellular proteins that carry leucine-rich repeat (LRR) and nucleotide-binding site (NBS) domains, motifs also found in other receptor and signal transduction proteins. In common with other receptors, it is generally considered that NBS-LRR R proteins have a modular structure with separate recognition and signalling domains, whereby the LRR is the candidate recognition domain and the N-terminal region including the NBS, the major signalling domain. Functional analysis of recombinant R proteins indicates that recognition specificity indeed resides in the LRR (Dodds *et al.*, 2001; Ellis *et al.*, 1999). Moreover, the LRR is the most variable region in closely related NBS-LRR proteins and is under selection to diverge (Meyers *et al.*, 1998; Noël *et al.*, 1999; Van der Vossen *et al.*, 2000). However, evidence is accumulating that LRRs also contribute to signalling through negative regulation involving putative intramolecular interactions (Bendahmane *et al.*, 2002; Hwang *et al.*, 2000; Moffet *et al.*, 2002).

Currently, five *R* genes have been cloned from potato, including the *S. demissum*- and *S. bulbocastanum*-derived late blight resistance genes *R1* and *RB*, respectively (Ballvora *et al.*, 2002; Bendahmane *et al.*, 1999, 2000; Song *et al.*, 2003; Van der Vossen *et al.*, 2000). Here, we report the positional cloning of *Rpi-blb1*, a CC-NBS-LRR plant *R* gene derived from *S. bulbocastanum*. *Rpi-blb1*, when expressed in potato or tomato, confers full resistance to a range of *P. infestans* isolates carrying multiple virulence factors. Race specificity has not yet been found. The potential significance of single *R* genes from the wider gene pool for cultivated *Solanaceae* is demonstrated.

Results

Evaluation of late blight resistance in S. bulbocastanum

A first and crucial step in determining the genetic basis of late blight resistance in *S. bulbocastanum* was the identification of susceptible individuals for the generation of intraspecific mapping populations. Five *S. bulbocastanum* accessions, originating from different areas in Mexico, were screened for *P. infestans* susceptibility in detached leaf assays using a complex isolate (IPO655-2A). Although the accessions predominantly produced resistant individuals, fully susceptible individuals were encountered at a low frequency in three of the five accessions tested. A series of intraspecific crosses were subsequently made between resistant individuals from each accession with a susceptible genotype from BGRC accession 8006, and segregation of the resistance was analysed. Resistance in the F_1 population resulting from a cross between a resistant plant from

Table 1 Characteristics of *P. infestans* isolates for which the Blb8005-8 clone showed a typical HR in detached leaf assays

Isolate ID	Country of origin	Isolation year	Host	Mating type	RACE[a]
IPO98014	the Netherlands	1998	Potato	A1	1.2.3.4.7.11
IPO428-2	the Netherlands	1992	Potato	A2	1.2.3.4.5.6.7.8.9.10.11
IPO82001	Belgium	1982	Potato	A2	1.2.3.4.5.6.7.10.11
IPO655-2A	the Netherlands	1992	Potato	A2	1.2.3.4.5.6.7.8.9.10.11
PIC96001	Mexico	1996	Potato	A1	n.d.
PIC96002	Mexico	1996	Potato	A2	n.d.
IPO01900	the Netherlands	2001	*S. sisymbriifolium*	A1	1.2.3.7.8.11
GNVM48-2	the Netherlands	2000	Potato	A1	n.d.
GNVM49-1	the Netherlands	2000	Potato	A2	n.d.
GNVM62-1	the Netherlands	2000	Potato	A1	n.d.

[a]Numbers indicate *R*-gene differentials for which isolates showed compatible interactions in detached leaf assays.

BGRC accession 8005 (individual 8005-8) and the susceptible plant from BGRC accession 8006 (individual 8006-9) segregated in a ratio not significantly different from 1 : 1. Of the 42 F_1 genotypes screened, 22 were resistant, 16 were susceptible and 4 initially displayed unclear phenotypes. However, these data indicated that the resistance in 8005-8 inherited as a dominant allele at a single locus. In the expectation that additional late blight resistance loci will be found in *S. bulbocastanum*, the locus identified in 8005-8 was named *Rpi-blb1*.

To determine the specificity of the *Rpi-blb1* locus, we screened the resistant genotype 8005-8 with 10 different *P. infestans* field isolates with varying complexity and aggressiveness (Table 1). Included in this panel of isolates were some of the highly aggressive isolates that can nowadays be collected in Dutch potato fields. the Netherlands forms a region with a high level of disease pressure because of the presence of both mating types A1 and A2 (Flier and Turkensteen, 1999; Flier *et al.*, 2003). *Rpi-blb1* conferred full resistance to all the tested isolates thereby inducing a typical HR reaction, suggesting the involvement of an *R* gene that currently has an unmatched specificity.

Genetic and physical mapping of the Rpi-blb1 *locus*

Screening of the original 42 F_1 progeny plants, derived from the intraspecific cross between *S. bulbocastanum* genotypes 8005-8 and 8006-9, with the chromosome 8 marker CT88 (Tanksley *et al.*, 1992) indicated that *Rpi-blb1* mapped to the same genetic region on chromosome 8 as the *S. bulbocastanum*-derived *Phytophthora* resistance locus *RB* (Naess *et al.*, 2000). To investigate this further, restriction fragment length polymorphism (RFLP) markers flanking CT88 (TG513 and CT64, respectively; Tanksley *et al.*, 1992) were developed into cleaved amplified polymorphic sequence (CAPS) markers (Table 2) and tested on 512 F_1

Table 2 Overview of markers and primers used for mapping Rpi-blb1

Marker	F/R[a]	Primer sequence[b]	Annealing temperature (°C)	Enzyme[c]
CT88	F	GGCAGAAGAGCTAGGAAGAG	57	*Mbo*I
	R	ATGGCGTGATACAATCCGAG		
	F	TTCAAGAGCTTGAAGACATAACA	60	a.s.
	R	ATGGCGTGATACAATCCGAG		
TG513	F	CGTAAACGCACCAAAAGCAG	58	a.s.
	R	GATTCAAGCCAGGAACCGAG		
CT64	F	ACTAGAGGATAGATTCTTGG	56	*Cfo*I
	R	CTGGATGCCTTTCTCTATGT		
B139R	F	GATCAGAAGTGCCTTGAACC	56	TaqI
	R	CAAGGAGCTTGGTCAGCAG		
SPB33L	F	ATTGCACAGGAGCAGATCTG	59	*Hin*fI
	R	TGTAAGAGAGCAAGAGGCAC		
SPB42L	F	AGAGCAGTCTTGAAGGTTGG	58	*Cfo*I
	R	GATGGTAACTAAGCCTCAGG		
B149R	F	GACAGATTTCTCATAAACCTGC	58	*Mse*I/*Xba*I
	R	AATCGTGCATCACTAGAGCG		
RGA1–4	F	TGTGGAGTAAGAGAGGAAGG	62	*Ssp*I/*Mse*I
	R	TCAGCTGAGCAGTGTGTGG		

[a]Orientation of the primer: F, forward; R, reverse.
[b]Primer sequences according to IUB codes.
[c]a.s., allele specific.

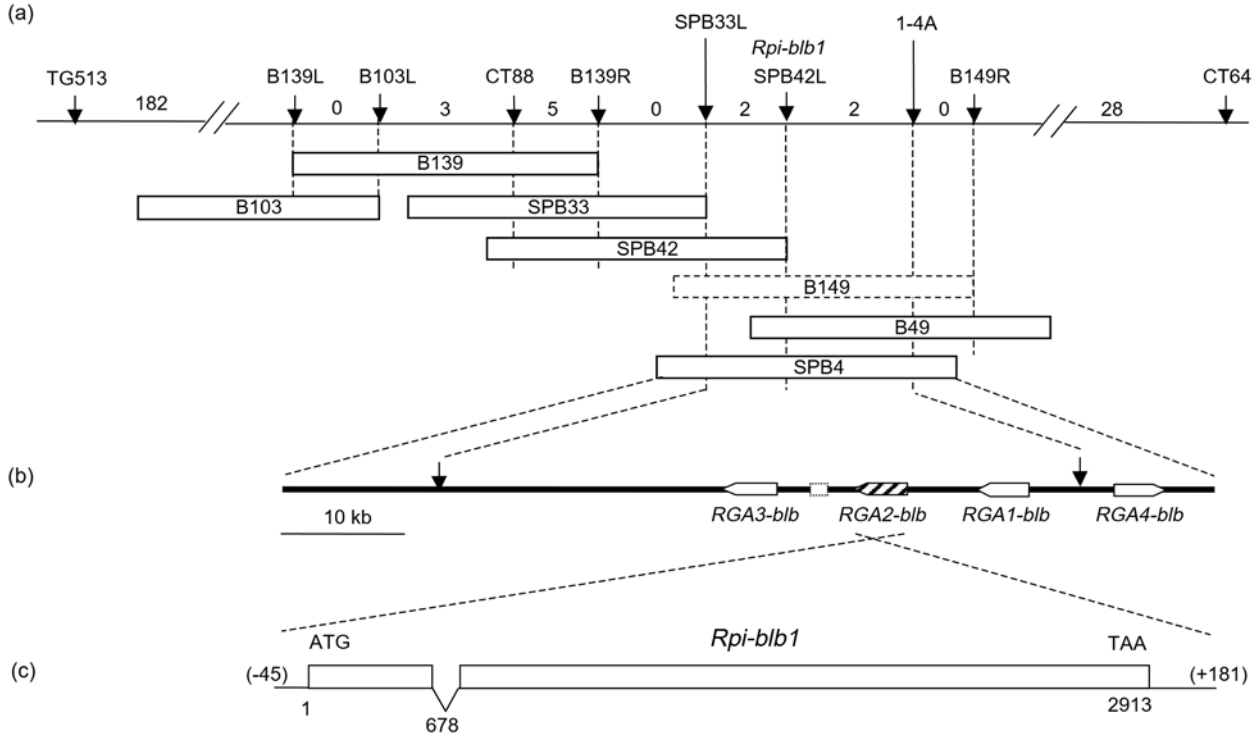

Figure 1. Genetic and physical map of the *Rpi-blb1* locus.
(a) Genetic and physical map of the *S. bulbocastanum* genomic region containing *Rpi-blb1*. Vertical arrows indicate the relative positions of markers linked to resistance. Numbers above the horizontal line indicate the number of recombinants identified between the flanking markers in 2109 progeny plants. Rectangles represent BAC clones. The dotted rectangle represents a BAC clone derived from the recessive *rpi-blb1* haplotype.
(b Relative positions of candidate genes for late blight resistance on BAC SPB4.
(c) Schematic representation of the *Rpi-blb1* gene structure. Horizontal lines indicate exons. Open boxes represent coding sequence. Lines angled downwards indicate the position of a 678-nt long intron sequence. Numbers in-between brackets indicate the length of the 5′- and 3′-UTRs of the putative *Rpi-blb1* transcript.

individuals. In this screen, 5 CT64-CT88 recombinant genotypes and 41 CT88-TG513 recombinant genotypes were identified. A single recombination event was found between the *Rpi-blb1* locus and marker CT88.

For further fine mapping of the *Rpi-blb1* locus, a total of 2109 F_1 individuals were screened for recombination between markers TG513 and CT64. The recombinant genotypes (219/2109) were subsequently screened with CAPS markers derived from left (L) and right (R) border sequences of bacterial artificial chromosome (BAC) clones isolated from a BAC library prepared from the resistant *S. bulbocastanum* parent genotype 8005-8 (Table 2). The BAC library was initially screened with marker CT88. BAC clones identified with this marker were used as seed BACs for a subsequent chromosome walk to the *Rpi-blb1* locus. These data together with the disease resistance data of each recombinant, obtained through detached leaf assays, positioned the *Rpi-blb1* locus within a 0.1-cM genetic interval between markers SPB33L and B149R (4/2109 recombinants), an interval physically spanned by the overlapping BAC clones SPB4 and B49 (Figure 1a). Within this interval, resistance co-segregated with the BAC end marker SPB42L, the sequence of which was highly homologous to partial NBS fragments (Q97, Q137, Q152 and Q153) from tomato (Pan *et al.*, 2000). Southern analyses of BAC clones spanning the SPB33L–B149R interval using a ^{32}P-labelled PCR fragment of marker SPB42L as a probe revealed the presence of at least four copies of this NBS sequence within the *Rpi-blb1* interval. Moreover, all of these copies were present on BAC SPB4. Sequencing and annotation of the complete insert of this BAC clone identified four complete *R* gene analogues (RGA) of the NBS-LRR class of plant *R* genes and a remnant part of such a gene (Figure 1b). Although two recombinant genotypes indicated that *RGA4-blb* was not a candidate for *Rpi-blb1*, all four RGAs were selected for complementation analysis.

Complementation analysis

Genomic fragments of 7–10 kbp harbouring *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb* were subcloned from BAC SPB4 into the binary plant transformation vector pBINPLUS (Van Engelen *et al.*, 1995) and transferred to a susceptible potato cultivar through *Agrobacterium*-mediated transformation. In view of clone instability and low transformation frequencies encountered with

Table 3 Complementation of late blight susceptibility in potato and tomato

Genotype[a]	AGL0[b]			LBA4404			UIA143		
	T_0[c]	RGA[d]	R[e]	T_0	RGA	R	T_0	RGA	R
IMP (RGA1-blb)	17	15	0	–	–	–	9	8	0
IMP (RGA2-blb)	–	–	–	31	6	6	14	12	9
IMP (RGA3-blb)	–	–	–	6	0	–	5	5	0
IMP (RGA4-blb)	19	18	0	12	1	0	–	–	–
IMP (vector)	8	8	0	–	–	–	10	9	0
MM (RGA2-blb)	–	–	–	–	–	–	11	9	7

[a]Primary transformants obtained from transformation of the susceptible potato and tomato genotypes Impala (IMP) and Moneymaker (MM), respectively, with T-DNA constructs containing the *Rpi-blb1* gene candidates *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb.*
[b]*A. tumefaciens* strains used for transformation.
[c]Number of primary transformants resistant to kanamycin.
[d]Number of kanamycin-resistant primary transformants containing a complete gene construct.
[e]Number of primary transformants resistant to *P. infestans*. Resistance was tested in detached leaf assays using the complex isolates IPO655-2A and IPO428-2.

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.*, 1983), second and third transformation experiments were carried out independently using the *A. tumefaciens* strains AGL0 (Lazo *et al.*, 1991) or UIA143 (Farrand *et al.*, 1989; Table 3). Primary transformants harbouring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays. Only the genetic construct harbouring *RGA2-blb* was able to complement the susceptible phenotype; 15 out of 18 *RGA2-blb* containing primary transformants were resistant (Table 3) whereas all *RGA1-blb, RGA3-blb* and *RGA4-blb* containing primary transformants were susceptible to *P. infestans*. As the *RGA2-blb* transformants showed similar resistance phenotypes as the *S. bulbocastanum*-resistant parent (Figure 2), *RGA2-blb* was designated the *Rpi-blb1* gene.

Rpi-blb1 *is functional in tomato*

To investigate whether *Rpi-blb1* could also complement the susceptible phenotype in tomato, primary transformants of cultivar Moneymaker harbouring the *Rpi-blb1* gene construct were initially tested with the potato-derived *P. infestans* isolates IPO655-2A and IPO428. Seven out of nine primary transformants were resistant (Table 3). As the tested potato *P. infestans* isolates were less virulent on tomato than on potato, the primary transformants were also tested with a *P. infestans* isolate collected from late blight diseased plants of cultivar Moneymaker in a home garden. Even though this isolate was significantly more aggressive on Moneymaker than the isolates IPO655-2A and IPO428 (data not shown), all seven primary transformants remained resistant. These results illustrate the potential effectiveness of the *Rpi-blb1* gene not only against complex isolates derived from potato but also against those specialised on tomato.

Rpi-blb1 *gene structure and putative amino acid sequence*

The size and structure of the *Rpi-blb1* gene were determined by comparing the genomic sequence derived from the insert of pRGA2-blb with cDNA fragments generated by 5′ and 3′ rapid amplification of cDNA ends (RACE). RACE identified 5′ and 3′ *Rpi-blb1*-specific cDNA fragments

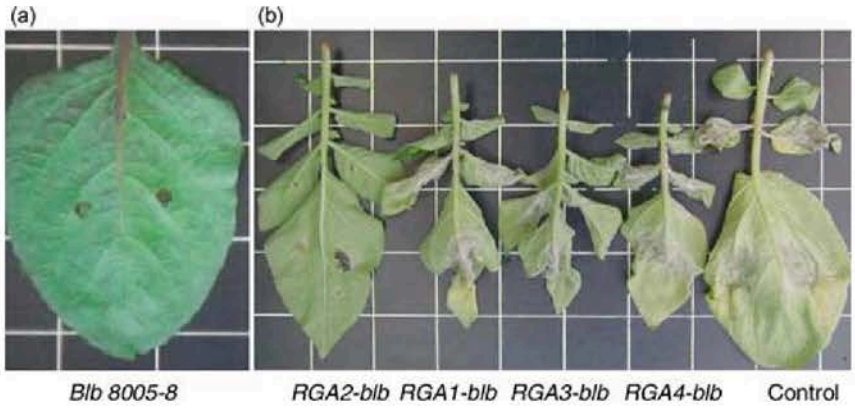

Figure 2. Detached leaf disease assays.
(a) Resistant phenotype of the *S. bulbocastanum* 8005-8 parent genotype 6 days post inoculation (dpi) with *P. infestans* sporangiospore droplets.
(b) Genetic complementation for late blight resistance in potato. Typical disease phenotypes of leaves derived from transgenic potato plants harbouring *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb* and control plants 6 dpi with *P. infestans* sporangiospore droplets.

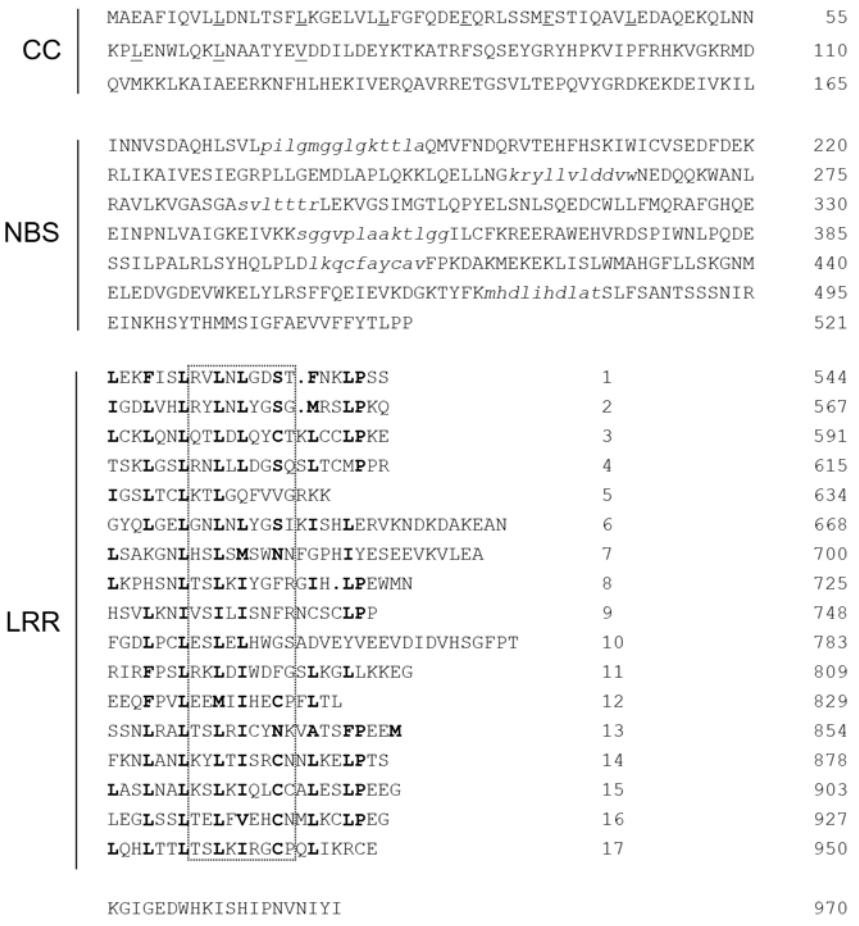

Figure 3. Deduced Rpi-blb1 protein sequence. The amino acid sequence deduced from the DNA sequence of *Rpi-blb1* is divided into three domains (CC, NBS and LRR). Hydrophobic residues in the CC domain that form the first and fourth residues of heptad repeats of potential coiled-coil domains are underlined. Conserved motifs in R proteins are written in lowercase and in italic in the NBS domain. Residues matching the consensus of the cytoplasmic LRR are indicated in bold in the LRR domain. Dots in the sequence have been introduced to align the sequence to the consensus LRR sequence of cytoplasmic LRRs. The LRR subdomain xxLxLxxxx is framed.

comprising 5′ and 3′ untranslated regions (UTRs) of 45 and 181 nucleotides (nt), respectively. The *Rpi-blb1* gene contains a single intron of 678 nt starting 428 nt downstream of the ATG start codon of the gene (Figure 1c). The coding sequence of the *Rpi-blb1* transcript is 2910 nt.

The deduced open-reading frame (ORF) of the *Rpi-blb1* gene encodes a predicted polypeptide of 970 amino acids with an estimated molecular weight of 110.3 kDa (Figure 3). Several functional motifs present in *R* genes of the NBS-LRR class of plant *R* genes are apparent in the encoded protein. As illustrated in Figure 3, the Rpi-blb1 protein belongs to the coiled-coil subset of NBS-LRR resistance proteins (Pan *et al.*, 2000). The N-terminal half of the Rpi-blb1 protein contains a potential coiled-coil region between amino acids 10 and 72 and six conserved motifs indicative of a nucleotide-binding site (Van der Biezen and Jones, 1998). The C-terminal half of Rpi-blb1 comprises a series of 17 LRRs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx observed in other cytoplasmic R proteins (Jones and Jones, 1997). The third LRR contains the xLDL motif that is conserved in the third LRR of many NBS-LRR proteins (Axtell *et al.*, 2001; Banerjee *et al.*, 2001). PROSITE analysis (Hofmann *et al.*, 1999) identified 8 *N*-glycosylation, 1 cAMP- and cGMP-dependent protein kinase phosphorylation, 12 protein kinase C phosphorylation, 8 casein kinase II phosphorylation, 10 *N*-myristoylation and 3 amidation putative sites in the deduced Rpi-blb1 amino acid sequence.

To identify *in silico* homologues of the *Rpi-blb1* gene, BLAST searches (Altschul *et al.*, 1990) were carried out with the coding sequence of the *Rpi-blb1* gene. BLASTN searches identified a number of sequences with significant homology to short stretches of the *Rpi-blb1* gene. Nucleotides 549–1245 of the coding sequence of the *Rpi-blb1* gene share 81–90% sequence identity to partial NBS fragments from tomato (e.g. Q194, Q137, Q198 and Q199; Pan *et al.*, 2000). These sequences vary in length between 525 and 708 nt and are PCR fragments, which were identified by systematically scanning the tomato genome using (degenerate) primer pairs based on ubiquitous NBS motifs (Leister *et al.*, 1996; Pan *et al.*, 2000). Nucleotides 76–805 of the coding sequence of the *Rpi-blb1* gene share 91% sequence identity with an expressed sequenced tag (EST) from potato (EMBL database Accession no. BG890602). No hits were identified that share homology with *Rpi-blb1* gene sequences downstream of nucleotide 1245, comprising the LRR region. At

Table 4 Sequence variability and nucleotide substitution rates in different regions of *Solanaceous R* gene paralogues

Comparison	Region[a]	Nucleotide substitutions[c] aa[b]	K_a	K_s	K_a/K_s
Rpi-blb1 versus *RGA3-blb*	Gene	81	0.099	0.163	0.61
	CC-NBS	91	0.043	0.122	0.35
	LRR	71	0.169	0.207	0.81
	xxLxLxxxx	63	0.280	0.335	0.84
Rpi-blb1 versus *RGA1-blb*	Gene	76	0.132	0.270	0.49
	CC-NBS	79	0.112	0.345	0.33
	LRR	72	0.155	0.201	0.77
Rpi-blb1 versus *RGA4-blb*	Gene	70	0.171	0.297	0.58
	CC-NBS	75	0.142	0.359	0.36
	LRR	66	0.207	0.237	0.86
Mi-1.2 versus *Mi-1.1*	Gene	91	0.048	0.037	1.31
	CC-NBS		0.043	0.029	1.48
	LRR		0.064	0.057	1.11
I2 versus *I2C-2*	Gene	93	0.064	0.091	0.70
	CC-NBS		0.038	0.101	0.37
	LRR		0.085	0.083	1.02
Sw5B versus *Sw5A*	Gene	97	0.024	0.018	1.34
	CC-NBS		0.012	0.009	1.30
	LRR		0.056	0.040	1.41
Gpa2 versus *Rx1*	Gene	88	0.060	0.041	1.46
	CC-NBS		0.041	0.050	0.82
	LRR		0.080	0.032	2.50

[a]Regions analysed are defined in Figure 4.
[b]Percentage amino acid (aa) sequence identity.
[c]The rates of non-synonymous (K_a) and synonymous (K_s) nucleotide substitutions per non-synonymous/synonymous site were calculated using the DIVERGE program in the GCG software package.

the amino acid level, similarity with any known sequence did not exceed 36% sequence identity. The highest BLASTX score was obtained with an NBS-LRR gene derived from rice (*Oryza sativa*; 36.5% amino acid sequence identity). The most homologous NBS-LRR gene derived from *Solanaceae* species is a member of the *I2* locus in tomato, which confers resistance to race 2 of the soil-borne fungus *Fusarium oxysporum* f sp. *lycopsersici* (*I2C-1*: 27% sequence identity; Ori *et al.*, 1997; Simons *et al.*, 1998).

Comparison of the protein sequences encoded by the four members of the *Rpi-blb1* gene cluster on BAC SPB4, revealed that amino acid sequence identity within the *Rpi-blb1* gene cluster varies between 70 and 81%. Within the cluster, Rpi-blb1 shares the highest overall homology with RGA3-blb (81% identity; Table 4). To further deduce sequence variation within the *Rpi-blb1* gene family, additional (susceptible) alleles of the *Rpi-blb1* gene cluster were isolated. The 8005-8-derived BAC clone B149 and two other existing BAC libraries, derived from *S. tuberosum* and *S. tarijense,* respectively, were screened with primers designed around or in the vicinity of the start and stop codons of the *Rpi-blb1* gene. BAC clones containing putative *Rpi-blb1* homologues were further screened with the *Rpi-blb1* flanking markers SPB33L and B149R (Figure 1) to identify those BAC clones that were derived from the same genetic interval as BAC clone SPB4. Putative *Rpi-blb1* homologous PCR fragments derived from BAC clone B149 or from *S. tuberosum* or *S. tarijense* BAC clones for which it was possible to generate a positive PCR signal of the correct size with the primers of markers SPB33L and/or B149R were subsequently cloned and sequenced. In this way, we were able to compare the deduced protein sequences of the *Rpi-blb1* haplotype with the deduced protein sequences of four additional *Rpi-blb1* homologues (RGA_B149-blb, RGA_SH10-tub, RGA_SH20-tub and RGA_T118-tar; Figure 4). Interestingly, two pairs of protein sequences shared a significantly higher degree of similarity than the paralogues of the *Rpi-blb1* haplotype. RGA1-blb and RGA_B149-blb are nearly identical to each other and RGA_SH20-tub and RGA_T118-tar are 96.5% identical, suggesting that they are orthologues and occupy allelic positions. Comparison of the eight sequences identified a total of 51 Rpi-blb1-specific amino acid residues (51/970; 5.25%; Figure 4). The majority are located in the LRR region (36/51; 70%) and are centred on the β-strand/β-turn motif xxLxLxxxx (framed region; Figure 3), which is predicted to encode the putative solvent exposed residues of the repeats (Figure 4). These data are compatible with the idea that the LRR region of R proteins mainly defines ligand specificity.

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Rpi-blb1    MAEAFIQVLLDNLTSFLKGELVLLFGFQDEFQRLSSMFSTIQAVLEDAQEKQLNNKPLEN   60
RGA3-blb            V                                             D        60
SH10-tub             I                  N       I                 D        60
RGA1-blb         L        F IQ   G V   EK  KK      M             KY AIK    60
B149-blb                  F IQ   G V   EK  KK      M             KY AIK    60
SH20-tub              E I   IQ   G  L  END ENI  R                KD AIK    60
T118-tar              E I   IQ   G  L  EN  ENI  R                KD AIK    60
RGA4-blb         L    E     IGDK   I   EK CEK   V        Q       KD AIE    60

Rpi-blb1    WLQKLNAATYEVDDILDEYKTKATRFSQSEYGRYHPKVIPFRHKVGKRMDQVMKKLKAIA  120
RGA3-blb                              L                             N     120
SH10-tub                                 AY                         N     120
RGA1-blb          V A        DC  E A  K AVL     RT T CY      KEM E  D     120
B149-blb          V A        DC  E A  K AVL     RT T CY      KEM E  D     120
SH20-tub            V K   L   C -- A LE  RL CH   A V    I   IKEM E  D     118
T118-tar            A K   L   C -- A LE  RL  H   A V    I   IKEM E  D     118
RGA4-blb          S A       G C NE I  E  RL F   GI N    I R  KEI E  D  S  120

Rpi-blb1    EERKNFHLHEKIVERQAVR--RETG-----------------------------------  143
RGA3-blb            Q   I    AT--    -----------------------------------  143
SH10-tub                I      --    -----------------------------------  143
RGA1-blb       R    D R I    A -- Q  -----------------------------------  143
B149-blb       R    D R I    A -- Q  -----------------------------------  143
SH20-tub    K  TD       I   VA --P   -----------------------------------  141
T118-tar    K  TD       I   VA --P   -----------------------------------  141
RGA4-blb       RK  FL   T    AAAT   VGWQWGWARLEYKRLLLGVLMRIMSLRMHVSTCSTL  180

Rpi-blb1    -----------------SVLTEPQVYGRDKEKDEIVKILINNVSDAQHLSVLpilgmggl  186
RGA3-blb    -----------------                              K              186
SH10-tub    -----------------              E                              186
RGA1-blb    -----------------F     K    E  E            YSEEVP            186
B149-blb    -----------------F     K    E  E            YSEEVP            186
SH20-tub    -----------------F             E            N  E              184
T118-tar    -----------------P             E            N LE              184
RGA4-blb    YEFKFYLCTPKVGARRCF     K       E           NV EE P F  I       240

Rpi-blb1    gkttlaQMVFNDQRVTEHFHSKIWICVSEDFDEKRLIKAIVESIEGRPLLGEMDLAPLQK  246
RGA3-blb         S           R YP       D                 KS S-D          245
SH10-tub                  I                     L   I                     246
RGA1-blb                  I    NL   V   D                 KS G-D          245
B149-blb                  I    NL   V   D                 KS G-D          245
SH20-tub                       YP       D        EN IGN  - SS DVK   SF    243
T118-tar                       YP       D        ET IGN  - SS DVK   SF    243
RGA4-blb            I   E   K  NP   V   D         T IGN  - SSPHVE   SF    299

Rpi-blb1    KLQELLNGkryllvlddvwNEDQQKWANLRAVLKVGASGAsvltttrLEKVGSIMGTLQP  306
RGA3-blb               F           H                F                     305
SH10-tub               F                            F  A                  306
RGA1-blb               F           E  D       I      I I      I        L  305
B149-blb               F           E  D       I      I I      I        L  305
SH20-tub       Q                      D   V                               303
T118-tar       Q                      D   A                               303
RGA4-blb                        D LE   K     T   R   I A                  359

Rpi-blb1    YELSNLSQEDCWLLFMQRAFGHQEEINPNLVAIGKEIVKKSGgvplaaktlggiLCFKRE  366
RGA3-blb           P    F                           C              R      365
SH10-tub                   I C                                     R      366
RGA1-blb     Q             K    C  T TS K ME        C            L R      365
B149-blb     Q             K    C  T TS K ME        C            L R      365
SH20-tub     Q      D      I    R     S                          L R      363
T118-tar     Q      D      I   YR     S                          L R      363
RGA4-blb     H     PH SL         Q K A              C            L R      419

Rpi-blb1    ERAWEHVRDSPIWNLPQDESSILPALRLSYHQLPLDLkqcfaycavfPKDAKMEKEKLIS  426
RGA3-blb                                   H     R   V        T  A  N  A  425
SH10-tub              E      E R           H     R            T           426
RGA1-blb     SE       E        N V         H     R            T I   Y  A  425
B149-blb     SE       E        N V         H     R            T I   Y  A  425
SH20-tub    K E       E        M           H   A R            T    K V    423
T118-tar    K E       E        M    V      H     R            T    K V    423
RGA4-blb     SE      NE  S                 H     R            T  I  N  T  479

Rpi-blb1    LWMAHGFLLSKGNMELEDVGDEVWKELYLRSFFQEIEVKDGKTYFKmhdlihdlATSLFS  486
RGA3-blb    F            L          N             ES                      485
SH10-tub             LE KLQP    N  S   C         A C          H           486
RGA1-blb         S              N   N              S                M     485
B149-blb         S              N   N         G    S                M     485
SH20-tub              RR L     RN G N             RY N                    483
T118-tar              RR L      N   N             RY N                    483
RGA4-blb                 L      N   N            A S N    I               539
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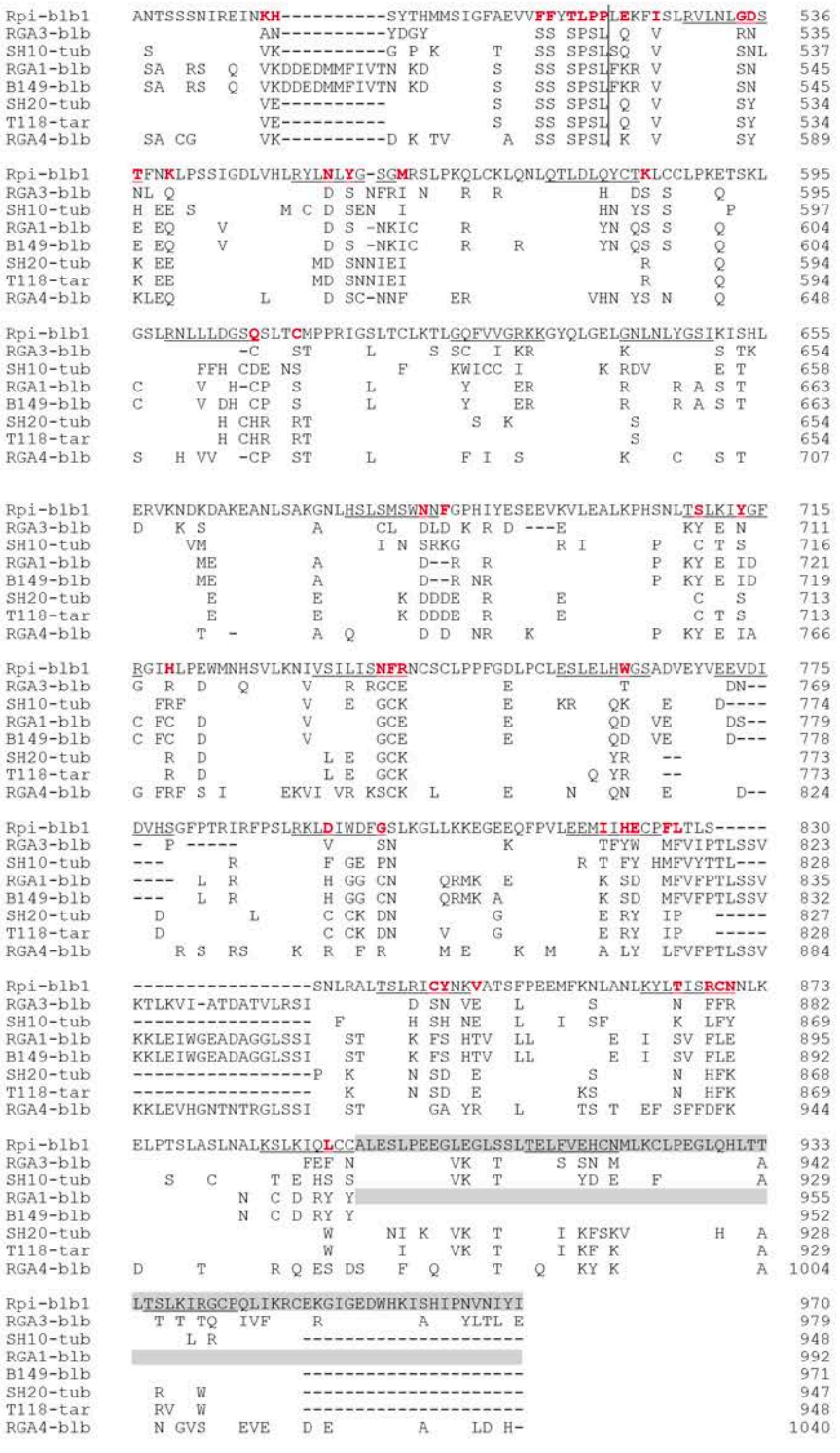
Figure 4. Alignment of the deduced protein products encoded by *Rpi-blb1*, *RGA1-blb*, *RGA3-blb*, *RGA4-blb*, *RGA_B149-blb*, *RGA_SH10-tub*, *RGA_SH20-tub* and RGA_*T118-tar*.
The complete amino acid sequence of Rpi-blb1 is shown and amino acid residues from RGA1-blb, RGA3-blb and RGA4-blb that differ from the corresponding residue in Rpi-blb1. Dashes indicate gaps inserted to maintain optimal alignment. Amino acid residues that are specific for Rpi-blb1, when compared to those at corresponding positions in RGA1-blb, RGA3-blb and RGA4-blb, RGA_B149-blb, RGA_SH10-tub, RGA_SH20-tub and RGA_T118-tar, are highlighted in red. The regions of the LRRs that correspond to the β-strand/β-turn motif xxLxLxxxx are underlined. Conserved motifs in the NBS domain are indicated in lowercase. A vertical line indicates the division between CC-NBS and LRR region. The shaded rectangle indicates the region at the C-termini of Rpi-blb1 and RGA1-blb that are identical.

Evolution of the Rpi-blb1 *locus*

Studies of plant *R* gene sequences involving the comparison of amino acid substitution rates (K_a) to synonymous (K_s) substitution rates has revealed that the LRR region of *R* genes and more specifically the β-strand/β-turn motif is subject to divergent selection (reviewed in Bergelson *et al.*, 2001). Under the assumption that synonymous changes are selectively neutral, values of the ratio K_a/K_s greater than 1 provide strong evidence for positive selection for amino acid substitution. Paired comparisons of synonymous and non-synonymous nucleotide substitutions between

Figure 4. continued



different regions of Rpi-blb1 with those of RGA1-blb, RGA3-blb and RGA4-blb revealed that although rates of amino acid replacement changes are higher in the LRR region, K_a/K_s values for the LRR domain remain smaller than 1. Higher rates of amino acid replacement changes in the LRR region are masked by striking high levels of synonymous divergence (K_s for the entire LRR) among paralogues of 20.1–23.7% (Table 4). For comparison, synonymous divergence in the LRRs of the most related paralogues of the *Solanaceous R* gene clusters *Mi* (Milligan *et al.*, 1998), *I2* (Ori *et al.*, 1997; Simons *et al.*, 1998), *Sw5* (Spassova *et al.*, 2001) and *Gpa2/Rx* (Bendahmane *et al.*, 2000; Van der Vossen *et al.*,

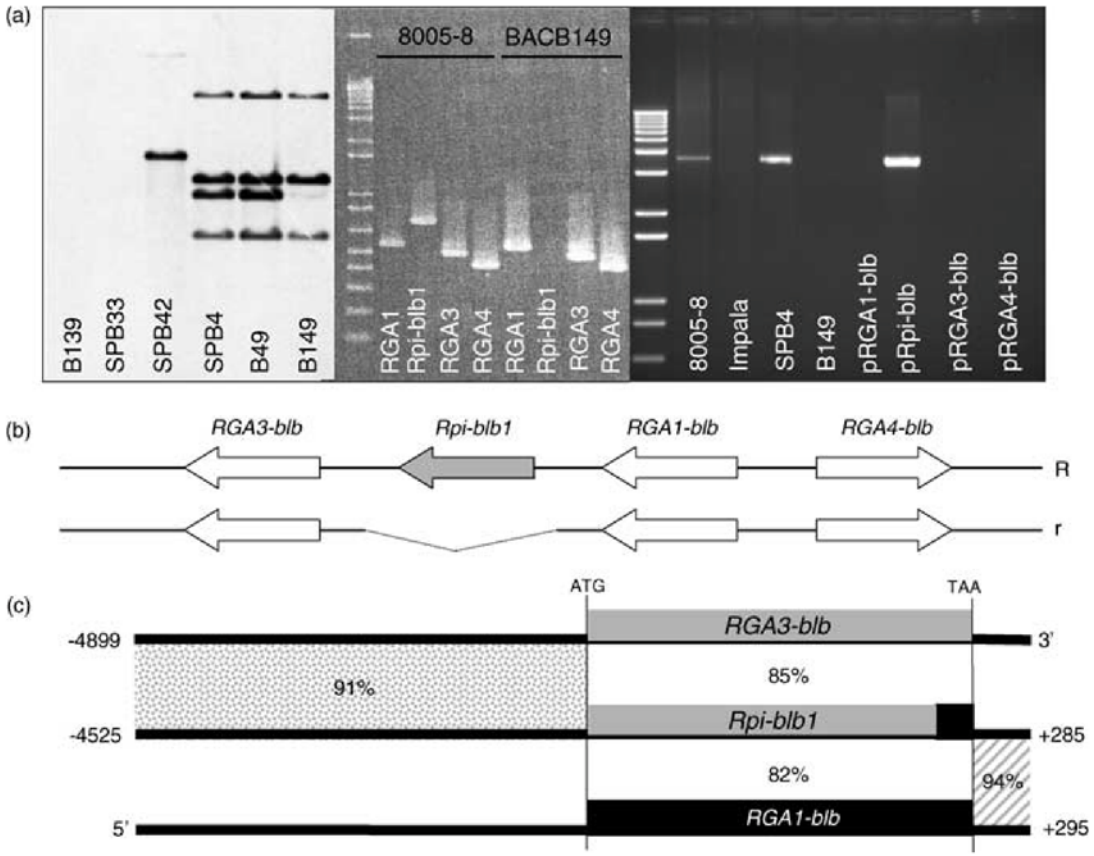


Figure 5. Genomic organisation of *Rpi-blb1* locus.
(a) Southern analysis of BAC DNA (left panel) and PCR analysis (central and right panel) of genomic DNA (8005-8, Impala), BAC DNA (B139, SPB33, SPB42, B49 and B149) or binary plasmid DNA (pRGA1-blb; pRpi-blb1, pRGA3-blb and pRGA4-blb) with gene-specific primer sets (RGA1, Rpi-blb1, RGA3 and RGA4).
(b) Schematic representation of the *Rpi-blb1* (R) and *rpi-blb1* (r) haplotype in the *S. bulbocastanum* genotype 8005-8.
(c) Sequence relationships between the coding and flanking sequences of *Rpi-blb1, RGA1-blb* and *RGA3-blb*. Homologous regions are shown in the same colour. Percentage nucleotide sequence identity is indicated. ATG and TAA indicate the relative positions of the start and stop codons of *Rpi-blb1*, respectively. The length of the homologous 5′ and 3′ flanking sequences are indicated.

2000) ranges between 3.2 and 8.3% (K_s for the LRR region; Table 4). The extraordinary high K_s values calculated for the *Rpi-blb1* gene cluster suggest that these genes are relatively old. Moreover, although amino acid diversity within the LRR region is centred on the xxLxLxxxx motif, the majority of nucleotide differences in this region have apparently not resulted in an amino acid change, leading to K_a/K_s ratio <1 (Table 4). Despite the relatively smaller genealogical antiquity of *Rpi-blb1* and *RGA3-blb*, exemplified by lower synonymous divergence in the CC-NBS region (12.2%) compared to *RGA1-blb* (34.5%) or *RGA4-blb* (35.9%), K_s values calculated for the LRR region are as high as those calculated for the paired comparisons between *Rpi-blb1* and *RGA1-blb* or *RGA4-blb* (Table 4). Altogether, these data suggest that the LRR region of *Rpi-blb1* may well be subject to purifying selection rather than to divergent selection.

Southern analyses of the BAC clones spanning the *Rpi-blb1* interval revealed that BAC SPB4 contained an extra copy of the SPB42L sequence compared to BAC B149 (Figure 5a). The latter BAC clone is derived from the homologous chromosome harbouring the putative susceptible *Rpi-blb1* allele. The finding that the RFLP was specific for BAC clones harbouring the *Rpi-blb1* gene and genetic constructs that complemented the susceptible phenotype in potato and tomato (data not shown), suggested that the *Rpi-blb1* gene was present as an extra copy on the *Rpi-blb1* bearing chromosome. To verify this, we designed several sets of *Rpi-blb1*-specific primers both within and flanking the coding region of *Rpi-blb1* and used these to screen BAC B149. These primer sets were able to amplify fragments of the expected size only from DNA templates containing the *Rpi-blb1* gene and not from B149 (Figure 5a), indicating that *Rpi-blb1* is present as an extra gene in the *Rpi-blb1* haplotype (Figure 5b).

Recombination and sequence exchange between paralogues at complex resistance loci may contribute to the evolution of novel resistance specificities by generating new combinations of polymorphic residues (Parniske *et al.*, 1997). We therefore examined the gene sequences for

shared polymorphisms that may be indicative of sequence exchange. Although alignment of informative polymorphic sites for paralogues of the *Rpi-blb1* haplotype did not reveal a patchwork arrangement of sequence identities, it did reveal an apparent recombination break point at the C-terminus of *Rpi-blb1.* Consistent sequence affiliation was observed throughout the ORFs of *Rpi-blb1* and *RGA3-blb*, except for the C-terminal region of the gene corresponding to the last 75 amino acids. This region of the *Rpi-blb1* gene is identical to the corresponding region of *RGA1-blb* (Figures 4 and 5). Comparison of the 5′ and 3′ sequences flanking the paralogues of the *Rpi-blb1* haplotype reveals sequence affiliation at the 5′-end between *Rpi-blb1* and *RGA3-blb* and at the 3′-end between *Rpi-blb1* and *RGA1-blb* (Figure 5c). The 4522 bp preceding the translation start codon of *Rpi-blb1* are highly homologous (91%) to the 4899 bp upstream of *RGA3-blb*. Sequence homology at the 3′-end is retained between *Rpi-blb1* and *RGA1-blb* until 285 and 295 bp after the stop codon of *Rpi-blb1* and *RGA1-blb*, respectively. The *Rpi-blb1* gene has apparently evolved through intragenic recombination between the ancestral genes of *RGA3-blb* and *RGA1-blb*.

Discussion

Development of disease-resistant cultivars is an effective way to control diseases if sufficient genetic variation for resistance is available. When sources of resistance are limited, breeders look to the secondary gene pool for species that can serve as such and that can be hybridised with cultivated species. However, because of the heterozygous nature of potato, introgression of resistance from wild species through recurrent backcross programmes is very inefficient. Moreover, several wild *Solanum* species displaying high levels of late blight resistance, e.g. *S. bulbocastanum*, are sexually incompatible with cultivated potato. Isolation of the genes responsible for resistance could be a faster means of exploiting potentially durable late blight resistance in wild *Solanum* species. Several resistance genes from species in the Solanaceae, the *Pto* gene of tomato (Rommens *et al.*, 1995), the *N* gene of tobacco (Whitham *et al.*, 1994), the *Cf-9* gene of tomato (Hammond-Kosack *et al.*, 1998) and the *Bs2* gene of pepper (Tai *et al.*, 1999), have successfully been transferred as transgenes to other *Solanaceous* species. In cloning the *Rpi-blb1* gene, we demonstrate the efficient interspecific transfer of broad-spectrum late blight resistance from *S. bulbocastanum* to cultivated potato and tomato.

The *Rpi-blb1* resistance locus was mapped in an intraspecific *S. bulbocastanum* population to the same genetic location on chromosome 8 as the previously reported late blight resistance locus *RB* (Naess *et al.*, 2000). Physical mapping of the *Rpi-blb1* locus identified a BAC clone comprising a tightly linked cluster of four *R* gene homologous sequences of the CC-NBS-LRR class of plant *R* genes. Complementation analysis showed that one of these candidate genes, *RGA2-blb*, was able to facilitate a typical HR in the otherwise susceptible cultivar Impala in more than 80% of the primary transformants carrying an intact genetic construct. This *RGA* was therefore designated the *Rpi-blb1* gene. Interestingly, the coding sequence of *Rpi-blb1* is identical to that of the *RB* gene, which has also recently been cloned (Song *et al.*, 2003). This suggests that *Rpi-blb1* and *RB* are identical genes. However, when comparing flanking sequences and the intron of these two genes, single nucleotide polymorphisms or single nucleotide insertions can be found at a frequency ranging between 0.4 and 0.8%. We therefore conclude that *Rpi-blb1* and *RB* are allelic, although functionally equivalent.

Significant DNA homology (81–90%) between *Rpi-blb1* and partial NBS sequences from tomato, which have been mapped to loci on chromosomes 2, 8 and 9 of tomato (Pan *et al.*, 2000), indicates that *Rpi-blb1* is probably a member of a Solanaceae NBS-LRR gene family, which apparently is not restricted to the *Rpi-blb1* gene cluster on chromosome 8. Map positions of *Rpi-blb1* homologous sequences on chromosomes 2 and 9 of tomato correspond to the resistance loci *Tm-1* and *Tm-2*, respectively, that encode resistance to tomato mosaic virus (Levesque *et al.*, 1990; Pan *et al.*, 2000; Young *et al.*, 1988). This suggests that *Rpi-blb1* gene family members also function against taxa of plant pathogens other than oomycetes. Interestingly, quantitative trait loci (QTL) associated with resistance to *P. infestans* (Oberhagemann *et al.*, 1999) and *Erwinia carotovora* ssp. *atroseptica* (Zimnoch-Guzwska *et al.*, 2000) also correspond to the *Rpi-blb1* homologous gene cluster on chromosome 2.

As *R* genes of the NBS-LRR class lack predicted transmembrane segments or signal peptides, perception of cognate *Avr* products probably occurs in the cytoplasm. The most straightforward interpretation of *R* gene-mediated elicitor recognition invokes a direct intermolecular interaction. However, experimental evidence for direct binding is limited (Jia *et al.*, 2000). This suggests that either the interaction is transient or the elicitor interacts indirectly with the *R* gene product through a virulence target (Banerjee *et al.*, 2001; Dangl and Jones, 2001; Kooman-Gersmann *et al.*, 1996; Leister and Katagiri, 2000; Mackey *et al.*, 2002; Van der Biezen and Jones, 1998). According to the 'guard model' (Van der Biezen and Jones, 1998), resistance is triggered when the R protein detects modifications to its guardee, which is presumably targeted and modified by the Avr factor to create a favourable environment for the pathogen. Accordingly, R proteins that guard virulence targets are more likely to be maintained in the plant population than R proteins that rely on a direct physical interaction between R protein and Avr factor (Van der Hoorn *et al.*, 2002). In this respect, it is tempting to speculate on the age of the *Rpi-blb1* gene cluster and on the mode of

action of the Rpi-blb1 protein. The extraordinary high levels of synonymous divergence among paralogues of the *Rpi-blb1* haplotype reflects longevity of the *Rpi-blb1* gene cluster and thereby the possible occurrence of balancing selection at this locus in natural *S. bulbocastanum* populations. This would implicate a high relative contribution of the matching *Avr* gene to the virulence of *P. infestans*. This is in line with the broad spectrum of the resistance conferred by *Rpi-blb1* and the observation that amino acid replacement changes have not accumulated faster than synonymous changes in those regions of the gene that, in well-studied *R* genes, have been pinpointed as the major determinants of recognition specificity for Avr factors (Ellis *et al.*, 2000).

Evolution of new pathogen specificity has been traced to shifts in solvent-exposed LRR residues that are caused by single-base changes, insertion or deletion events, and by unequal-exchange meiotic recombination events within *R* genes or between closely linked homologous *R* genes in a cluster (Ellis *et al.*, 2000). Comparison of the Rpi-blb1 protein sequence with those of seven other members of the Rpi-blb1 protein family identified, in addition to various indels, a total of 51 Rpi-blb1-specific amino acid residues, the majority of which (70%) are located in the LRR region. Targeted or random mutagenesis of *Rpi-blb1* is necessary to determine which of these residues are essential for recognitional and/or signalling capacity of the Rpi-blb1 protein. Recent structure–function analysis of the CC-NBS-LRR protein encoded by *Rx* implies that conserved motifs in the NBS and LRR regions of Rx regulate a signalling domain in the amino-terminal region possibly through intramolecular interactions (Bendahmane *et al.*, 2002). Interestingly, one of the Rpi-blb1-specific amino acid residues is situated at the same position as an Rx gain-of-function mutation close to the RNBS-D (CFLY) motif that is conserved in R proteins (Meyers *et al.*, 1999).

The majority of *R* genes are members of tightly linked multigene families. This is thought to facilitate the generation of novel *R* gene specificities through re-assortment of sequence polymorphism by (un)equal inter- and/or intragenic meiotic recombination between family members resulting in duplication, partial deletion or formation of new chimaeric genes. Comparative sequence analysis of several *R* gene haplotypes supports this idea (reviewed in Hulbert *et al.*, 2001). The two homologous chromosomes of *S. bulbocastanum* 8005-8 contain different numbers of RGAs, the *Rpi-blb1* gene being present as an extra copy in the *Rpi-blb1* haplotype. Shared polymorphisms between *Rpi-blb1* and the two flanking paralogues *RGA3-blb* and *RGA1-blb* indicate that *Rpi-blb1* is likely to have evolved through unequal intragenic meiotic recombination between ancestral genes of *RGA3-blb* and *RGA1-blb*. The presence of a truncated RGA between *RGA3-blb* and *Rpi-blb1* suggests that additional unequal recombinational events have occurred in the evolution of the *Rpi-blb1* gene cluster.

The cloning of *Rpi-blb1* has paved the way to develop late blight-resistant potato and tomato varieties through a transgene approach. A drawback to the use of *R* genes to obtain resistance in crop plants is that their effects often appear not to be durable because of shifts in *Avr* gene frequencies in pathogen populations. Indirect evidence that *Rpi-blb1*-mediated resistance might be stable is the observation that it confers full resistance to a range of *P. infestans* isolates carrying multiple virulence factors. Race specificity has also not been observed with breeding material containing the *RB* locus. Somatic hybrids of *S. bulbocastanum* and *S. tuberosum* and backcrossed germplasm were found to be highly resistant to late blight, even under extreme disease pressure in repeated trials (Helgeson *et al.*, 1998). One reason *Rpi-blb1* may remain effective is if the loss of the corresponding *Avr* gene from the pathogen has a significant cost in terms of fitness or pathogenicity (Leach *et al.*, 2001). An example of this is the isolated *Bs2* gene from pepper (Tai *et al.*, 1999), whose cognate *Avr* gene that codes for a virulence factor is widespread in *Xanthomonas campestris* pathovars (Kearney and Staskawicz, 1990). The possible occurrence of balancing selection at the *Rpi-blb1* locus may however imply the existence of resistance-breaking isolates. Cloning of *Avr-blb1* and subsequent analysis of its frequency and variation within *Phytophthora* species will help in predicting the stability of the *Rpi-blb1*-derived resistance in crop plants. However, to date, no *Avr* gene from *Phytophthora* has been cloned. Another way to increase the stability of late blight resistance is by combining *R* genes that actually require the pathogen to lose or mutate several *Avr* genes simultaneously. This would be especially important when introducing *R* genes that, in natural populations, are under balancing selection. Manipulation of *R* genes as transgenes facilitates the pyramiding of *R* genes at a single linkage block in the genome. This could promote durability of the complex by preventing the single deployment of the component genes in other cultivars by various breeding programmes.

Experimental procedures

Disease assay

The phenotypes of *S. bulbocastanum*, *S. tuberosum* and tomato genotypes for resistance to *P. infestans* were determined by detached leaf assays (El-Kharbotly *et al.*, 1994). Each leaf was inoculated with two droplets (25 μl each) of sporangiospore solution (30 000–50 000 sporangiospores per millilitre) on the abaxial side. After 6 days, the leaves were evaluated for the development of *P. infestans* disease symptoms. Plants with leaves that clearly showed sporulating lesions 6 days after inoculation were considered to have a susceptible phenotype, whereas plants with leaves showing no visible symptoms or necrosis at the site of inoculation

in the absence of clear sporulation were considered to be resistant. Plants initially displaying unclear phenotypes could, in nearly all cases, be classified as resistant or susceptible after a second test. The assays were performed with *P. infestans* isolates mentioned in Table 1.

Plant DNA marker screening

Genomic DNA was extracted from young leaves according to Bendahmane *et al.* (1997). For PCR analysis, 15-μl reaction mixtures were prepared containing 0.5 μg DNA, 15 ng of each primer, 0.2 mM of each dNTP, 0.6 units Taq polymerase (15 U μl^{-1}, SphaeroQ, Leiden, the Netherlands), 10 mM Tris–HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatin. The PCRs were performed using the following cycle profile: 25-sec DNA denaturation at 94°C, 30-sec annealing and 40-sec elongation at 72°C. As a first step in PCR amplification, DNA was denatured for 5 min at 94°C and finalised by an extra 5-min elongation step at 72°C. The amplification reactions were performed in a Biometra® T-Gradient or Biometra® Uno-II thermocycler (Westburg, Leusden, the Netherlands). Depending on the marker, the PCR product was digested with an appropriate restriction enzyme to generate polymorphisms. An overview of the markers including primer sequences, annealing temperature and restriction enzymes is given in Table 2. Subsequently, the (digested) PCR products were analysed by electrophoresis in agarose or acrylamide gels.

BAC library construction and screening

A resistant clone of *S. bulbocastanum* (blb) BGRC accession 8005, heterozygous for the *Rpi-blb1* locus, was used as source DNA for the construction of the 8005-8 BAC library. High-molecular weight DNA preparation and BAC library construction were carried out as described by Rouppe van der Voort *et al.* (1999). Approximately 130 000 clones with an average insert size of 100 kbp, which corresponds to 15 genome equivalents, were obtained. A total of approximately 83 000 clones were individually stored in 216 384-well microtitre plates at −80°C. Another 50 000 clones were stored as bacterial pools containing approximately 1000 white colonies. These were generated by scraping the colonies from the agar plates into LB medium containing 18% glycerol and 12.5 μg ml^{-1} chloramphenicol using a sterile glass spreader. These so-called super pools were also stored at −80°C. Marker screening of the BAC library harbouring the individually stored BAC clones was carried out as described by Rouppe van der Voort *et al.* (1999). For the screening of the BAC library stored as 50 pools of approximately 1000 clones, plasmid DNA was isolated from each pool of clones using the standard alkaline lysis protocol and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plates containing chloramphenicol (12.5 μg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones subsequently identified as described above. Names of BAC clones isolated from the super pools carry the prefix SP (e.g. SPB33).

DNA sequencing and computer analysis

The DNA sequence of BAC clone SPB4 was determined by shotgun sequence analysis as described by Van der Vossen *et al.* (2000). The automated assembly of the shotgun reads was carried out using the PHRED–PHRAP programs (Ewing and Green, 1998; Ewing *et al.*, 1998). A total of 835 reads provided an overall BAC sequence coverage equal to 5x. Gaps between contigs were closed by primer walking or through a combinatorial PCR approach. The sequence was finally edited at PHRED quality 40 (1 error every 10 000 nt) by manual inspection of the assembly using the Gap4 contig editor and re-sequencing of all low-quality regions. The complete sequence of the insert of BAC SPB4 consisted of 77 283 nt. Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997) and GENEMARK (Lukashin and Borodovsky, 1998). Homology searches were carried out using the BLAST program (Altschul *et al.*, 1990). DNAstar (Lasergene, Madison, WI, USA) and Genetics Computer Group (Madison, WI, USA) software packages were used for sequence alignments and sequence comparisons. Nucleotide substitution rates were calculated using the DIVERGE program in the Genetics Computer Group software package.

Subcloning of candidate genes

Candidate RGAs were subcloned from BAC clone SPB4 as follows. Aliquots of approximately 1 μg BAC DNA were digested with 1, 0.1 or 0.01 U of *Sau3*AI restriction enzyme for 30 min. The partially digested BAC DNA was subjected to CHEF electrophoresis at 4°C in 0.5× TBE using a linear increasing pulse time of 1–10 sec and a field strength of 6 V cm^{-1} for 16 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, USA) according to the manufacturer. The size-selected DNA was ligated to the *Bam*HI-digested and dephosphorylated binary vector pBINPLUS (Van Engelen *et al.*, 1995) followed by transformation to ElectroMAX *E. coli* DH10B competent cells (Life Technologies, UK). A total of 480 clones were PCR screened for the presence of RGA sequences using primers SPB42LF and SPB42LR or RGA4F and RGA4R (Table 2). Positive clones were selected for further characterisation. Identification of clones harbouring *RGA1-blb, RGA2-blb, RGA3-blb* or *RGA4-blb* was carried out by sequencing the SPB42L PCR fragments derived from positive clones. The relative position of the RGAs within a subclone was determined by sequencing the ends of the clone and subsequent comparison of the sequences to the complete BAC insert sequence.

Agrobacterium tumefaciens-mediated transformation of potato and tomato

Binary plasmids harbouring the candidate genes were transformed to *A. tumefaciens* strains LBA4404 (Hoekema *et al.*, 1983), AGL0 (Lazo *et al.*, 1991) or UIA143 (Farrand *et al.*, 1989), the latter containing the helper plasmid pCH32 (Hamilton *et al.*, 1996). Overnight cultures of the transformed *A. tumefaciens* strains were used to transform potato tuber discs (cv. Impala) or tomato leaf discs (cv. Moneymaker) according to standard protocols (Fillati *et al.*, 1987; Hoekema *et al.*, 1989). Three plants per transformed regenerant were transferred to the greenhouse.

Rapid amplification of amplification of cDNA ends (RACE)

The 5′- and 3′-ends of the *Rpi-blb1* cDNA were determined by RACE using the GeneRacer™ kit (Invitrogen™, the Netherlands). 3′

RACE was carried out with the nested primers GSP1 (5′-GAGGAATCCATCTCCCAGAG) and GSP2 (5′-GTGCTTGAAGAGATGATAATTCACGAG) in combination with the GeneRacer™ 3′ primer and GeneRacer™ 3′ nested primer, respectively. 5′ RACE was carried out on cDNA synthesised with the primer GSP3 (5′-GTCCATCTCACCAAGTAGTGG) using primers GSP4 (5′-GAAATGCTCAGTAACTCTCTGG) and GSP5 (5′-GGAGGACTGAAAGGTGTTGG) in combination with the GeneRacer™ 5′ primer and GeneRacer™ 5′ nested primer, respectively.

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The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato

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Summary

The necessity to develop potato and tomato crops that possess durable resistance against the oomycete pathogen *Phytophthora infestans* is increasing as more virulent, crop-specialized and pesticide resistant strains of the pathogen are rapidly emerging. Here, we describe the positional cloning of the *Solanum bulbocastanum*-derived *Rpi-blb2* gene, which even when present in a potato background confers broad-spectrum late blight resistance. The *Rpi-blb2* locus was initially mapped in several tetraploid backcross populations, derived from highly resistant complex interspecific hybrids designated ABPT (an acronym of the four *Solanum* species involved: *S. acaule, S. bulbocastanum, S. phureja* and *S. tuberosum*), to the same region on chromosome 6 as the *Mi-1* gene from tomato, which confers resistance to nematodes, aphids and white flies. Due to suppression of recombination in the tetraploid material, fine mapping was carried out in a diploid intraspecific *S. bulbocastanum* F1 population. Bacterial artificial chromosome (BAC) libraries, generated from a diploid ABPT-derived clone and from the resistant *S. bulbocastanum* parent clone, were screened with markers linked to resistance in order to generate a physical map of the *Rpi-blb2* locus. Molecular analyses of both ABPT- and *S. bulbocastanum*–derived BAC clones spanning the *Rpi-blb2* locus showed it to harbor at least 15 *Mi-1* gene homologs (MiGHs). Of these, five were genetically determined to be candidates for *Rpi-blb2*. Complementation analyses showed that one ABPT- and one *S. bulbocastanum*-derived MiGH were able to complement the susceptible phenotype in both *S. tuberosum* and tomato. Sequence analyses of both genes showed them to be identical. The Rpi-blb2 protein shares 82% sequence identity to the Mi-1 protein. Significant expansion of the *Rpi-blb2* locus compared to the *Mi-1* locus indicates that intrachromosomal recombination or unequal crossing over has played an important role in the evolution of the *Rpi-blb2* locus. The contrasting evolutionary dynamics of the *Rpi-blb2/Mi-1* loci in the two related genomes may reflect the opposite evolutionary potentials of the interacting pathogens.

Keywords: ***Rpi-blb2*, disease resistance, *Phytophthora infestans*, *Solanum bulbocastanum*, potato, late blight.**

Introduction

The oomycete *Phytophthora infestans*, the causal agent of late blight, remains the most important pathogen in major potato producing regions of the world. It has a potential to devastate crops in a couple of weeks if meteorological conditions are conducive to the onset and spread of an epidemic. To a lesser extent, late blight is also problematic in tomato cultivation (Fry and Goodwin, 1997). Disease management is currently based on the application of fungicides. Frequent seasonal spraying with fungicides imposes high input costs to the farmer, is detrimental to the environment and imposes a pressure on the pathogen for developing resistance to the active ingredients of the crop protectants applied. During the 20th century, breeding of new potato cultivars with high levels of durable resistance to

P. infestans has been considered an alternative to the use of fungicides. Breeders have introgressed from the wild potato species *Solanum demissum* the dominant resistance (*R*) genes *R1*, *R2*, *R3*, *R4* and *R10* up to cultivar level, but races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen, 1993). By the end of the 1950s, most breeders had switched to the use of sources of germplasm with partial/quantitative resistance (Hawkes, 1978). The underlying paradigm was that this type of resistance is *R* gene independent and assumed to be of polygenic nature, which could make it more durable than resistance based on monogenic inherited *R* genes (Turkensteen, 1993). However, partial resistance is day-length dependent and is strongly correlated with late maturity under long-day conditions (Howard, 1970). Also, erosion of general resistance has been reported in case studies (Flier *et al.*, 2003). Genetic studies using molecular markers have shown that general resistance is often based on two or three major quantitative trait loci (QTL), the genome positions of which often correspond to loci harboring *R* gene clusters (Gebhardt and Valkonen, 2001; Grube *et al.*, 2000). The goal of some breeders now is to identify additional major *R* genes conferring broad-spectrum resistance to late blight and to combine these in modern potato varieties. In contrast to introgression breeding, isolation of such genes from *Solanum* species and their stable transformation as *cis* genes into existing potato or tomato varieties is by far the fastest means of exploiting potentially durable late blight resistance present in the *Solanum* gene pool.

Identification of numerous functional *R* genes from model and crop species has revealed that the majority of these genes encode cytoplasmic proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains and that they often belong to complex loci comprised of arrays of related genes (reviewed in Martin *et al.*, 2003). This structure–function relationship has led to the development of homology-based approaches aimed at the identification of structurally related sequences, termed *R* gene homologs (RGHs), from many host species including *Solanaceae* (Leister *et al.*, 1996; van der Linden *et al.*, 2004; Pan *et al.*, 2000). In a comparative study of genomic organization of *R* genes and RGHs in three solanaceous crop genera, tomato, potato and pepper, Grube *et al.* (2000) observed significant conservation of *R* gene loci, despite limited positional correspondence of phenotypically defined genes conferring resistance to related or identical pathogens. This suggests that the chromosomal locations of *R* gene clusters may be quite broadly conserved through speciation and that comparative genomics can be instrumental for the rapid identification of genes similar to those already mapped in related genera.

Seven functional *R* genes from potato have currently been cloned and all belong to the NBS-LRR class of plant *R* genes (Ballvora *et al.*, 2002; Bendahmane *et al.*, 1999, 2000; Huang *et al.*, 2005; Paal *et al.*, 2004; Song *et al.*, 2003; van der Vossen *et al.*, 2000, 2003). Three of these confer resistance to late blight. The *Solanum demissum*-derived genes *R1* and *R3a* on chromosomes 5 and 11, respectively, confer race-specific resistance whereas the identical genes *Rpi-blb1* and *RB*, further referred to as *Rpi-blb1*, on chromosome 8 from the wild potato species *Solanum bulbocastanum* confer high levels of resistance to a range of *P. infestans* isolates with complex race structures (Helgeson *et al.*, 1998; Song *et al.*, 2003; van der Vossen *et al.*, 2003). *Solanum bulbocastanum* from Mexico and Guatemala is a diploid tuber bearing species that has long been known for its high levels of resistance to late blight (Niederhauser and Mills, 1953). Transfer of its resistance to the gene pool of cultivated potato has been successful by carrying out a tedious and time-consuming breeding scheme involving ploidy manipulations and a series of bridge crosses. The resulting *P. infestans* resistant interspecific hybrids were designated ABPT, an acronym of the four *Solanum* species involved: *S. acaule, S. bulbocastanum, Solanum phureja* and *Solanum tuberosum* (Figure S1) (Hermsen and Ramanna, 1973). Over 20 years of resistance phenotyping of ABPT-derived germplasm under diverse conditions suggested the presence of broad-spectrum late blight resistance, prompting us to analyze its genetic basis. Here, we describe the genetic mapping and cloning of a second late blight *R* gene derived from *S. bulbocastanum*. The *Rpi-blb2* gene was initially mapped in several ABPT-derived tetraploid backcross populations to the same region on chromosome 6 as the *Mi-1* gene from tomato. Subsequently, *Mi-1* gene homolog (MiGH) specific markers were developed to aid in the fine mapping of *Rpi-blb2* in a diploid intraspecific *S. bulbocastanum* F1 population and in the identification and isolation of candidate genes on bacterial artificial chromosome (BAC) clones spanning the locus. The *Rpi-blb2* gene is able to complement the susceptible phenotype in both cultivated potato and tomato. The Rpi-blb2 protein shares 82% sequence identity with the Mi-1 protein, which in tomato confers resistance to three species of root knot nematodes (*Meloidogyne* spp.) as well as to the potato aphid *Macrosiphum euphorbiae* (Milligan *et al.*, 1998; Rossi *et al.*, 1998; Vos *et al.*, 1998) and to both B and Q biotypes of whitefly *Bemisia tabaci* (Nombela *et al.*, 2003).

Results

Evaluation of resistance in ABPT-derived backcross clones and mapping populations

Late blight resistance in the ABPT-derived BC2 clone ARF 87-601 was initially evaluated in a field trial in the Toluca area in Mexico in 1991. A plot of clone ARF 87-601 with seven

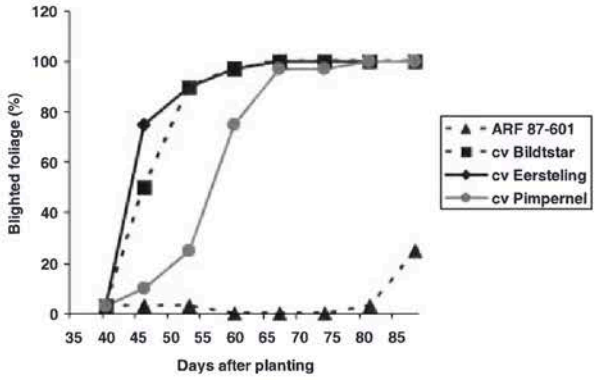


Figure 1. Disease progress curves for clone ARF 87-601 and susceptible control cultivars (cv) Bildtstar, Eersteling and the partial resistant control cultivar Pimpernel in a field test for foliar resistance to late blight in Toluca Valley, Mexico.
At eight time points after planting, the percentage blighted foliage due to a natural late blight infection was scored on the 1 to 9 International Potato Center (CIP) scale (Estrada-Ramos *et al.*, 1983).

plants was evaluated in comparison to plots with control cultivars Bildtstar, Eersteling and Pimpernel, which according to the ratings for resistance to late blight in the Dutch national list of recommended potato cultivars of 1988, scored 3, 3 and 8 respectively on a scale from 3 to 8 of increasing resistance. Cultivar Pimpernel is considered as a source of partial resistance (Colon *et al.*, 1985). About forty days after planting, natural infection by *P. infestans* established. The development of late blight in the foliage was subsequently monitored eight times during the growing season (Figure 1). At seventy four days after planting, foliage of the control cultivars was completely or nearly completely blighted, whereas clone ARF 87-601 showed no visible symptoms.

For the development of mapping populations, clones ARF 87-601, ARF 87-507 and ARF 87-801 were subsequently used for further backcrossing with late blight susceptible cultivars and breeding clones of *S. tuberosum*. This resulted in one tetraploid BC3 population (ARG 95-15), two tetraploid BC4 populations (ARG 95-3 and ARP 96-11) and a diploid BC4 population (DP1; Figure S2). Clear segregation for late blight resistance in ABPT-derived progeny and mapping populations was observed during successive years of field testing in the Netherlands, suggesting the presence of a dominant resistance allele at a single locus. Typically, resistant clones showed no or hardly any sporulating lesions, whereas susceptible clones showed completely blighted foliage. Notably, detached leaf assays with ABPT-derived progeny were found to be less accurate for phenotyping than screening under field conditions. Approximately 17% of the resistant haplotype plants and 3% of the susceptible haplotype plants were miscored as susceptible or resistant, respectively, in the detached leaf assays.

Mapping of the Rpi-blb2 locus to the Mi-1 gene cluster on chromosome 6

To identify Amplified Fragment Length Polymorphism (AFLP) markers linked to resistance, a bulked segregant analysis (BSA; Michelmore *et al.*, 1991) was carried out with 160 primer combinations on two resistant and two susceptible DNA pools, each containing genomic DNA of eight resistant or susceptible ARG 95-15 progeny plants, respectively. This resulted in the identification of 58 AFLP markers potentially linked to resistance. When a number of these markers were tested on progeny plants of ARG 95-3, linkage to resistance was also observed in this population, suggesting that in both populations the resistance was determined by the same locus, which we designated *Rpi-blb2*.

To determine the position of the *Rpi-blb2* locus on the genetic map of potato, two cosegregating AFLP markers, E40M58 and E46M52, were developed into cleaved amplified polymorphic sequence (CAPS) markers. Cleaved amplified polymorphic sequence marker E40M58 was subsequently tested on 46 progeny plants of the reference mapping population CxE (van Eck *et al.*, 1995). By applying JOINMAP linkage analyses (Stam, 1993), we mapped E40M58 8 cM distal to the chromosome 6 specific marker GP79 (Gebhardt *et al.*, 1991), positioning *Rpi-blb2* on the short arm of chromosome 6. This was further confirmed through the conversion of the chromosome 6 specific Restriction Fragment Length Polymorphism (RFLP) markers CT119 and CT216 into CAPS markers, which cosegregated with resistance in all four mapping populations.

Subsequently, for recombinant screening purposes, the cloned AFLP markers E46M52 and E40M58 were extended by thermal asymmetric interlaced (TAIL) Polymerase Chain Reaction (PCR) on genomic DNA of AR 91-1263 and ARD 1197-16, respectively, and converted into the sequence SCAR markers E46M52e and E40M58e. E46M52e was polymorphic in all the mapping populations, whereas E40M58e was amplified only in ARG 95-3 and DP1. As the parental clones of the different mapping populations were derived from different ABPT clones, we extended the AFLP fragment a second time but now from AR 91-1292, the resistant parental clone of ARP 96-11, resulting in E40M58e2. Sequence alignment of the two extended fragments showed that only the first 37 bp of the extended fragments were identical. When both extended markers were tested on genomic DNA derived from *S. bulbocastanum* (BGRC 8005 and 8006), only E40M58e amplified a fragment, indicating that part of the sequence of E40M58e2 was not derived from *S. bulbocastanum*. This observation suggested that E40M58e was located on the border of the *S. bulbocastanum* introgression fragment in clone AR 91-1292 and that the position of the *Rpi-blb2* locus was proximal to marker E40M58e.

In an attempt to identify functionally relevant markers linked to resistance, an RGH-fingerprinting technique was

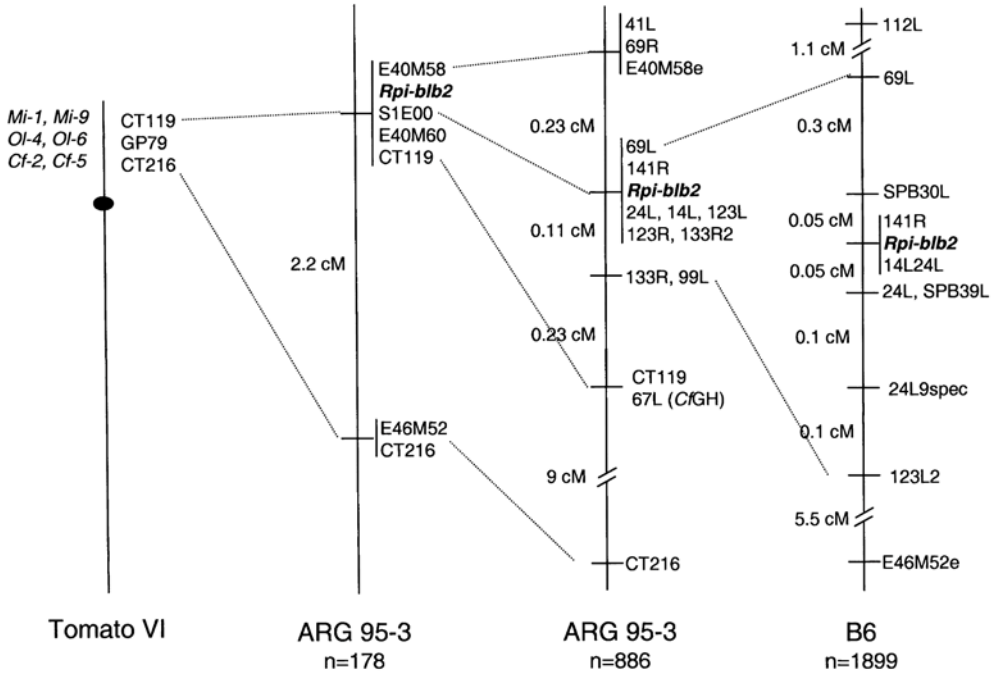


Figure 2. Genetic linkage maps of the *Rpi-blb2* locus generated in two mapping populations, ARG95-3 and B6.
Long vertical lines represent either chromosome 6 of tomato or short stretches of the distal part of chromosome 6 of potato harboring the *Rpi-blb2* locus. Indicated to the left is a schematic representation of tomato chromosome 6, showing the relative positions of resistance loci and markers identified on the short arm of this chromosome. Numbers left of the remaining vertical lines indicate genetic distances (cM). Relative positions of mapped loci are indicated by horizontal lines. Names of the mapped loci are indicated to the right of the horizontal lines. Vertical lines connected to the right side of the horizontal lines group loci that cosegregate. The size of each mapping population is indicated at the bottom of each map.

applied. Using the P- loop based primer S1 from Leister *et al.* (1996) in combination with the Eco00 AFLP primer, an RGH specific marker, S1E00 was identified that cosegregated with resistance and markers E40M58e and CT119 in the ARG 95-3 mapping population (Figure 2). Cloning and sequencing of the 75-bp-long S1E00 fragment did not reveal homology to St3313, an RGH sequence previously mapped to the short arm of chromosome 6 in potato (Leister *et al.*, 1996), but rather to the *Mi-1* gene from tomato.

Physical mapping of the ABPT-derived Rpi-blb2 locus

The diploid clone ARD 1197-16, heterozygous for the *Rpi-blb2* locus, was used as source DNA for the construction of a BAC library (hereafter, referred to as the 1197-16 BAC library), consisting of 104.832 clones with an average insert size of 100 kb (approximately 12 genome equivalents). In order to build a BAC contig across the *Rpi-blb2* locus, the 1197-16 BAC library was initially screened with markers E40M58e, S1E00 and CT119, which are all linked in coupling phase to resistance. E40M58e identified BAC clones 69 and 141, S1E00 identified BAC clones 14, 24, 123 and 133 and CT119 identified BAC clone 67. Sequencing of the left (L) and right (R) borders of these BAC clones led to the development of markers 69R, 69L, 141R, 24L, 14L, 24R, 123L, 123R, 133R and 67L, all of which were linked in coupling phase to resistance. Screening of the identified BAC clones with these BAC-end specific markers indicated that the S1E00 positive BAC clones in fact did not all overlap with each other, implying that S1E00 was a repetitive sequence. This together with the finding that the sequences of 24L and 123R were highly homologous to various regions of the *Mi-1* gene indicated that the *Rpi-blb2* locus harbored a cluster of Mi-GHs. Subsequent screening of the 1197-16 BAC library with the BAC-end specific markers 69R, 24L, 24R, 123L and 133R identified BAC clones 36, 211, 242, 191 and 99 (Figure 3), leading to the development of markers 36L and 99L. Finally, the BAC contig comprising BAC clones 141, 69 and 36 was distally extended with BAC clones 41 and 112, leading to the development of markers 41L and 112L (Figure 3).

Fine mapping of the ABPT-derived *Rpi-blb2* locus was carried out on 2283 ARG 95-3 and 568 ARP 96-11 clones, which were tested in a field trial for resistance to late blight. Finally, only 886 ARG 95-3 and 170 ARP 96-11 clones with a clear resistant phenotype were selected for recombinant screening with SCAR marker CT216 and CAPS marker 41L or 36L, respectively. In total, 85 (9.6 cM) and 22 (12.9 cM) recombinants were obtained in mapping populations ARG 95-3 and ARP 96-11, respectively, three of which positioned the *Rpi-blb2* locus between markers E40M58e and 133R (Figure 2). Interestingly, the left (L) BAC-end sequence of BAC clone 67, which was identified with marker CT119 and shares

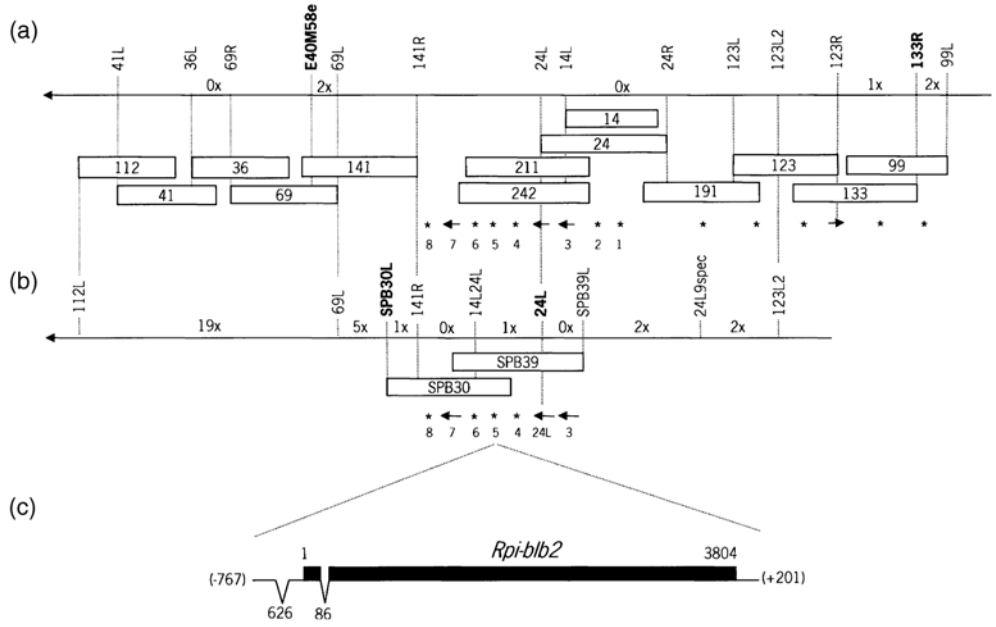


Figure 3. Genetic and physical strategy used to clone the *Rpi-blb2* gene.
High-resolution genetic and physical maps of the *Rpi-blb2* locus in (a) the ABPT-derived plant material and (b) the *S. bulbocastanum*-derived material. Vertical spotted lines indicate the relative positions of markers linked to resistance. Numbers above the horizontal lines are the numbers of recombinants identified between the flanking markers in 886 ARG95-3 and 1899 B6 progeny plants, respectively. Rectangles represent bacterial artificial chromosome (BAC) clones. Relative positions of *Mi-1* gene homologs (MiGHs) are indicated by asterisks and horizontal arrows, the latter indicating the relative orientations there where known.
(c) Schematic representation of the *Rpi-blb2* gene structure. Horizontal lines represent exons. Black rectangles represent open reading frames. Lines angled downwards indicate the positions of introns. Numbers between parentheses indicate the length of the 5′- and 3′- untranslated regions (UTRs) of the putative *Rpi-blb2* transcript.

significant DNA sequence homology (94%) to *Cf-5* from tomato (Dixon *et al.*, 1998), was mapped 0.33 and 1.76 cM proximal to *Rpi-blb2* in ARG 95-3 and ARP 96-11, respectively.

To estimate the number of MiGHs present in the above defined *Rpi-blb2* interval, BAC clones spanning the interval were screened by Southern analysis using markers 123R, 14L, or 24L as probes. These probes share high homology to different regions of the *Mi-1* gene. The results indicated that the BAC contig comprising BAC clones 211, 24, 191, 123 and 133 contained at least approximately 12 MiGHs. Additional MiGH specific markers were developed by cloning and sequencing PCR fragments generated from BAC clones 24 and 123 with the primer combination 14LR and 24LF. Based on the alignment of these sequences, a set of primers was designed, univ14L and univ24L (Table 1), with the aim to amplify the corresponding region of as many as possible MiGHs within the *Rpi-blb2* interval. This universal primer set was subsequently used to develop the MiGH specific SCAR/CAPS markers 14L24L and 24L9spec (Figure 2).

Genetic and physical mapping of Rpi-blb2 *in a* Solanum bulbocastanum *intraspecific mapping population*

To delimit the *Rpi-blb2* locus further, the intraspecific *S. bulbocastanum* mapping population B6 was developed (Figure S3). Repeated detached leaf assays on 47 progeny plants showed a clear 1:1 segregation of susceptibility:resistance in this population. Screening of these plants with markers 112L and E46M52e indicated that the resistance locus was located in the same region as the ABPT-derived *Rpi-blb2* locus. Subsequently, 1899 progeny plants were subjected to a recombinant screen. A total of 138 112L/E40M52e recombinant plants were identified (7.26 cM) and screened with all the available markers within the interval, positioning *Rpi-blb2* between markers 69L and 24L (Figure 2).

As the generated ABPT-derived BAC contig spanning the *Rp-blb2* locus still contained a gap between 141R and the 24L positive BAC clones (Figure 3), a second BAC library was made using high molecular weight DNA of the resistant *S. bulbocastanum* parental clone of mapping population B6. Approximately 100 000 clones with an average insert size of 100 kb were generated and stored as 50 bacterial pools containing approximately 2000 colonies. Screening of this pooled BAC library with markers 141L and 24L led to the isolation of the two overlapping BAC clones SPB30 and SPB39, which spanned the 141R–24L marker interval. Bacterial artificial chromosome end sequences of both BAC clones were used to develop the markers SPB30L and SPB39L (Figure 3). Screening of the recombinants within the 69L–24L interval delimited *Rpi-blb2* between SPB30L and 24L (Figures 2 and 3).

Table 1 Overview of markers used for mapping *Rpiblb2*

Marker	Orientation of the primer	Sequence	Annealing temp (°C)	Restriction enzyme
E46M52	F	TTGTGGTTATCGATGAGAAT	56.5	SCAR (b)
	R	GAAACAACAGCAGGATAGTGAG		
E46M52e	F	TTGTGGTTATCGATGAGAAT	61	SCAR (a,b); MboI (c)
	R	GAAACAACAGCAGGATAGTGAG		
E40M58	F	GAATTCAGCACAAATACCAA	50	DdeI (a)
	R	TTAACGTTTACTATCACGAG		
E40M58e	F	GTAGAAACAGCAGCCTCATAAGC	55	SCAR (a)
	R	TTCTGCCTAATTGCCCTGTG		
S1E00	F	GGGGTTGGGAAGACAACGACAC	50	
	R	AATTCCAAGATACAGTCAAATAC		
41L	F	AGGCAGGATTAACAGTAGAAG	58	TaqI (a)
	R	CATGCTTTTAGGAAGAAGCTC		
36L	F	TTGAGACAAAGCAGCTCCAC	59	ApoI (a,b)
	R	ACGTTTCTCACACCTACAGG		
69L	F	TGATGGCACGTTTGATCGTG	61	TaqI (a,b); HpaII (c)
	R	TAAGATCCAAACCAGCCACC		
69R	F	CCTTATCACACATGTGGCTAC	58	RsaI (a,b); ApoI (c)
	R	ATTGAAACGGAGGAAGTACAAC		
141R	F	TTCTTCATATGGCAGACCAAC	60	RsaI (a,b); DdeI (c)
	R	CTACTCTGCTGACATGCAGG		
24L	F	GAGATTCTCAAAGGTGTCTTCC	60	SCAR (a,b,c)
	R	AACCTGTGCTTTCCCATTCG		
24R	F	CTTTCACAAGCGTCACTTTGG	58	SCAR (a,b)
	R	TAAAAAGAATCAACAGGGCAAC		
14L	F	ACGACTGCTCAAAGTTGGCC	58	SCAR (a,b,c)
	R	CCAAGAAGCCAGTTGAGAGC		
123L	F	GTAGATTACACTATGGATATGG	60	SCAR (a,b)
	R	CAGTTAGCAGCAATGTCAGC		
123L2	F	CATTCAACTAGGCCAAAAGTGG	59	SCAR (a,b); DraI (c)
	R	CCAGGTAGGTGTTTTCTTCC		
123R	F	GTTCTAAGTCAGATGCCACC	62	SCAR (a,b)
	R	AAGTGCTCCAACACGAGCC		
133R	F	TGAGTTCTCTTACCCTGCG	60	SCAR (a,b)
	R	GGATATCCAGCATCAATGCC		
133R2	F	GGTGAGCCTCCTTGCATTCC	60	SCAR (a,b)
	R	CCTGAGGGAAGATGTCACG		
99L	F	CCTAGTTTAGAGTGAGTAGAC	58	SCAR (a,b)
	R	GTGATATATTGCTCAAGGATCC		
67L	F	GATTAGTGTAGATCTTAGCTTG	62	MboI (a,b)
	R	AAATCTCTCTCACAATTATCCC		
112L	F	CTATTGACTGAACCTGCTGAG	56	HaeIII (a); HinfI (c)
	R	TGAAGTCATTTAGTCCACAGC		
CT216	F	CGTAGTCCATCTGAAGCTCC	65	SCAR (a,b)
	R	TCTTCTTCTGCTAGTCGTCG		
CT119	F	ACTATTCTCACGTAAGGGGACAC	60	HindIII (a,b)
	R	GTGTACATGTATGAAACTCTAGC		
14L24L	F	AGAAAGCTCACCAGTGGACC	60	CfoI (c)
	R	ATTTATGGCTGCAGAGGACC		
SPB30L	F	CAAGTTACGGCAACCAAGAG	57	HpaII (c)
	R	CTTTGACACAGTGTTAGAATGC		
SPB39L	F	CGTGATCTAGGAGTTACGAC	52	SCAR (c)
	R	CTTATTTTAAATACAAGACATCTGG		
24L9spec	F	AGAAAGCTCACCAGTGGACC	56	HhaI (c)
	R	CAGAGGAAAGTCAACCAACG		

F, forward primer; R, reverse primers; a, ARG95-3; b, ARP96-11; c, B6.

Complementation analyses

For complementation purposes, all MiGHs present on BAC clones SPB30, SPB39, 242, 211 and 24 were subcloned as approximately 10 kb genomic DNA fragments into the binary vector pBINPLUS (van Engelen *et al.*, 1995). MiGH harboring subclones were identified by screening subclone libraries of each BAC clone with the primer combination 14L24LF/R. Based on the restriction pattern of the 14L24L fragments digested with the enzymes RsaI, TaqI, AluI, DpnII or MseI, eight different MiGHs were identified. Subclones harboring the 24L specific MiGH were not detected with the universal primers 14L24LF/R. These were selected using the 24L specific primers 24LF and 24LR, bringing the total number of identified MiGHs on these BAC clones to nine (Figure 3). Subclones with MiGHs from the SP30L–24L interval were transferred to the susceptible potato cultivars Impala and Kondor through *Agrobacterium* mediated transformation using strain UIA143 (Farrand *et al.*, 1989) or AGL0 (Lazo *et al.*, 1991). Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays using the complex isolates IPO655-2A and IPO82001 (Table 2). Genetic constructs harboring MiGH5, derived either from the ABPT material or from *S. bulbocastanum*, were able to complement the susceptible phenotype in both cultivars. In total, 34 out of 36 MiGH5 containing primary transformants were resistant whereas all other MiGH containing primary transformants were susceptible to *P. infestans* (Table 2; Figure 4). A selection of primary transformants containing MiGH5 was analyzed for copy number by Southern analysis, identifying four single copy integrations in cultivar Impala and six in cultivar Kondor. Of these, nine were resistant. MiGH5 was therefore designated the *Rpi-blb2* gene.

Primary transformants of cultivar Moneymaker harboring the *Rpi-blb2* gene construct were also produced and tested with the potato-derived isolates IPO82001 and IPO655-2A. The disease resistance assay revealed that *Rpi-blb2* is also able to complement the susceptible tomato phenotype (Table 2; Figure 4).

Rpi-blb2 gene structure and putative amino acid sequence

The inserts of two MiGH5 containing binary subclones, from BAC clone 211 and SPB39 from the ABPT and *S. bulbocastanum* sources respectively were sequenced by a primer walk strategy. The complete sequences of the inserts of clones 211-F/C12 and SPB39-20 consisted of 7967 and 9949 nucleotides (nt), respectively. The sequence of clone 211F/C12 was identical to the corresponding sequence within clone SPB39-20. The size and structure of the gene was determined by comparing the genomic sequence with 5′ and 3′ rapid amplification of cDNA ends (RACE) products. The *Rpi-blb2* gene contains 5′ and 3′ untranslated regions (UTRs) of 767 and 201 nt, respectively, and two introns at similar positions as those in *Mi-1*. Intron 1 is 626 nt long and is positioned within the 5′ UTR ending 32 nt upstream of the ATG start codon. Intron 2 is 86 nt long starting 43 nt downstream of the ATG start codon of the gene. The coding sequence of the *Rpi-blb2* transcript is 3804 nt.

The deduced open reading frame of the *Rpi-blb2* gene encodes a predicted polypeptide of 1267 amino acids with an estimated molecular weight of 146 kDa (Figure 5). The N-terminal half of the Rpi-blb2 protein contains three potential coiled-coil (CC) domains (amino acids 24–62, 326–374 and 413–434) and six conserved motifs indicative of an NBS motif (van der Biezen and Jones, 1998). The C-terminal half of Rpi-blb2 comprises a series of 13 irregular LRRs that can be aligned according to the consensus sequence hxxhxxLxxLxLxxC/N/Sx(x)LxxLPxx observed in other cytoplasmic R proteins, whereby h can be L, I, M, V or F, and x any amino acid residue.

The *Rpi-blb2* coding sequence shares 89.7% homology with *Mi-1* from tomato. At the amino acid level Rpi-blb2 shares 82% sequence identity with Mi-1.1 and 81% with Mi-1.2. Through ClustalW alignment of the deduced amino acid sequences of Rpi-blb2, Mi-1.1 and Mi-1.2, 197 amino acid residues were identified that are unique to Rpi-blb2 (Figure 5). Of these, 12 are encoded by three insertions; a single amino acid insertion at position 317, a three amino acid insertion comprising residues 33–35 and an eight amino acid insertion comprising residues 95–102 (Figure 5). Of the remaining 185 amino acids unique to Rpi-blb2, 111 (60%) are non-conservative substitutions. This means that the relevant amino acid does not belong to the same group as the corresponding amino acid in Mi-1.1 or Mi-1.2 (Figure 5), whereby the amino acid groups are classified based on ionic charges. When comparing the ratio of non-conserved versus conserved substitutions in the different domains of Rpi-blb2 (CC domain, NBS domain and LRR domain; Figure 5), we observed that the non-conservative nature of the substitution was highest in the LRR domain (42/61; 69%) and lowest in the NBS-domain (25/52; 48%). Within the LRR domain, these types of substitutions are centered on the xxLxLxxxx motif, which is predicted to encode the putative solvent exposed residues of the repeats (Figure 5). This fits the model that the LRR region of R proteins mainly defines resistance specificity. However, the relatively high non-conservative substitution rate in the CC domain (44/72; 61%) together with the concentration of insertions within this region also suggest an active role for this region. Correct intramolecular interactions between N- and C-terminal domains of the Mi-1 protein are essential for proper functioning of the protein (Hwang and Williamson, 2003; Hwang *et al.*, 2000). Highlighted in Figure 5 are 26 amino acids in the LRR region of the Mi-1 protein that are crucial for correct functioning of the protein. At 23 positions, alteration of the Mi-1.2 amino acid to that of Mi-1.1 resulted in the loss of both nematode resistance

Table 2 Complementation of late blight susceptibility in potato and tomato

			cv Impala		cv Kondor		Tomato cv Moneymaker	
BAC-library	Source BAC	Genotype[a]	MiGH-containing plants/transformants	R plants/MiGH-containing plants	MiGH-containing plants/transformants	R plants/MiGH-containing plants	MiGH-containing plants/transformants	R plants/MiGH-containing plants
ARD 1197-16	24	R_0 (MiGH1)	12/15[b]	0/12				
			8/10[c]	0/8				
	24	R_0 (MiGH2)	8/11[b]	0/8				
			5/6[c]	0/5				
	24	R_0 (MiGH3)	11/13[b]	0/11				
			5/7[c]	0/5				
	211	R_0 (MiGH4)	5/7[b]	0/5	10/12[b]	0/10		
	242	R_0 (MiGH4)	5/7[b]	0/5	8/8[b]	0/8		
	211	R_0 (MiGH5)	5/7[b]	4/5	12/13[b]	12-dec		
	211	R_0 (MiGH6)						
	211	R_0 (MiGH-24L)						
Blb 2002	SPB39	R_0(MiGH4)	5/6[b]	0/5	3/3[b]	0/3		
	SPB39	R_0 (MiGH5)	11/15[b]	11/11	8/8[b]	7′/8	24/25[b]	22/24
	SPB39	R_0 (MiGH6)	3/3[b]	0/3	6/6[b]	0/6		
	SPB30	R_0 (MiGH7)	3/4[b]	0/3	9/9[b]	0/9		
	SPB30	R_0 (MiGH8)	1/1[b]	0/1				
	SPB39	R_0 (24L)						
		Ro (pBINPLUS)	3/3	0/3	8/10	0/8		

[a]R_0 genotypes are primary transformants obtained from transformation of the susceptible potato cultivars Impala or Kondor with T-DNA constructs containing the Rpi-blb2 gene candidates RGC1 to RGC8 and RGC24L or an empty pBINPLUS vector. *Agrobacterium tumefaciens* strains UIA143[b] or AGL0[c] were used for transformation of the *Phytophthora infestans* susceptible potato cultivars Impala and Kondor or susceptible tomato cultivar Moneymaker.

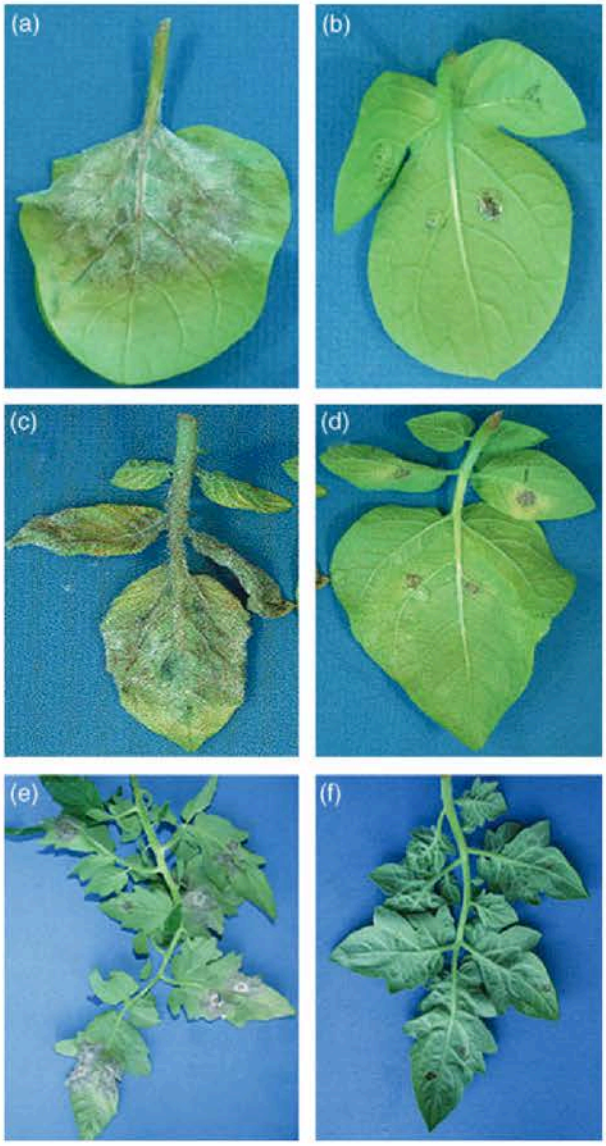


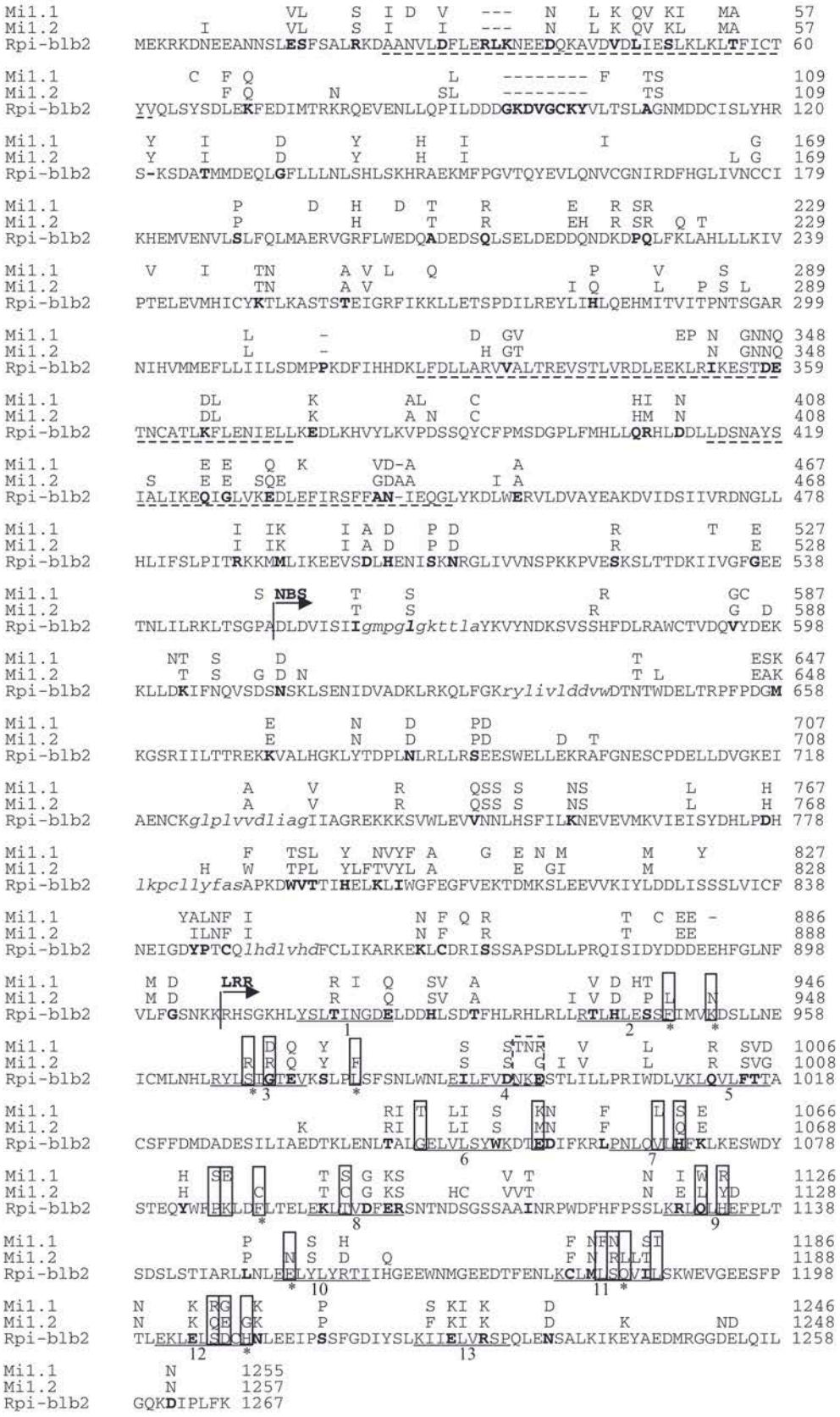
Figure 4. Genetic complementation for late blight susceptibility.
Typical disease phenotypes 6 days after inoculation with a sporangiospore suspension of *Phytophthora infestans* isolate 655-2A. Indicated are leaves derived from: (a) cv Kondor; (b) a primary transformant of cv Kondor harboring the MiGH5 (*Rpi*-blb2) from bacterial artificial chromosome (BAC) SPB39; (c) cv. Impala; (d) a primary transformant of cv Impala harboring MiGH5 (*Rpi*-blb2) from BAC 211; (e) tomato cv. Moneymaker; (f) a primary transformant of tomato cv Moneymaker harboring *Rpi-blb2*.
Panels (a), (c) and (e) depict typical susceptible responses with extensive sporulating lesions of *P. infestans*.
Panels (b), (d) and (f) depict typical resistance reactions observed at the sites of inoculation on transgenic plants harboring *Rpi-blb2*.

mediated by *Mi-1.2* and the ability of the *Mi-DS3* chimeric gene construct to cause cell death in a transient expression assay on *Nicotiana* benthamiana leaves (Hwang and Williamson, 2003). Their data suggest a role of these amino acids in recognition of signaling molecules or interacting proteins, or changes in protein folding, stability or localization. Interestingly, Rpi-blb2 carries the Mi-1.1 specific amino acid at eight of these positions (Figure 5), suggesting therefore a different function for these amino acids in the Rpi-blb2 protein context. Moreover in the former study, a cluster of three amino acids was identified that appeared to be crucial for nematode recognition and not for cell death (Figure 5). Interestingly, Rpi-blb2 harbors two of the three Mi-1.2 specific amino acids at the corresponding position.

Discussion

Here, we report on the cloning of the late blight resistance gene *Rpi-blb2* from the wild potato species *S. bulbocastanum* and demonstrate that this gene is orthologous to the tomato *Mi-1* gene from tomato. The *Rpi-blb2* locus was initially mapped in several tetraploid BC populations, derived from highly resistant complex interspecific hybrids designated ABPT, to the distal end of the short arm of chromosome 6 using a standard marker BSA approach (Michelmore *et al.*, 1991). However, fine mapping in these BC populations was hampered by low recombination frequencies at the *Rpi-blb2* locus and the fact that only marker alleles linked in coupling phase to resistance could be used. To circumvent these intrinsic problems of genetics in tetraploid potato, a diploid intraspecific *S. bulbocastanum* mapping population was developed in which late blight resistance cosegregated with *Rpi-blb2* locus specific markers previously developed in the BC populations. Higher recombination frequencies in the diploid *S. bulbocastanum* resistant parent together with the additional informative nature of marker alleles linked in repulsion phase to resistance enabled the genetic mapping of BAC-end markers and thus the efficient tiling of BAC clones across the *Rpi-blb2* locus. Instrumental in the cloning process of *Rpi-blb2* was also the syntenous nature of *Rpi-blb2* to the *Mi-1* locus, revealing the probable nature of the gene and thus prompting the development of candidate gene specific markers. By switching to the wild species level and through comparative genomics, problems usually encountered during the positional cloning of an *R* gene in potato were successfully circumvented.

Recently, comparative analyses of the *S. demissum*-derived complex locus *R3*, conferring race-specific resistance to *P. infestans* with the corresponding complex locus *I2* from tomato, conferring resistance to the fungus *Fusarium oxysporum* f. sp. *lycopersici*, revealed a significant expansion of the *R3* locus compared with the *I2* locus (Huang *et al.*, 2005). It was argued that the contrasting evolutionary fates of the two syntenic loci reflect the opposite evolutionary potential of the interacting pathogens (McDonald and Linde, 2002). The similar chromosomal positions of the *Rpi-blb2* and *Mi-1* gene clusters in potato and tomato warrants the comparative analyses of these two loci. *Mi-1* originates from the wild tomato species *Lycopersicon peruvianum* and has been introgressed into many tomato lines. In nematode-



resistant tomato, *Mi-1* and six homologs are grouped into two clusters separated by 300 kb (Vos *et al.*, 1998). *Mi-1* resides in the proximal cluster with two additional MiGHs. Recently, it was shown that the *Mi-1* locus from susceptible tomato carried the same number and distribution of MiGHs as the resistant locus (Seah *et al.*, 2004). The similarity in structure and organization of the *Mi-1* loci in *L. peruvianum* and *Lycopersicon esculentum* could suggest that the locus has not evolved much since the divergence of *L. esculentum* and *L. peruvianum*. In contrast, the *Mi-1* gene cluster in *S. bulbocastanum*, which harbors *Rpi-blb2*, seems to have expanded significantly since speciation of *Solanum* and *Lycopersicon*. Although the two-cluster structure is roughly conserved, the number of homologs per cluster has significantly increased. The distal cluster contains *Rpi-blb2* with eight additional MiGHs, whereas the proximal cluster contains at least six MiGHs (Figure 3). The distal cluster harboring *Rpi-blb2* is inverted compared with the proximal cluster, the proximal cluster having the same orientation as the *Mi-1* harboring cluster in tomato (Figure 3). Although the orientation of the distal cluster in tomato is unknown, the conservation of the two-cluster structure in both species suggests that the original duplication event occurred in the ancestral progenitor species of *Solanum* and *Lycopersicon*. As we also have preliminary data that indicate that the locus in *S. tuberosum* is also expanded compared with *L. peruvianum* and *L. esculentum*, we conclude that intrachromosomal recombination or unequal crossing over has played an important role in the evolution of the *Rpi-blb2* locus in potato. Intergenic and intragenic recombination at *R* gene loci has been described extensively and is thought to be a major mechanism for generating novel resistance specificities (reviewed in Hulbert *et al.*, 2001).

The short arm of chromosome 6 can be defined as a hot spot for resistance. In tomato, other dominant resistance genes that reside in the same region as *Mi-1* are *Mi-9*, conferring heat-stable resistance to root-knot nematodes (Ammiraju *et al.*, 2003), *Ol-4* and *Ol-6* for resistance to *Oidium neolycopersici* (Bai *et al.*, 2005), and the cloned *Cf-2* and *Cf*-5 genes for resistance to *Cladosporium fulvum* (Dixon *et al.*, 1996, 1998). In addition, quantitative resistance traits *Ty-1* for resistance to tomato yellow leaf curl virus (Zamir *et al.*, 1994) and *Bw-5* for resistance to *Ralstonia solanacearum* (Thoquet *et al.*, 1996) also reside in this region. In potato, in addition to *Rpi-blb2*, QTLs for resistance to *P. infestans* and *Erwinia carotovora* have been described in this region (Gebhardt and Valkonen, 2001). Clearly, the *Mi-1* locus in tomato and potato is not only agronomically of interest but also scientifically. Further comparison of the structure of this locus in the *Solanum* and *Lycopersicon* accessions harboring the above described dominant or quantitative resistance traits is likely a rich source of information on the genetic mechanisms associated with the evolution of *R* genes, thereby opening up an important experimental system for comparative studies, with potential economic benefits.

The most straightforward prediction of the gene-for-gene model (Flor, 1971) is that R proteins recognize single pathogen avirulence (Avr) or effector proteins and that recognition involves a direct binding between the two proteins. However, more recent findings are consistent with the so-called guard model for R-Avr interactions. This model predicts that R proteins detect modifications of host proteins targeted by effectors, rather than the effectors themselves (van der Biezen and Jones, 1998; Dangl and Jones, 2001). In light of this model, host genes identified through mutational screens as being required for *R* gene function or proteins that interact with effector proteins in Y2H screens and/or co-immunoprecipitation studies, are possible candidates for virulence targets (Mackey *et al.*, 2002; Rooney *et al.*, 2005; Shao *et al.*, 2003). In case of the Mi-1 protein, mutations at the *Rme* locus of tomato have been shown to suppress Mi-1 function both in the nematode and potato aphid resistance (de Ilarduya *et al.*, 2001). Classic receptor-ligand models would predict that all pathogens recognized by the Mi-1 protein have evolved conserved ligands. However, it seems more likely that Mi-1 recognizes the modification of a conserved host factor, possibly the Rme protein, which is targeted and modified by several unrelated effectors. The finding that Rpi-blb2 and Mi-1 are highly homologous (81% amino acid sequence identity) and that the corresponding genes have a common ancestral origin could suggest that they function through the same signaling pathways. The question that now arises is whether both proteins have evolved to guard the same or similar virulence targets or that the *P. infestans* effector(s) recognized by *Rpi-blb2* is (are) somehow related to those from root-knot nematodes, potato aphids and white flies. The observation that the Rpi-blb2 protein in fact contains one-third of the Mi-1.1 specific amino acid residues that when present in an Mi-1.2 context lead to loss of function (Hwang and Williamson, 2003) may suggest that neither is the case. Further insight into the above questions awaits functional analyses of *Rpi-blb2* in an *rme* background and/or the cloning

Figure 5. Alignment of the deduced protein products encoded by *Rpi-blb2*, *Mi-1.1* and *Mi-1.2*.
The complete amino acid sequence of Rpi-blb2 is shown and amino acid residues from Mi-1.1 and Mi-1.2 that differ from the corresponding residue in Rpi-blb2. Dashes indicate gaps inserted to maintain optimal alignment. The positions of putative coiled-coil (CC) domains are underlined with a dashed line. The N-terminal boundaries of the NBS and LRR region are indicated. Conserved motifs in the NBS domain are indicated in lower-case italics. The regions of the leucine-rich repeats (LRRs) that correspond to the β-strand/β-turn motif xxLxLxxxx are underlined. Rpi-blb2 specific amino acid residues comprising a non-conservative amino acid substitution between Rpi-blb2 and Mi-1.1 and Mi1.2 are highlighted in bold. Amino acid residues in the LRR region of the Mi-1 protein that are crucial for correct functioning of the protein are boxed (Hwang and Williamson, 2003). Boxed amino acid positions where Rpi-blb2 carries the Mi-1.1 specific amino acid are indicated with an asterisk.

of the effectors from the relevant pathogens. Recently, the first *Avr* gene from *P. infestans*, *Avr3a*, was identified using association genetics (Armstrong *et al.*, 2005). *Avr3a* is part of an ancestral oomycete locus and encodes a protein that is recognized in the host cytoplasm, where it triggers *R3a*-dependent cell death.

Like *Rpi-blb1*, *Rpi-blb2* confers broad-spectrum resistance to *P. infestans*. In more than 20 years of late blight resistance breeding involving annual screenings on inoculated fields in the Netherlands, potato clones harboring the *Rpi-blb2* gene showed no or hardly any sporulating lesions. The same observation was performed when such clones were tested under organic farming conditions at multiple sites in regions of the Netherlands where more virulent and aggressive isolates are being found (Flier *et al.*, 2003). The few sporulating lesions that were found only appeared at the end of the growing season at crop maturity. This phenomenon was also observed for the *Rpi-blb1* gene containing plant material (Helgeson *et al.*, 1998). Interestingly, detached leaf assays with ABPT-derived progeny were found to be less accurate for phenotyping than screening under field conditions. Although the apparent partial resistance phenotype observed under detached leaf assay conditions suggests a resistance mechanism similar to what can be observed in clones with enhanced levels of quantitative resistance, under field conditions, the *Rpi-blb2* based resistance is complete and behaves as a dominant trait. It remains to be seen if the use of these broad-spectrum late blight *R* genes is durable under conditions of large-scale agricultural production. Anyway, the cloning of these genes opens the way to efficient gene pyramiding or polyculture strategies (Niederhauser *et al.*, 1996), although durability prediction criteria need to be developed that will allow for the combination of genes that have the greatest potential for conferring durable resistance. For this purpose, it is essential to better understand how late blight *R* gene loci have evolved in the *Solanum* gene pool, how they are combined in natural *Solanum* habitats, the resistance mechanisms by which they confer resistance and, most important of all, to understand the biological function of the effectors that they recognize. Only then can we start thinking of durably exploiting *R* genes in the battle against late blight.

Experimental procedures

Plant material and development of mapping populations

The complex interspecific hybrid clones designated ABPT were made by Hermsen and co-workers (Figure S1; Hermsen and Ramanna, 1973). The *Phytophthora infestans* resistant clones ARF 87-507, ARF 87-801 and ARF 87-601 represent offspring from a second backcross (BC2) with the complex interspecific ABPT clones 55 or 60 and were used to develop the tetraploid mapping populations ARG 95-15 (BC3), ARG 95-3 and ARP 96-11 (BC4) and the diploid mapping population DP1 (Figure S2). Diploid population DP1 was obtained by crossing the resistant clone ARD 1197-16, with the susceptible diploid clone ARD 93-2090. The resistant diploid clone ARD 1197-16 was induced from AR 92-1197. This tetraploid clone AR 92-1197 was derived from a BC3 offspring derived from clone ARF 87-601.

The diploid *S. bulbocastanum* mapping population, designated B6, was developed by crossing a *P. infestans* resistant clone Blb 2002 with a susceptible clone Blb 48-5 (Figure S3). Results from reciprocal crosses of population B6 were combined. The resistant parental clone of population B6 was obtained from a cross between *S. bulbocastanum* clone Blb 93-D26-3 (accession numbers BGRC 8002, CGN 17690 and Pi 275187) as the female parent and *S. bulbocastanum* clone Blb 93-60-10 (accession numbers BGRC 8006 and Pi 275194) as the male parent. The susceptible parental clone of population B6 was obtained from a cross between *S. bulbocastanum* clones from accession numbers BGRC 8005 (CGN 17692 and PI 275193) and BGRC 8006.

Disease assays

Two different *P. infestans* isolates were obtained from Plant Research International B.V. (Wageningen, the Netherlands). Race structures and mating types were as follows: IPO82001, race structure 1.2.3.4.5.6.7.10.11, mating type A2; IPO655-2A. race structure 1.2.3.4.5.6.7.8.9.10.11, mating type A1 (Flier *et al.*, 2003).

Glasshouse-grown seedling tubers or field-grown seed potatoes were planted at trial sites in Marknesse, the Netherlands, from 1985 to 2002 and in the Toluca area of Mexico in 1991. For individual clones, plots were planted consisting of one to ten tubers. Approximately eight weeks after planting, the field at Marknesse was inoculated with a sporangiospore solution of *P. infestans* isolate IPO82001 and disease scores were collected 3 to 6 weeks after inoculation. Clones that were free or nearly free from late blight were classified as having a resistant phenotype, whereas clones with complete or nearly complete blighted foliage were classified as susceptible. Clones with intermediate reactions to late blight were classified as having an unknown phenotype. At the field trial in Mexico, natural infection had to occur. Once this natural infection by *P. infestans* was established, the percentage of blighted foliage of plants on each plot was scored 8 times on a 1–9 scale. Estimated percentages of blighted foliage from 1 to 9 were: 0, 3, 10, 25, 50, 75, 90, 97 and 100 (Estrada-Ramos *et al.*, 1983).

Detached leaf assays were carried out as described previously (van der Vossen *et al.*, 2003).

Plant DNA marker screening

Genomic DNA extractions and PCR analyses were carried out as described previously (van der Vossen *et al.*, 2003). An overview of the markers including primer sequences, annealing temperature and restriction enzymes if appropriate is given in Table 1.

AFLP fingerprinting and cloning and elongation of AFLP fragments

Template preparation and AFLP fingerprinting were performed essentially as described in Vos *et al.* (1995). Cloning of specific AFLP fragments was performed as described in Brugmans *et al.* (2003). Elongation of the sequence of an AFLP fragment was performed by TAIL PCR according to Liu and Whittier (1995). Essentially, elongation of AFLP fragments was performed using two or three nested specific primers (sp) in combination with an arbitrary degenerate

(AD) primer. The first PCR was performed with primers sp1 and an AD, the second with sp2 and an AD and the third with sp3 and an AD. The elongated fragments were cloned in pGEM-T (Promega, Leiden, the Netherlands) and sequenced.

R gene homolog fingerprinting

Template preparation was essentially performed as described in Vos *et al.* (1995). However, the second amplification step was carried out with the P-loop based primer S1 from Leister *et al.* (1996) in combination with the *Eco*RI + 0 AFLP primer. A 10-μl reaction mixture [0.5 μl ^{33}P-labelled S1 primer (10 ng μl^{-1}); 0.5 μl *Eco*R1 + 0 primer (10 ng μl^{-1}); 0.8 μl dNTPs (5 mM); 2 μl 10xGoldstar™ PCR buffer (Eurogentec, Seraing, Belgium); 1.2 μl $MgCl_2$ (25 mM); 0.06 μl Goldstar™ DNA polymerase (5 U μl^{-1}; Eurogentec); 14.94 μl MQ water] was added to a 10 μl diluted template (20× diluted in MQ water) and a PCR reaction performed using the following cycle profile: 45 sec DNA denaturation at 94°C, 45 sec primer annealing at 49°C and 2 min elongation step at 72°C (35 cycles). Prior to the cycling, the template DNA was denatured for 2 min at 94°C and the PCR was finalized by applying an extra 5 min elongation step at 72°C. The labelled PCR products were separated on a 6% polyacrylamide gel and the individual bands visualized by autoradiography according to standard procedures.

Bacterial artificial chromosome library construction and screening

Resistant clones ARD 1197-16 and Blb2002, both heterozygous for the *Rpi-blb2* locus, were used as source DNA for the construction of the ABPT-derived and *S. bulbocastanum*-specific BAC libraries, respectively. High molecular weight DNA preparation and BAC library construction were carried out as described in Rouppe van der Voort *et al.* (1999). Marker screening of the BAC library harboring the individually stored BAC clones was carried out as described in Rouppe van der Voort *et al.* (1999). The BAC library stored as superpools was screened as described in van der Vossen *et al.* (2003). Names of BAC clones isolated from the superpools carry the prefix SP (e.g. SPB39).

Subcloning of candidate genes and transformation to potato and tomato

Candidate MiGHs were subcloned from BAC clones 24, 211, 242, SPB30 and SPB39 as described previously (van der Vossen *et al.*, 2003). Binary plasmids harboring the candidate genes were transformed to *Agrobacterium tumefaciens* strains AGL0 (Lazo *et al.*, 1991) or UIA143 (Farrand *et al.*, 1989), the latter containing the helper plasmid pCH32 (Hamilton *et al.*, 1996). Overnight cultures of the transformed *A. tumefaciens* strains were used to transform potato tuber discs (cvs Impala and Kondor) or tomato leaf discs according to standard protocols (Fillati *et al.*, 1987; Hoekema *et al.*, 1989; van der Vossen *et al.*, 2003).

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends was carried out using the GeneRacer™ kit (Invitrogen™, Groningen, the Netherlands). 5′ rapid amplification of cDNA ends was carried out on cDNA synthesized with primer GSP4 (CTCAGCCATCAGTTGAAACAGAGA). Subsequently, primer GSP6 (GAGAGAGATTCAAGAGGAGGAAGC) was used in combination with the GeneRacer™ 5′ primer and the final amplification was carried out with GSP6 in combination with the GeneRacer™ 5′ nested primer. 3′ rapid amplification of cDNA ends was carried out with the nested primers GSP1 (GTGCTTCATTCAAACTCAAGGAG) and GSP2 (CTGAAC TAGAAAAACTCACTGTAGA) in combination with the GeneRacer 3′ primer. The final amplification was carried out with GSP3 (GTTTGAAAAGATTGCAATTGCATG) in combination with GeneRacer nested 3′ primer. Both 5′ and 3′ RACE amplification steps were carried out using Accuprime (Invitrogen) instead of the Taq polymerase supplied by the GeneRacer™ kit.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Schematic representation of the development of the complex interspecific hybrid clones designated as ABPT (an acronym of the four species involved: A, *Solanum acaule*; B, *Solanum bulbocastanum*; P, *Solanum phureja*; T, *Solanum tuberosum*).
2x, diploid (2n = 2x = 24); 3x, triploid; 4x, tetraploid; 6x, hexaploid; cv, cultivar.

Figure S2. Schematic representation of the development of the *Solanum tuberosum* mapping populations derived from ABPT (an acronym of the four species involved: *Solanum acaule*, *Solanum bulbocastanum*, *Solanum phureja*, *Solanum tuberosum*) clones 55 or 60.
(a) ARG 95-3 and DP1.
(b) ARG 95-15.
(c) ARP 96-11.
2x, diploid (2n = 2x = 24); 4x, tetraploid; cv, cultivar; BC, backcross. Codes in italics indicate mapping populations.

Figure S3. Schematic representation of the development of the diploid, intraspecific mapping population B6 of *Solanum bulbocastanum*.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Mapping and Cloning of Late Blight Resistance Genes from *Solanum venturii* Using an Interspecific Candidate Gene Approach

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Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato. Resistance (*R*) genes from the wild species *Solanum demissum* have been used by breeders to generate late-blight-resistant cultivars but resistance was soon overcome by the pathogen. A more recent screening of a large number of wild species has led to the identification of novel sources of resistance, many of which are currently being characterized further. Here, we report on the cloning of dominant *Rpi* genes from *S. venturii*. *Rpi-vnt1.1* and *Rpi-vnt1.3* were mapped to chromosome *9* using nucleotide binding site (NBS) profiling. Subsequently, a *Tm-2*^{2}-based allele mining strategy was used to clone both genes. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the coiled-coil NBS leucine-rich repeat (LRR) class of plant *R* genes and encode predicted peptides of 891 and 905 amino acids (aa), respectively, which share 75% amino acid identity with the *Tomato mosaic virus* resistance protein Tm-2^2 from tomato. Compared with Rpi-vnt1.1, Rpi-vnt1.3 harbors a 14-aa insertion in the N-terminal region of the protein and two different amino acids in the LRR domain. Despite these differences, *Rpi-vnt1.1* and *Rpi-vnt1.3* genes have the same resistance spectrum.

Late blight, one of the world's most devastating plant diseases, is caused by the oomycete *Phytophthora infestans*, causing an estimated yearly economic loss of $3.25 billion in potato-growing areas worldwide. Despite more than 150 years of resistance breeding, disease management still relies on the enormous application of fungicides. Breeding for late blight resistance was stimulated in the mid-nineteenth century by the disastrous consequences of *Phytophthora* epidemics in the United States and Europe. Only a few of the many existing landraces survived and these formed the basis for cultivar development. However, until 1910, the reduced aggressiveness of the pathogen since 1850 probably played a more prominent role in the survival of the potato crop in Europe than the first breeding activities. The rediscovery of the Mendelian laws of genetics subsequently directed the focus of potato resistance research on the use of wild *Solanum* spp. grown in South and Central America. Initially, special attention was given to *Solanum demissum* as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966). To date, at least 11 specificities (*R1–R11*) have been identified in *S. demissum*, four of which (*R1*, *R2*, *R3*, and *R10*) have been introgressed by breeders up to cultivar level; however, races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen 1993). By the end of the 1950s, most breeders switched to the use of sources of germplasm with partial or quantitative resistance, the underlying paradigm being that this type of resistance is resistant (*R*)-gene independent and assumed to be of polygenic nature and, thus, more durable (Turkensteen 1993). Extensive research in this area has led to the identification of many minor quantitative trait loci (QTL) (Bradshaw et al. 2006b; Collins et al. 1999; Costanzo et al. 2005); however, stacking of these QTL has proven to be very difficult, due to the outbreeding nature of cultivated potato. Moreover, this type of resistance is strongly correlated with late maturity under long day conditions (Howard et al. 1970). This, together with the finding that quantitative resistance is apparently also amenable to erosion (Flier et al. 2003), has stimulated breeders to reconsider the use of *R* genes, preferably through stacking of complementary genes.

A recent comprehensive survey of wild tuber-bearing *Solanum* spp. revealed that the genus *Solanum* harbors a wealth of late-blight-resistance sources that have yet to be exploited. Recent studies into the genetic basis of late-blight resistance in *S. demissum* showed that 8 of the 11 known specificities—*R3* (now known to be R3a and R3b), R5, R6, R7, R8, R9, R10, and R11—are located close to each other on chromosome 11 (Bradshaw et al. 2006a; El Kharbotly et al. 1994; Huang et al. 2004, 2005). Other mapped *R* genes from *S. demissum* include *R2* on chromosome *4* (Li et al. 1998) and *R1* on chromosome *5* (El Kharbotly et al. 1994; Leonards-Schippers et al. 1992). In addition to *S. demissum*, other wild *Solanum* spp. have been recognized as sources of late blight resistance. The described *R* loci include *Rpi-ber1* (initially named R_{ber}) from *S. berthaultii* on chromosome *10* (Ewing et al. 2000); *RB/Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from *S. bulbocastanum* on chromosome *8*, *6*, and *4*, respectively (Naess et al. 2000; Park et al. 2005; van der Vossen et al. 2003, 2005); *Rpi-pnt1* (initially named *Rpi1*) (Kuhl et al. 2001) from *S. pinnatisectum* on chromosome *7*; *Rpi-mcq1* (initially named *Rpi-moc1*) (Smilde et al. 2005)

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from *S. mochiquense* on chromosome *9*; and *Rpi-phu1* from *S. phureja* on chromosome *9* (Sliwka et al. 2006). In addition, several QTL involved in resistance to late blight have been reported, in both cultivated potato (Gebhardt and Valkonen 2001) and wild species, such as *S. microdontum* (Sandbrink et al. 2000; Tan at al. 2008), *S. paucissectum* (Villamon and Spooner 2005), and *S. phureja* on chromosome *7* and *12* (Ghislain et al. 2001).

In contrast to introgression breeding, isolation of *R* genes from *Solanum* spp. and their stable transformation into existing potato cultivars is by far the fastest means of exploiting potentially durable late-blight resistance present in the *Solanum* gene pool. Cloning of *R* genes is typically done through a positional cloning strategy. Currently, four *R* genes for late-blight resistance have been cloned and all belong to the coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) class of plant *R* genes: *R1* (chromosome *5*) and *R3a* (chromosome *11*) from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) and *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* on chromosomes *8* and *4*, respectively (Naess et al. 2000; van der Vossen et al. 2003, 2005). Once a functional gene is cloned from a specific *R* locus, one can try to clone functional alleles from the same or different species in order to determine allele frequency and allelic variation at a given locus. This was illustrated by the cloning of *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita*, respectively, which were shown to be functional alleles of *Rpi-blb1* (Vleeshouwers et al. 2008). Here, we demonstrate that NBS profiling (van der Linden et al. 2004), when combined with bulked segregant analysis (BSA) (Michelmore et al. 1991), is a powerful tool to generate candidate gene markers. They can predict the position of the *R* locus under study and, upon sequence information, form a starting point for cloning of the gene through a functional allele mining strategy, avoiding the map-based cloning approach. Depending on the resolution of relevant genetic mapping studies and the size of the candidate gene family, an allele mining approach can generate many candidate genes which need to be functionally analyzed.

To date, functional analysis of candidate *R* gene homologs (RGH) typically requires stable transformation of a susceptible genotype for complementation purposes. This is a time-consuming and inefficient approach because it takes at least several months to generate transgenic plants that can functionally be analyzed. In the current study, we have exploited the finding that *Nicotiana benthamiana* is susceptible to *P. infestans* (Becktell et al. 2006) to develop a transient complementation assay for *R* genes that confer resistance against *P. infestans* (H. Rietman, I. Hein, R. G. F., Visser, E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, *unpublished results*).

Here, we present an example of how we used the above-described approaches or techniques to clone two functionally equivalent *R* genes from *S. venturii*, a species with its origin in the Andean region of South America.

RESULTS

Genetic basis and spectrum of late blight resistance in *S. venturii*.

To determine the genetic basis of late blight resistance in *S. venturii*, 14 *S. venturii* accessions were screened in detached-leaf assays (DLA) with the *P. infestans* isolate IPO-C. Resistant genotypes selected from the *S. venturii* accessions CGN18108 and CGN18000 were used to generate the mapping populations 7698 and 7663, respectively. Following DLA with 52 F1 progeny plants of population 7698, 30 were scored as resistant and 22 as susceptible, suggesting the presence of a single dominant *R* gene (*Rpi-vnt1.1*). Of the 60 F1 progeny plants screened from population 7663, 24 were scored as resistant and 36 as susceptible, suggesting that vnt365-1 also contained a single dominant *R* gene (*Rp-vnt1.3*). Resistance genes were named in agreement with the concurrent work of Foster and associates (2009) who identified in the *S. venturii* accessions CGN18108, CGN18279, and CGN18000 three single dominant *R* genes, *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3*, respectively.

The resistance spectra of *Rpi-vnt1.1* and *Rpi-vnt1.3* were analyzed by challenging them with several isolates of different complexity and aggressiveness (Table 1). Both genes were only overcome by strain EC1, suggesting that they share the same resistance spectrum.

Mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* to chromosome *9*.

In an attempt to develop markers linked to *Rpi-vnt1.1* and *Rpi-vnt1.3*, we carried out a BSA in combination with NBS profiling in both mapping populations. This led to the identification of nine bulk-specific markers for *Rpi-vnt1.1* in 7698 and eight for *Rpi-vnt1.3* in 7663. On the full offspring, only two resistant bulk-specific fragments, one generated with the NBS2/*Rsa*I primer–enzyme combination and the other with NBS3/*Hae*III, cosegregated with resistance in both initial 7698 and 7663 populations of 52 and 60 F1 progeny plants, respectively. Therefore, these fragments were cloned and sequenced. When subjected to a BLAST analysis, both sequences turned out to be highly similar to the *Tm-2*^{2} gene on chromosome 9 of tomato (Lanfermeijer et al. 2003). The cloned NBS2/*Rsa*I and NBS3/*Hae*III fragments were 350 and 115 bp in size and shared 88.3 and 80.3% DNA sequence identity, respectively, with *Tm-2*^{2}, suggesting that *Rpi-vnt1.1* and *Rpi-vnt1.3* were *Tm-2*^{2} related and, thus, could lie on chromosome *9*. Genetic linkage of the NBS fragments to the *R* genes under study was verified by developing fragment-specific SCAR (sequence-characterized amplified region) markers in the *Rpi-vnt1.1* and *Rpi-vnt1.3* mapping populations. In this way, the NBS3-derived marker NBS3b was mapped relative to the chromosome-*9*-derived markers TG35, TG551, TG186, CT183, or T1421 (Fig. 1). Linkage of TG35 and TG186, and TG35 and TG551, to *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively, indicated that both genes were indeed derived from similar regions on chromosome *9* (Fig. 1). These findings were in line with the result of

Table 1. Description of *Phytophthora infestans* isolates used to determine the specificity of *Rpi-vnt1.1* and *Rpi-vnt1.3*

Isolate ID	Country of origin	Isolation year	Race	*Rpi-vnt1.1* and *Rpi-vnt1.3* phenotypes
90128	Geldrop, The Netherlands	1990	1.3.4.7.(8)	Resistant
H30P04	The Netherlands	Unknown	7	Resistant
IPO-C	Belgium	1982	1.2.3.4.6.7.10.11	Resistant
USA618	Toluca Valley, Mexico	Unknown	1.2.3.6.7.11	Resistant
VK98014	Veenkolonien, The Netherlands	1998	1.2.4.11	Resistant
IPO-428-2	The Netherlands	1992	1.3.4.7.8.10.11	Resistant
NL00228	The Netherlands	2000	1.2.4	Resistant
Katshaar	Katshaar, The Netherlands	Unknown	1.3.4.7.10.11	Resistant
F95573	Flevoland, The Netherlands	1995	1.3.4.7.10.11	Resistant
89148-09	The Netherlands	1989	0	Resistant
EC1	Ecuador	Unknown	3.4.7.11	Susceptible

concurrent work of Foster and associates (2009), who followed a map-based cloning approach to clone *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* genes.

In order to develop flanking markers to screen a large offspring for recombinant events, markers linked to TG35 or TG551 were selected from Solanaceae Genomics Network (SGN) and screened in both populations. Despite low levels of polymorphism, expressed sequence tag-based markers U276927 and U270442 have been developed and mapped in populations 7698 and 7663, respectively (Table 2; Fig. 1). U276927 could be mapped 1.8 centimorgans (cM) north of *Rpi-vnt1.1* whereas U270442 was mapped 3.5 cM south of *Rpi-vnt1.3*. Subsequently, recombinant offspring was identified using 500 individuals of population 7698 and 1,005 individuals of population 7663, using the flanking markers U276927/TG186 and NBS3B/U270442, respectively. This resulted in the mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* within a genetic intervals of 4 and 3.7 cM, respectively (Fig. 1).

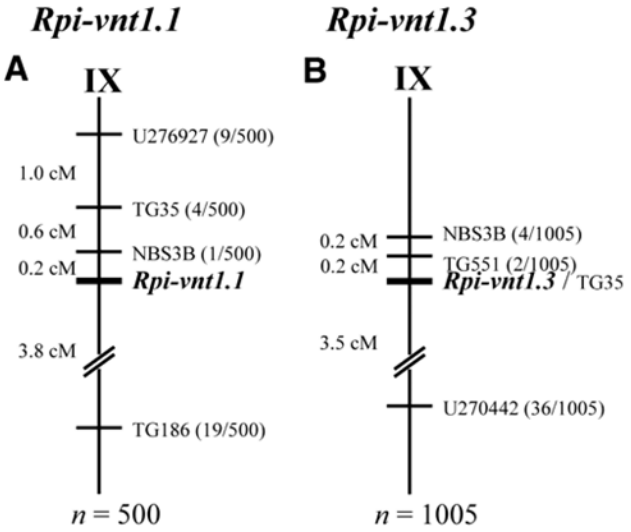

Fig. 1. Genetic linkage maps of chromosome *9* of the **a,** *Rpi-vnt1.1* and **b,** *Rpi-vnt1.3* loci mapped in the populations 7698 and 7663, respectively. Numbers on the left side indicate genetic distances (centimorgans). Relative positions of mapped loci are indicated by horizontal lines. The letter n represents the size of each population.

Tm-2²-based allele mining.

In view of the expected high DNA sequence homology between *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2²*, we adopted a homology-based allele mining strategy to clone the former two genes. The first step was to design degenerated primers incorporating the putative start and stop codons of candidate *Tm-2²* gene homologs (*Tm2*GH). Based on an alignment of all the available potato- and tomato-derived *Tm-2²*-like sequences in public sequence databases, we designed primers ATG-Tm2F and TGA-Tm2R (Table 3). However, no amplicons of the expected size were generated when this primer set was tested on the parental genotypes of both mapping populations. Because the ATG-Tm2F primer sequence was present in the cosegregating NBS profiling-derived marker sequences, three new reverse primers (REV-A, REV-B, and REV-C) were designed 100 bp upstream of the initial TGA-Tm2R primer site, in a region that was conserved in all the aligned *Tm-2²*-like sequences. When combined with either ATG-Tm2F or NBS3BF, a single amplicon of approximately 2.5 kb was specifically amplified from the resistant parental genotypes only (i.e., vnt7014-9 and vnt365-1). These fragments were cloned into the pGEM-T Easy vector and approximately 96 individual clones from each genotype were sequenced using a primer walk strategy. All the obtained sequences shared 75 to 80% similarity to *Tm-2²*. A total of five different classes could be distinguished within the vnt7014-9-derived sequences whereas the vnt365-1-derived sequences fell into only three different classes. These different classes were subsequently named NBS3B-like or non-NBS3B-like based on the degree of homology to the NBS3B sequence.

Table 2. Markers used to map *Rpi-vnt1.1* and *Rpi-vnt1.3*

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Enzyme[a]
NBS3B	Forward	ccttcctcatcctcacatttag	65	a.s.
	Reverse	gcatgccaactattgaaacaac	…	…
TG35	Forward	cacggagactaagattcagg	60	*Hha*I/*Xap*I (c)
	Reverse	taaaggtgatgctgatgggg	…	…
TG551	Forward	ccagaccaccaagtggttctc	58	*Taq*I (c)
	Reverse	aactttcagatatgctctgcag	…	…
TG186	Forward	aacggtgtacgagattttac	58	*Hph*I (c)
	Reverse	acctacatagatgaacctcc	…	…
U270442	Forward	ggatattatcttgcaacatctcg	55	*Xap*I (r)
	Reverse	cttctgatggtatgcatgagaac	…	…
U276927	Forward	gcattagcgcaattggaatccc	58	*Hph*I (c)
	Reverse	ggagagcattagtacaggcgtc	…	…

[a] Abbreviations: a.s. = allele specific, c = coupling phase, and r = repulsing phase.

Table 3. Primers used for various experiments as described in this article

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Experiments
NBS-GSP1	Forward	tccaaatattgtcgagttggg	Touch down	Genome walking
NBS-GSP2	Forward	gctttggtgcagacatgatgc	Touch down	Genome walking
REV-A	Reverse	ggttgtcgaagtaacgtgcac	55	*Tm2*-based allele mining
REV-B	Reverse	tgcacggatgatgtcagtatgcc	55	*Tm2*-based allele mining
REV-C	Reverse	caacttgaagttttgcatattc	55	*Tm2*-based allele mining
ATG-Tm2F	Forward	atggctgaaattcttctcacagc	55	*Tm2*-based allele mining
TAA-8bisR	Reverse	ttatagtacctgtgatattctcaac	55	*Tm2*-based allele mining
ATG2-Tm2F	Forward	atgaattattgtgtttacaagacttg	55	*Tm2*-based allele mining
TGA-Tm2R	Reverse	tgatattctcaactttgcaagc	55	*Tm2*-based allele mining
GSP1-5race	Reverse	gaacactcaaattgatgacagacatgcc	67	5′ rapid amplification of cDNA ends
GSP2-5race	Reverse	cccaaaccgggcatgccaactattg	67	5′ rapid amplification of cDNA ends

In order to retrieve the missing C-terminal part of the amplified *Tm2*GH, a 3′-genome walk was performed using primers NBS-GSP1 and NBS-GSP2 (Tables 2 and 3), which were designed approximately 100 bp upstream of the REV-A, REV-B, and REV-C primers, in order to generate an overlap of 100p between the cloned NBS3B-like sequences and clones generated with the genome walk. Three amplicons of approximately 200 bp were obtained from vnt7014-9 and a single clone of approximately 1 kb from vnt365-1. Following cloning, sequencing, and alignment to the cloned *Tm2*GH, all four clones seemed to fit to clone *Tm2*GH-vnt8b, because the overlapping 100 bp were an exact match. To be able to subsequently amplify full-length *Tm2*GH from the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, we designed a novel reverse primer (TAA-8bR) (Tables 2 and 3) based on the alignment of the full-length *Tm2*GH-vnt8b sequence with the *Tm-2*^{2} sequence from tomato (Fig. 2). The original TGA stop codon was not present in the *Tm2*GH-vnt8b sequence; therefore, we included the next in-frame stop-codon (TAA) which was situated 12 bp downstream.

Full-length amplification of *Tm2*GH from vnt7014-9 and vnt365-1 was subsequently pursued with high-fidelity *Pfu* Turbo polymerase using primers ATG-Tm2F and TAA-8bR. Amplicons of approximately 2.6 kb were cloned into the pGEM-T Easy vector and sequenced. Three different types of clones were obtained from vnt7014-9, one of which harbored an open reading frame (ORF) of the expected size (*Tm2*GH-vnt1b). All the clones obtained from vnt365-1 were identical to each other and contained the expected ORF. Clone *Tm2*GHvnt1.9 was chosen together with *Tm2*GH-vnt1b for further genetic analysis.

Before targeting *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 for complementation analysis, we needed to confirm that the selected *Tm2*GH indeed mapped to the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci. When tested as SCAR markers in the initial mapping populations, both markers cosegregated with resistance. Upon amplification with ATG-Tm2F and TAA-8bisR in the set of recombinants which defined the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, amplicons of the expected size were indeed only generated from late-blight-resistant recombinants, confirming that both *Tm2*GH were indeed good candidates for *Rpi-vnt1.1* and *Rpi-vnt1.3*. However, there were resistant recombinants, two in the *Rpi-vnt1.1* mapping population and one in the *Rpi-vnt1.3* mapping population, which did not give the expected polymerase chain reaction (PCR) product, suggesting that both loci could in fact harbor a tandem of two functional *R* genes.

Transient complementation using *Agrobacterium* transient transformation assays in *N. benthamiana*.

To investigate whether *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 were functional *R* genes, we inserted them into a Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press). *N. benthamiana* leaves were then infiltrated with *Agrobacterium tumefaciens* cultures containing the relevant clones. Two days postinfiltration, the leaves were challenged with *P. infestans* in a DLA. Two different isolates were used, EC1 and IPO-C, which show a differential response to *Rpi-vnt1.1* and *Rpi-vnt1.3*. Both genes confer resistance to IPO-C but allow for a compatible interaction in the case of EC1. Three independent transient complementation assays were carried out in triplicate with both isolates. For each replicate, leaf numbers 4, 5, and 6 (when counting from the bottom of the plant) were agroinfiltrated and subsequently challenged with *P. infestans*. At 6 days postinoculation with IPO-C, leaves transiently expressing *Tm2*GH-vnt1b or *Tm2*GH-vnt1.9 displayed an infection efficiency between 40 and 60% (Fig. 3A and B). The resistant control plants transiently expressing the functional *Rpi-sto1* gene (Vleeshouwers et al. 2008) showed a significantly lower infection efficiency ranging between 10 and 20%, (80 to 90% of the challenged leaves showed a hypersensitive response [HR]). In contrast, leaves expressing abptGH-a, a nonfunctional paralog of *Rpi-abpt* (Lokossou et al. in press) were fully susceptible (Fig. 3A and B). In the case of EC1, all agroinfiltrated leaves were susceptible except for those infiltrated with *Rpi-sto1*, which confers resistance to EC1 (Fig. 3A and C). These

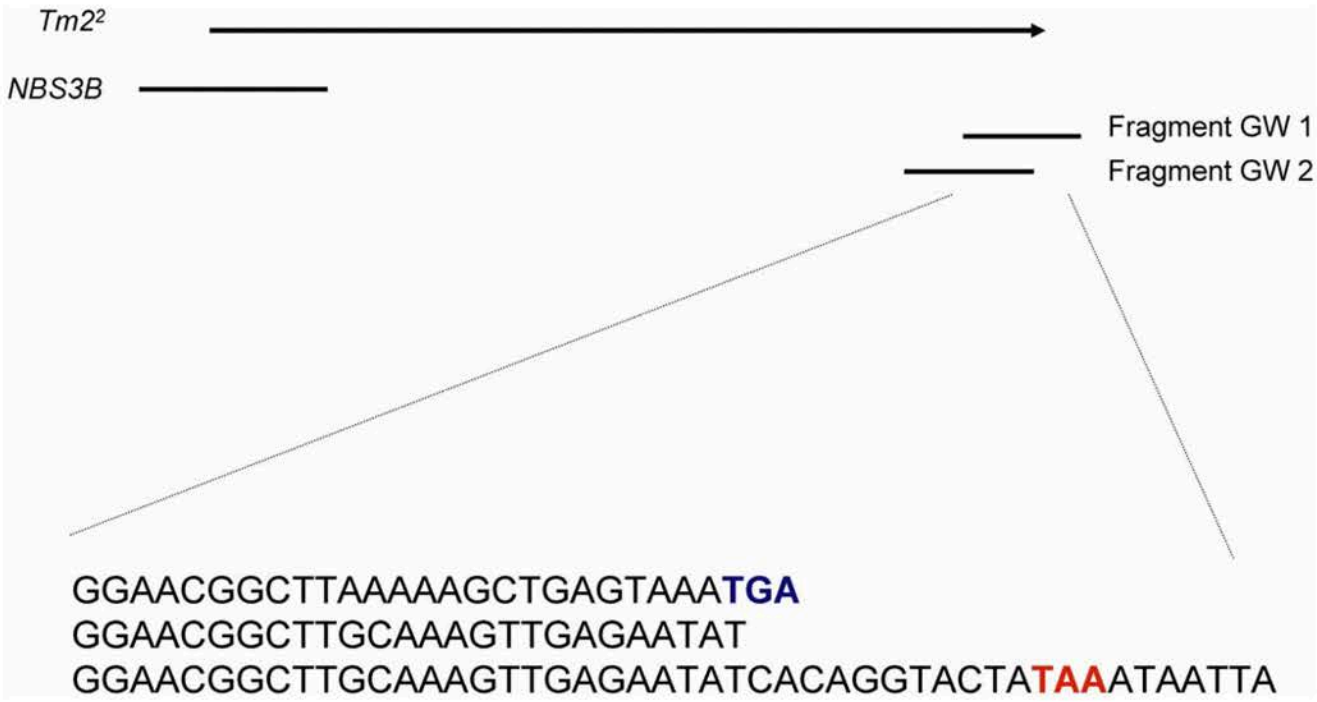

Fig. 2. Alignment of the *Tm-2*^{2} open reading frame with two polymerase chain reaction fragments obtained with a Genome Walking kit (GW1 and GW2). The stop codons of the *Tm-2*^{2} gene and *Rpi-vnt1.1 / vnt1.3* are presented in blue and red, respectively.

data matched with the resistance spectrum of *Rpi-vnt1.1* and *Rpi-vnt1.3*, suggesting that *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 indeed represented *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Complementation analysis through stable transformation of cv. Desiree.

To confirm the results obtained with the transient complementation assays in N. benthamiana, the binary Gateway constructs harboring *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. As a resistant control, we also transformed cv. Desiree with construct pSLJ21152, a binary construct harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence (Foster et al. 2009). Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. Surpris-

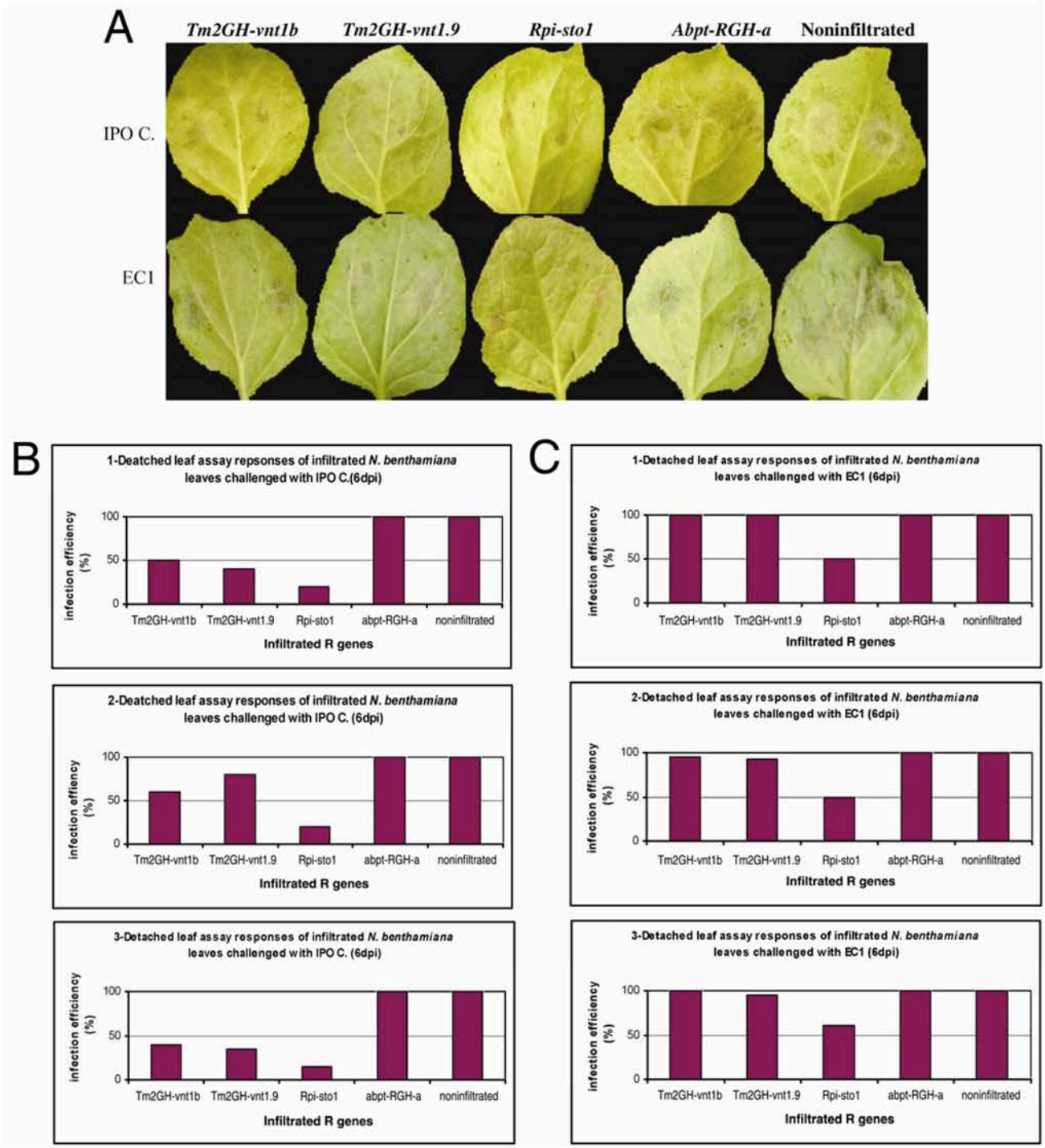

Fig. 3. Transient complementation assays in *Nicotiana benthamiana*. **A,** Typical detached leaf assay responses of *N. benthamiana* leaves infiltrated with either *Tm2*GH-vnt1b, *Tm2*GH-vnt1.9, *Rpi-sto1* (resistant control), or *abptGH-a* (susceptible control). The top row shows the response to IPO-complex (non-virulent isolate) whereas the bottom row shows the response to EC1 (virulent isolate). *Rpi-sto1* gives resistance to both isolates as expected. Pictures were taken 6 days postinoculation. **B** and **C,** Quantification of infection efficiency in the transient complementation assay as illustrated in A. *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 showed a higher infection efficiency, ranging from 40 to 60%, than the resistant control *Rpi-sto1* construct during an incompatible interaction with IPO complex isolate.

ingly, only the genetic construct harboring the 4.3-kb *Rpi-vnt1.1* fragment was able to complement the susceptible phenotype; eight of nine primary transformants were resistant. All 22 *Tm2*GH-vnt1b and 17 *Tm2*GH-vnt1.9 containing primary transformants were susceptible to *P. infestans*.

Alignment of the *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 sequences to the 4.3-kb *Rpi-vnt1.1* fragment revealed the presence of an additional ATG start codon 99 nucleotides (nt) upstream from the start codon that was used as basis for the PCR-based allele-mining experiments exploiting *Tm-2²* homology. This finding, together with the negative complementation results obtained with the *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 and the positive complementation result with 4.3-kb *Rpi-vnt1.1* fragment, suggest that the 5′ most upstream start codon represents the actual start of the functional *Rpi-vnt1.1* and *Rpi-vnt1.3* genes.

Transient complementation assays using extended allele mining products.

In an attempt to PCR amplify the putatively full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from vnt7014-9 vnt365-1, respectively, genomic DNA of both genotypes was subjected to long-range PCR using the primers ATG2-Tm2F and TAA-8bR (Table 3). Amplicons of the expected size were cloned into the pGEM-T Easy vector and sequenced. Clones obtained from vnt7014-9 were all the same and identical to the corresponding sequence in pSLJ21152 (Foster et al. 2009). Clones obtained from vnt365-1 were also all identical but contained an insertion of 42 nt in the 5′ extended region compared with those obtained from vnt7014-9. Both sequences were subsequently inserted into the Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press) and targeted for transient complementation analysis in *N. benthamiana*, together with the original *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 constructs and pSLJ21152. Both full-length genes showed infection efficiency comparable with the 4.3-kb genomic clone harboring the *Rpi-vnt1.1* gene (10 to 30% of the challenged leaves showed typical symptoms of late blight), whereas the shorter gene constructs again displayed significantly higher infection efficiency (65 to 75% of infected leaves), indicating that the full-length amplicons derived from vnt7014-9 and vnt365-1 represent *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively (Fig. 4A, B, and C).

Complementation analysis through stable transformation of cv. Desiree with the full-length PCR products.

To confirm the results obtained with the second transient complementation assays in *N. benthamiana*, the binary Gateway constructs harboring the full-length candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. As expected, both construct were able to complement the susceptible phenotype (Fig. 5). In all, 13 of 15 and 14 of 20 primary transformants with *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL, respectively, were resistant. This result confirmed the results observed in the transient assay. Therefore, the cloned PCR products *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL were demonstrated to be *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Gene structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*.

The 5′-terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3* was determined by comparing the genomic sequences with cDNA fragments generated by 5′ rapid amplification of cDNA ends (RACE). For *Rpi-vnt1.1* and *Rpi-vnt1.3*, RACE identified 5′ cDNA fragments comprising 5′ untranslated regions of 83 and 43 nt, respectively. The ORF of *Rpi-vnt1.1* and *Rpi-vnt1.3* encode predicted peptides of 891 and 905 amino acids, respectively. In addition to the 14-amino-acid insertion in the N-terminal region of *Rpi-vnt1.3*, only two other amino acids differ between *Rpi-vnt1.1* and *Rpi-vnt1.3*. At positions 548 and 753, *Rpi-vnt1.1* harbors an asparagine and arginine residue whereas the corresponding residues in *Rpi-vnt1.3* are tyrosine and lysine, respectively (Fig. 6). However, the substituted residues have the same characteristics. Asparagine and tyrosine belong to the group of hydrophobic residues whereas arginine and lysine are positively charged residues. The protein sequences of both genes harbor several conserved motifs of the CC-NBS-LRR class of R proteins (Fig. 6). A CC domain is located in the N-terminal parts of the proteins between amino acids 1 and 183 for *Rpi-vnt1.1* and between 1 and 198 for *Rpi-vnt1.3*. In the first 183 or 198 residues, two pairs of putative heptad motifs composed of hydrophobic residues could be recognized in *Rpi-vnt1.1* and *Rpi-vnt1.3* sequences, respectively. An NBS-ARC (apoptosis, *R* gene products, CED-4) domain could be recognized in the amino acid stretch between residues 183 or 198 and 472 or 486, respectively (P-loop, Kinase-2, GLPL) (Van der Biezen and Jones 1998). The C terminal part of *Rpi-vnt1.1* and *Rpi-vnt1.3* comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale et al. 2006). A PROSITE analysis (Hofmann et al. 1999) identified 4 N-glycosylation sites, 7 casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites, 6 N-myristoylation sites, and 1 cAMP- and cGMP-dependent protein kinase phosphorylation site.

At the protein level, Rpi-vnt1.1 and Rpi-vnt1.3 share 73% amino acid sequence identity with Tm-2². Interestingly, the lowest percentage of similarity was found in the LRR domain, where Rpi-vnt1.1 and Rpi-vnt1.3 share 62% amino acid sequence identity with Tm-2². In contrast, the CC and NBS-ARC domains of Rpi-vnt1.1 and Rpi-vnt1.3 share 86.5% amino acid sequence identity with the corresponding regions of Tm-2².

Mapping of *Tm2*GH in the ultradense genetic map of potato.

As previously described, two groups of *Tm2*GH were found based on the degree of homology to the NBS3B sequence: NBS3B-like and non-NBS3B-like. The *Rpi-vnt1.1* and *Rpi-vnt1.3* candidate genes *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9, respectively, were from the NBS3B-like group and segregated with resistance. In an attempt to determine the chromosomal positions of other *Tm2*GH belonging to the non-NBS3B-like sequences group, we used the primers ATG-Tm2-F combined with REV-C to develop *Tm2*GH-specific markers in the diploid reference mapping population, a cross between the diploid potato genotypes SH83-92-488 (SH) and RH89-039-16 (RH) (SH × RH), which was previously used to generate the ultradense genetic map of potato (Isidore et al. 2003; van Os et al. 2006). Following digestion of the PCR amplicons derived from SH, RH, and five F1 progeny plants with different restriction enzymes, we identified four enzymes to generate CAPS markers that segregated from RH (*Cfr*131, *Hin*1II, *Alu*I, and *Hha*I) and one that segregated from SH (*Hpy*F10IV). Subsequent mapping of these markers in the complete SH × RH mapping population positioned them at the bottom of chromosome *9*, a region of the chromosome that is similar to the late-blight *R* gene *Rpi-mcq1* (Fig. 7A and B) (Lanfermeijer et al. 2003; Sliwka et al. 2006; Smilde et al. 2005; Trognitz and Trognitz 2005). The mapped fragments shared between 85 and 90% homology (DNA level), with *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2²* revealing the presence of a third *Tm-2²*-like cluster at

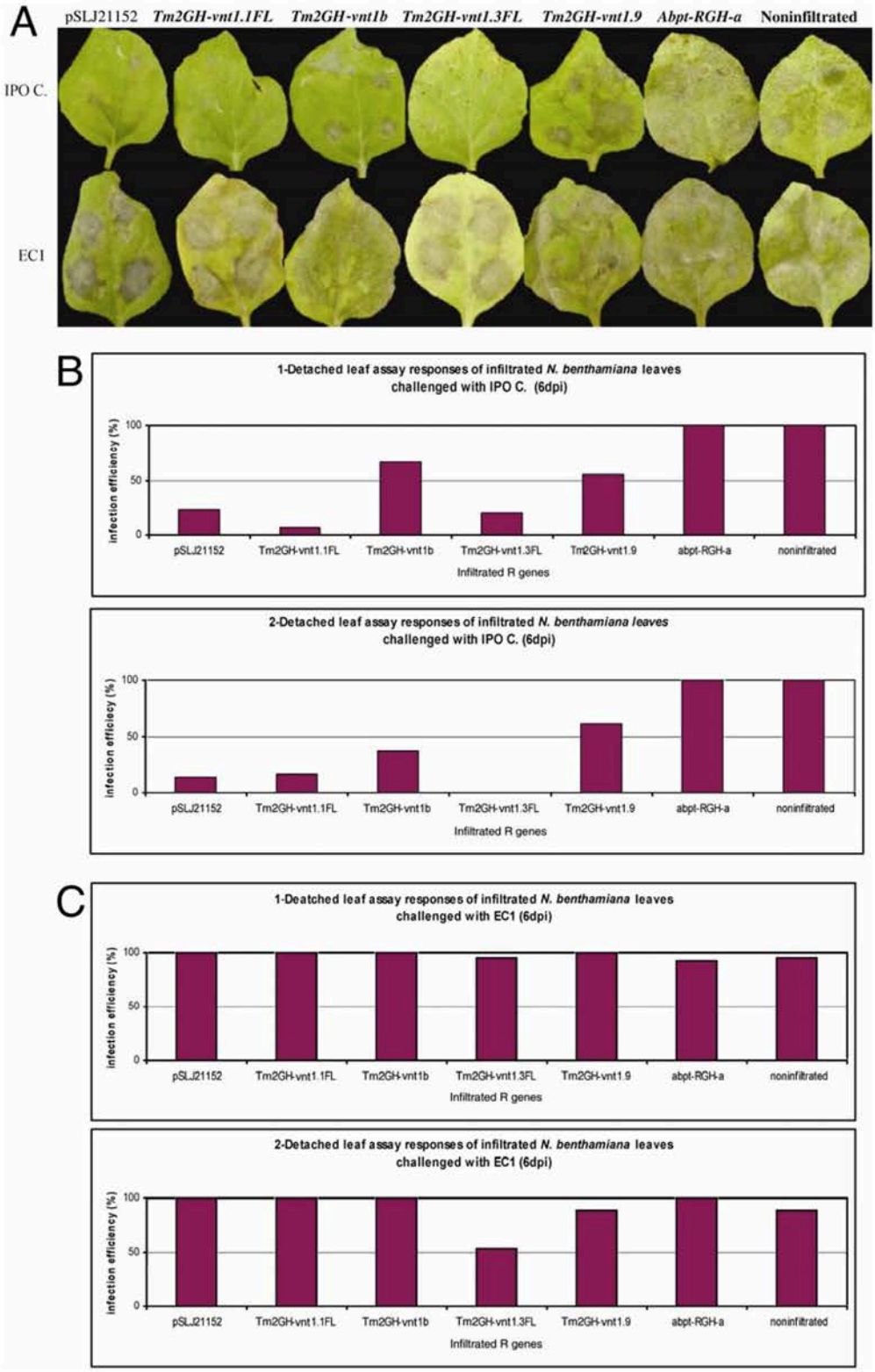

Fig. 4. Functional comparison of the N-terminal part of the truncated and full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* gene constructs. **A,** Transient complementation assays in *Nicotiana benthamiana* were repeated to compare infection efficiency of the full-length candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL with the truncated ones, *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9. The construct pSLJ21152, harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame (ORF), and terminator sequence was used as resistant control. The nonfunctional resistance gene *abpt*-RGH-a was chosen as susceptible control. Candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL show an infection efficiency as low as the resistant control (construct pSLJ21152) whereas the truncated candidate genes show a higher infection efficiency. **B** and **C,** Quantification of infection efficiency in the transient complementation assay as illustrated in A. *Tm2*GH-vnt1.1FL (full length of *Rpi-vnt1.1* candidate gene) and *Tm2*GH-vnt1.3FL (full length of *Rpi-vnt1.3* candidate gene) showed a level of infection similar to the resistant control, pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence).

the distal end of the long arm of the chromosome *9*. Alignment of mapped fragments shows that they all cluster with *Tm2*GHs in a different group than *Rpi-vnt1.1* and *Rpi-vnt1.3*. Therefore, *Rpi-mcq1* is likely to be a *Tm2*GH as well.

DISCUSSION

Here, we report on the cloning of two putatively allelic late-blight *R* genes, *Rpi-vnt1.1* and *Rpi-vnt1.3*, from the wild potato species *S. venturii*, using a candidate gene allele-mining approach. The candidate gene family was identified through NBS profiling (van der Linden et al. 2004) in combination with a BSA approach. Sequences of NBS marker bands genetically linked to the resistance phenotypes suggested that the target genes were located in an area of the potato genome that harbored *R* gene homologs that were highly homologous to the *Tomato mosaic virus* resistance (ToMV) gene *Tm-2*^{2} from tomato, which resides on the long arm of chromosome *9* (Ganal et al. 1989; Young et al. 1988). Putative genomic location of *Rpi-vnt1.1* and *Rpi-vnt1.3* allowed for the targeted selection of chromosome-*9*-specific markers (e.g., TG35 or TG551) to align both linkage groups to potato and tomato chromosome *9*. Subsequently, all publicly available *Tm-2*^{2} homologous sequences were aligned to design a set of primers with which we could PCR amplify putatively full-length gene candidates from relevant resistant genotypes. Candidate genes that were genetically closely linked to the *R* loci of interest were cloned in between the promoter and terminator sequences of the recently cloned *Rpi-blb3* gene (Lokossou et al. in press) and targeted for complementation analyses, either through *Agrobacterium* transient transformation assays (ATTA) in *N. benthamiana* or by stable *Agrobacterium*-mediated transformation of the susceptible potato cv. Desiree. Results of transient assays carried out with the initial truncated amplicons in *N. benthamiana* using the appropriate differential isolates suggested that we had indeed cloned two functional *R* genes. However, transgenic Desiree plants transformed with the same gene constructs were fully susceptible to *P. infestans*, whereas those transformed with a 4.3-kb genomic fragment, which was subcloned by Foster and co-workers from a BAC (bacterial artificial chromosome) clone that spanned the Rpi-vnt1.1 locus (Foster et al. 2009), displayed the expected resistance spectrum. Alignment of the initial truncated amplicon sequences to those of the functional genomic fragment revealed the presence of an additional in-frame ATG start codon 99 nt upstream of the start codon that was initially used as basis for the PCR-based allele-mining experiments, suggesting that the functional Rpi-vnt1.1 protein had an extended N-terminus of 33 amino acids compared with Tm-2^2. Subsequent analysis of the full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from the relevant parental genotypes revealed that *Rpi-vnt1.3* contained an additional 42-nt insertion in the 5′ extended region compared with *Rpi-vnt1.1*. Full-length versions of both *Rpi-vnt1.1* and *Rpi-vnt1.3* were shown to confer resistance using transient and stable complementation assays. Although the 5′ truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* were not able to confer resistance when expressed as stable transgenes, they were able to induce significant levels of resistance when expressed transiently in ATTA experiments. We speculate that absence of the N-termini of the R proteins may be compensated by the relatively high expression inherent to ATTA experiments. Rpi-vnt1.1 and Rpi-vnt1.3 belong to the CC-NBS-LRR class of plant R proteins. The CC domain, typically containing two or more α helices, which interact to form a super coil structure, is involved in protein–protein interactions (Liu et al. 2006). In the case of R proteins, it seems that the CC domain is involved in downstream signaling rather than in recognition (Van der Biezen 2002; Warren et al. 1999). Alignment of several R protein sequences recently led to the identification of a conserved EDVID motif among CC-NBS-LRR proteins. This motif was shown to be required for interaction between the CC domain and NBS-ARC or LRR domain (Rairdan et al. 2008). Moreover, this interaction is dependent on a wild-type P-loop motif (Moffett et al. 2002). A model proposed by Takken and co-workers (2006) suggests that the CC domain plays a role as an interactive platform for downstream signaling partners. Upon binding of ATP to the NB domain, a conformational change occurs in the CC domain, which releases the LRR signaling potential. Although the CC domain plays a role in downstream signaling, it cannot trigger the HR on its own. Another model proposed by Moffett and coworkers (2002) suggests that the CC domain may bind to an effector molecule which is released upon conformational changes within the R protein leading to the activation of downstream partners. The N-termini of Rpi-vnt1.1 an Rpi-vnt1.3 harbor four putative α helices that could interact to form two dimers. The first helice is present in the additional 99 nt upstream of the start codon that corresponds to the start codon of the *Tm-2*^{2} gene. The truncated versions of Rpi-vnt1.1 and Rpi-vnt1.3 lack this first helice, thereby destabilizing a possible interaction of the CC domain with the NB or LRR domain leading to less efficient downstream signaling. Overexpression of the truncated protein may override the negative effect of destabilized intramolecular interactions and, thus, have only minor effects on the triggering of the HR upon pathogen attack. This is in line with previous findings that overexpression of functional *R* genes (e.g., *Rx*, *RPM1* and *RPS2*) using the 35S

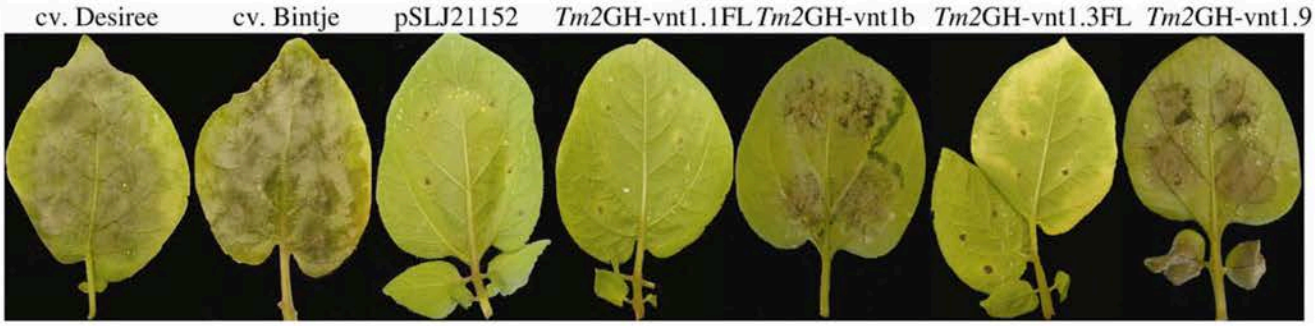

Fig. 5. Genetic complementation of cv. Desiree using full-length versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* (*Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL, respectively). Cv. Bintje, cv. Desiree, *Tm2*GH-vnt1b, and *Tm2*GH-vnt1.9 were used as susceptible controls and pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame, and terminator sequences) as resistant control. Primary transformants were challenged with IPO-complex (nonvirulent isolate) and scored 6 days postinoculation. *Tm2*GH-vnt1.1FL primary transformant -16 and *Tm2*GH-vnt1.3FL primary transformant -15 were chosen to illustrate detached leaf assay results. As expected, Bintje, Desiree, *Tm2*GH-vnt1b, and *Tm2*GH-vnt1.9 showed late-blight symptoms whereas pSLJ21152 and the full-length primary transformants were fully resistant to IPO-complex.

promoter can lead to cell death responses even in the absence of the pathogen (Belkhadir et al. 2004; Tao et al. 2000).

Complementation analysis of candidate *R* genes is usually done by stable transformation of susceptible potato cultivars. This approach is time consuming, requiring several months to confirm the function of the candidate genes. In the case of an allele-mining approach, one can expect to identify many paralogous candidate resistant genes, which calls for a quick and efficient complementation assay. Previous studies reported that resistance observed on *N. benthamiana* to *P. infestans* was mediated by the recognition of the elicitor protein INF1 (Kamoun et al. 1998). A transgenic *P. infestans* line, engineered to silence INF1 (Van West et al. 1999), was shown to be virulent on *N. benthamiana*. However, recent screens with a diverse set of *P. infestans* isolates showed that most isolates are, in fact, able to infect *N. benthamiana* (H. Rietman, I. Hein, R. G. F., Visser,

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  1 MNYCVYKTWAVDSNTKANSTSFLSSFSYFPFLILTFRKKKFNEKLKEMAEILLTAVINKS Rpi-vnt1.3
  1 .............--------------................................. Rpi-vnt1.1
  1 ---------------------------------------------.......S..... Tm2²

 61 IEIAGNVLFQEGTRLYWLKEDIDWLQREMRHIRSYVDNAKAKEVGGDSRVKNLLKDIQQL Rpi-vnt1.3
 47 ..................................................... Rpi-vnt1.1
 14 V.....L.I...K.............................A.............E. Tm2²

121 AGDVEDLLDEFLPKIQQSNKFICCLKTVSFADEFAMEIEKIKRRVADIDRVRTTYSITDT Rpi-vnt1.3
107 ..................................................... Rpi-vnt1.1
 74 .........D...........NY...RS.................V....I.K..N.I.. Tm2²

181 SNNNDDCIPLDRRRLFLHADETEVIGLEDDFNTLQAKLLDHDLPYGVVSIVGMPGLGKTT Rpi-vnt1.3
167 ..................................................... Rpi-vnt1.1
134 D......VL..............I...D....M......NQ..H................ Tm2²

241 LAKKLYRHVCHQFECSGLVYVSQQPRAGEILHDIAKQVGLTEEERKENLENNLRSLLKIK Rpi-vnt1.3
227 ..................................................... Rpi-vnt1.1
194 .......LIRD.................S...L.....I....QKM.....D......... Tm2²

301 RYVILLDDIWDVEIWDDLKLVLPECDSKIGSRIIITSRNSNVGRYIGGDFSIHVLQPLDS Rpi-vnt1.3
287 ..................................................... Rpi-vnt1.1
254 ...F....V...................V..................ES.L.A....E. Tm2²

361 EKSFELFTKKIFNFVNDN-WANASPDLVNIGRCIVERCGGIPLAIVVTAGMLRARGRTEH Rpi-vnt1.3
347 ................-..................................... Rpi-vnt1.1
314 ..............DDN.S.............N..G...................E.... Tm2²

420 AWNRVLESMAHKIQDGCGKVLALSYNDLPIALRPCFLYFGLYPEDHEIRAFDLTNMWIAE Rpi-vnt1.3
406 ..................................................... Rpi-vnt1.1
374 .........G..V....A.............S.....................I...... Tm2²

480 KLIVVNTGNGREAESLADDVLNDLVSRNLIQVAKRTYDGRISSCRIHDLLHSLCVDLAKE Rpi-vnt1.3
466 ..................................................... Rpi-vnt1.1
434 .F....S..R....D..E.............L.....N..................... Tm2²

540 SNFFHTEHYAFGDPSNVARVRRITFYSDDNAMNEFFHLNPKPMKLRSLFCFTKDRCIFSQ Rpi-vnt1.3
526 ........N..................................................... Rpi-vnt1.1
494 ......A.D.....G....L........-..V.I...RS...LE...V....A..PS...H Tm2²

600 MAHLNFKLLQVLVVVMSQKGYQHVTFPKKIGNMSCLRYVRLEGAIRVKLPNSIVKLKCLE Rpi-vnt1.3
586 ..................................................... Rpi-vnt1.1
553 ..YFD....HT.......SFQAY..I.S.F...T....L....N.CG.........TR.. Tm2²

660 TLDIFHSSSKLP-FGVWESKILRHLCY------TEECYCVSFASPFCRIMPPNNLQTLMW Rpi-vnt1.3
646 ............-..............------.......................... Rpi-vnt1.1
613 .I..DRR.LIQ.PS......H......RDYGQACNS.FSI.SFY.NIYSLH......... Tm2²

713 VDDKFCEPRLLHRLINLRTLCIMDVSGSTIKILSALSPVPKALEVLKLRFFKNTSEQINL Rpi-vnt1.3
699 ........................................R..................... Rpi-vnt1.1
673 IP...F.............K.G.LG..N..V.M..IF...L........S.SSDP....K. Tm2²

773 SSHPNIVELGLVGFSAMLLNIEAFPPNLVKLNLVGLMVDGHLLAVLKKLPKLRILILLWC Rpi-vnt1.3
759 ..................................................... Rpi-vnt1.1
733 ..Y.H.AK.H.NVNRT.A..SQS.....I..T.ANFT..RYI.....TF....K.KMFI. Tm2²

833 RHDAEKMDLSGDS----FPQLEVLYIEDAQGLSEVTCMDDMSMPKLKKLFLVQGPNISPI Rpi-vnt1.3
819 .............----.......................................... Rpi-vnt1.1
793 KYNE...A...EANGYS.......H.HSPN.......T..V.........L.T-.--FH-C Tm2²

889 SLRVSERLAKLRISQVL                                          Rpi-vnt1.3
875 .................                                          Rpi-vnt1.1
849 GISL....K..SK                                              Tm2²
```

Fig. 6. Alignment of Rpi-vnt1.1, Rpi-vnt1.3, and Tm-2^2 protein sequences. In the coiled-coil domain, four putative heptad motifs and the conserved motif EDVDID are underlined in bold or italic, respectively. Conserved motifs within the NBS-ARC (nucleotide-binding site apoptosis, *R* gene products, CED-4) domain are underlined in italic. Residues in bold represent the differences between *Rpi-vnt1.1* and *Rpi-vnt1.3* in the leucine-rich repeat domain.

E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, *unpublished results*). Previous studies, focused on potato–*Phytophthora* gene-for-gene interactions, showed that co-infiltration of cognate avirulence (*Avr*) and *R* genes in *N. benthamiana* leaves leads to the triggering of an HR typical for incompatible interactions (Bos et al. 2006), indicating that all the necessary *P. infestans* resistance signaling components are present in *N. benthamiana.* We have exploited these findings to develop a transient complementation assay for late-blight *R* genes using ATTA in combination with DLA. Despite the successful use of the transient assay in the cloning of the functional *Rpi-blb1* orthologs *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008), the results obtained with the truncated *Rpi-vnt1.1* and *Rpi-vnt1.3* genes indicate that one needs to be careful with the interpretation of the results, and that stable transformation of a susceptible potato cultivar remains the ultimate functional assay of a gene.

Chromosomes *9* of tomato and potato seem to be hot spots for resistance because several *R* genes, conferring resistance to a broad range of pathogens, are located on this chromosome. In tomato, in addition to *Tm-2*^{2}, four other *R* genes have been mapped on chromosome *9*; *Sw5* at the distal end of the long arm, conferring resistance to *Tomato spotted wilt virus* (Spassova et al. 2001); *Ve1* and *Ve2*, conferring resistance to *Verticillium dahliae* (Simko et al. 2004); and *Frl*, conferring resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Vakalounakis et al. 1997). Interestingly, in tomato, three major QTL for resistance to *P. infestans* have been described, one of which, *Ph3*, is located at the distal end of the long arm of chromosome *9* (Chunwongse and Black 2002). In potato, *Nx-Phu*, a potato gene for hypersensitive resistance to *Potato virus X* (Tommiska et al. 1998); the late blight *R* genes *Rpi-mcq1* (Smilde et al. 2005); and *Rpi-phu1* (Sliwka et al. 2006) reside on chromosome *9*. In the current study, we mapped *Tm2*GHs at the distal end of the long arm of chromosome *9*, a region that coincides with the *Rpi-mcq1* locus (Fig. 6), suggesting that these two genes are likely be *Tm2*GH. *Rpi-phu1* is also likely to be *Tm2*GH because it was mapped in the region harboring *Tm2GH* clusters. In addition to the above-described *R* loci, several QTL have also been described on chromosome *9* of potato that confer foliage resistance to *P. infestans* (*Pin9a*), resistance to the root cyst nematode *Globodera pallida* (*Gpa6*), and tuber and leaf resistance to *Erwinia carotovora* subsp. *atroseptica* (*Eca9A*) (Gebhardt and Valkonen 2001). To what extent *Tm2*GHs are also involved in quantitative resistance remains unclear.

Alignment of the Rpi-vnt1.1 and Rpi-vnt1.3 protein sequences to those of the currently cloned genes *R1*, *R3a*, *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* (Ballvora et al. 2002; Huang et al. 2005) (Lokossou et al. in press) revealed very little homol-

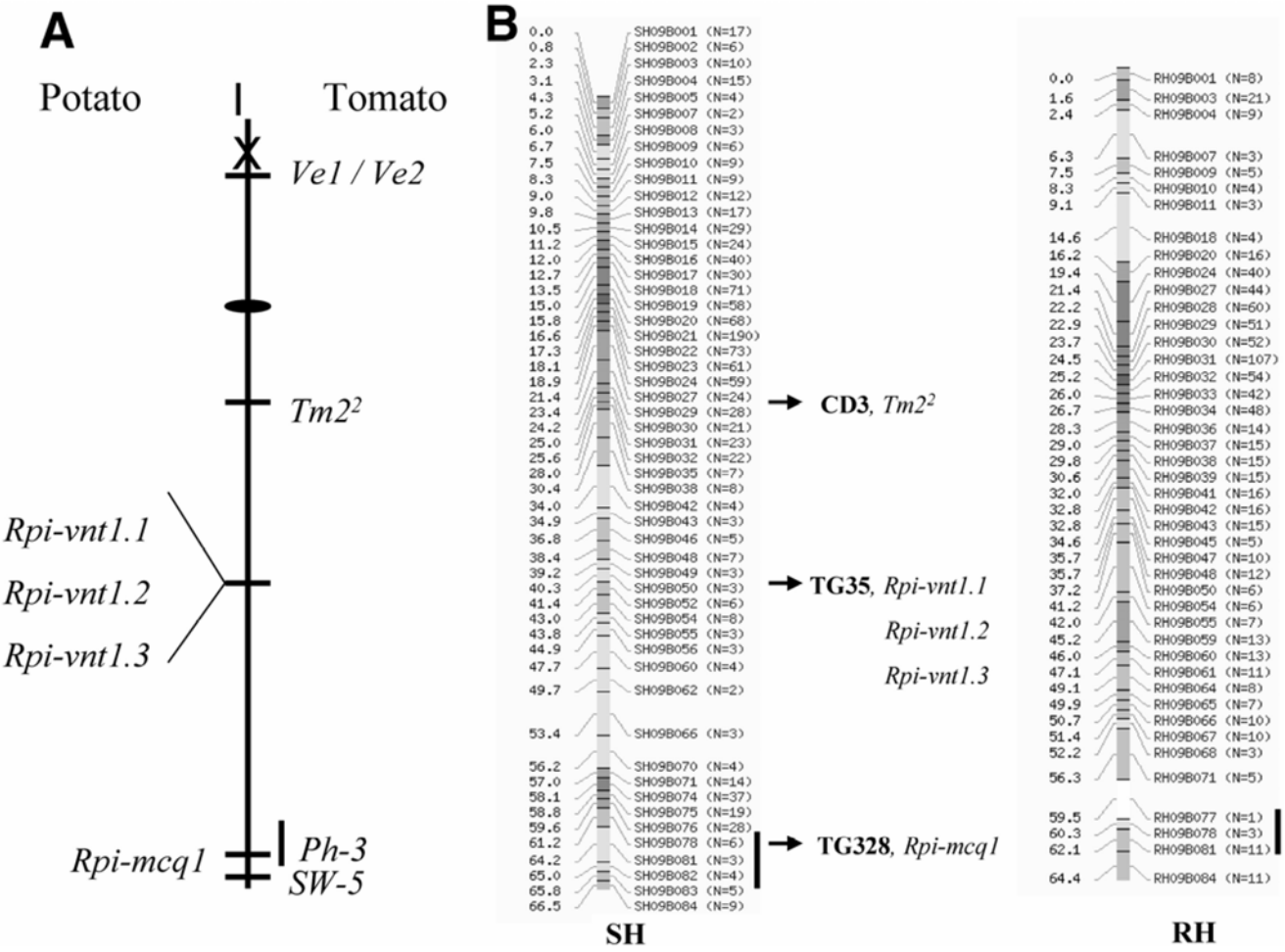

Fig. 7. Relative genetic positions of *Tm2*GH in potato and tomato. **A,** Schematic linkage maps of potato and tomato showing five resistance gene clusters. **B,** Ultra Dense potato genetic map of chromosome *9* divided in BINs from the parents SH83-92-488 (SH) and RH89-039-16 (RH). The thick vertical bars show the location of *Tm2*GHs belonging to the non-NBS3B-like group in the genome of SH and RH. The distal end of the long arm of the chromosome *9* from SH and RH harbors *Tm2*GHs in BINs 78-82 and BINs 77-81, respectively. This region coincides with the late-blight-resistant loci *Rpi-mcq1* and *Ph-3* from potato and tomato, respectively.

ogy to those genes, although all belong to the CC-NBS-LRR class of plant *R* genes. This is the first time that *Tm2*GH are shown to be involved in late-blight resistance. Alignment of the protein sequences of the chromosome-*9*-derived *R* genes (*Tm-2*^{2}, *Sw5a*, *Sw5b*, *Ve1*, and *Ve2*) suggests the presence of three distinct gene families, *Tm2*-like, *Sw5*-like, and *Ve*-like. The *Ve* genes are located on the short arm of chromosome *9* whereas the *Sw5* genes are located at the distal end of the long arm (Fig. 6). *Tm-2*^{2} and the cloned or mapped *Rpi* genes reside at three *Tm2*GH clusters that are spread across the long arm of chromosome *9* (Fig. 6), suggesting that *Tm2*GH is the predominant gene family on this chromosome arm. The overall homology between the identified *Tm2*GH ranges between 74 and 99.8% amino acid identity, suggesting that they have a common origin and that they have arisen through gene duplication events. Gene duplication and sequence exchange between RGH are major mechanisms that shape *R* gene diversity in plants (Kuang and Woo 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Comparison of the protein sequence of *Tm-2*^{2} with those of *Rpi-vnt1.1* and *Rpi-vnt1.3* revealed a higher homology within the CC and NB-ARC domains (86.5%) than the LRR domain (62%), which is in line with the fact that they confer resistance to completely different pathogens, ToMV and *P. infestans*, respectively, and that the LRR domain primarily determines recognition specificity and, thus, is subject to diversifying selection (Ellis et al. 2000). The functions of the CC and NBS domains, on the other hand, are likely to be more conserved, encompassing the fine tuning of intramolecular interactions (Moffett et al. 2002) and the regulation of downstream signaling (Belkhadir et al. 2004; Van der Biezen 2002; Warren et al. 1999).

The rapid breakdown of the first introgressed *Rpi* genes from *S. demissum* previously stimulated breeders to reconsider their breeding goals and, subsequently, efforts toward improving late-blight resistance were focused on increasing partial resistance by using race-nonspecific sources of resistance. However, under long-day conditions, breeders using this strategy have achieved little progress, the major drawback being the correlation between foliage resistance and late foliage maturity. Nevertheless, we anticipate that breeding for late-blight resistance in potato aimed at substantially contributing to disease management requires, by one way or another, the deployment of *Rpi* genes. As more and more *Rpi* genes are identified and cloned, the chances increase that new *Rpi* genes reside at known and well-characterized loci, enabling the use of comparative genomics and, thus, the development of efficient allele-mining strategies. Moreover, ongoing potato and tomato genome sequencing projects by international consortia are providing a (complete) survey of the distribution of *R* gene clusters in the family *Solanaceae*, enabling even faster cloning of *Rpi* genes. Challenges that remain are how to predict *Rpi* gene durability and how to introduce durable combinations of *Rpi* genes into existing and future cultivars in the most efficient and sustainable manner. We believe that knowledge of effector diversity may provide clues about the putative durability of *Rpi* genes (Birch et al. 2008; Vleeshouwers et al. 2008). The recent discovery of a common RXLR motif in oomycete Avr effector proteins (Birch et al. 2006; Rehmany et al. 2005) promises to accelerate the discovery and functional characterization of late-blight *Rpi* genes and of *P. infestans Avr* genes and, thus, the engineering of durable late-blight-resistant potato cultivars in the future. Efficient stacking of *Rpi* genes from one or several species is essential; however, in practice, this will enlarge the linkage drag problems considerably. Their introduction by genetic modification is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing cultivars with a long history of safe use. Currently, *Rpi* genes of natural origin derived from sexually compatible species, so-called cisgenes (Schouten et al. 2006), can be introduced using marker-free transformation systems (Vetten et al. 2003), leading to cisgenic plants with only the gene or genes of interest and without linkage drag (Jacobsen and Schouten 2007). It implies an environmental benefit and will, therefore, hopefully meet with consumer acceptance.

MATERIALS AND METHODS

Plant material.

Accessions of *S. venturii* were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. Following screening with *P. infestans*, resistant genotypes vnt367-1 and vnt367-1 from accessions CGN18108 and CGN18000, respectively, were used to generate inter- or intraspecific mapping populations. The *Rpi-vnt1.1* mapping population 7698 was generated by crossing vnt7014-9 (resistant) (vnt367-1 [resistant] × vnt366-8 [susceptible]) × nrs735-2 (susceptible). The *Rpi-vnt1.3* mapping population 7663 was generated by crossing the resistant plant vnt365-1 (resistant) × nrs735-2 (susceptible). The genes observed in CGN18108 and CGN18000 (initially called *Rpi-oka1* and *Rpi-nrs1*, respectively) (*unpublished results*) were named after the species *S. okadae* and *S. neorossi*. However, DNA fingerprinting of a wide range of wild *Solanum* spp. showed that the GenBank accessions CGN18108 and CGN18000 clustered with other accessions of *S. venturii* (Jacobs 2008; Jacobs et al. 2008). Morphological inspection of the CGN18108 and CGN18000 accessions confirmed that the labels *S. okadae* and *S. neorossi* should indeed be replaced with the label *S. venturii*. Furthermore, all available GenBank accessions of *S. okadae* and *S. neorossi* were susceptible and did not amplify a PCR product with our *Rpi-vnt1.1*- or *-1.3*-specific primers (M. A. Pel, *unpublished results*).

Disease assays and agroinfiltration.

DLA on the *Solanum* spp. were carried out as described by Vleeshouwers and associates (1999). Leaves were inoculated with 10-µl droplets of inoculum (5×10^4 zoospores/ml) on the underside and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16 and 8 h, day and night, respectively. At 6 days postinoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

ATTA were performed in *N. benthamiana* followed by DLA using appropriate *P. infestans* isolates. Four-week-old plants were infiltrated with a solution of *A. tumefaciens* COR308 (Hamilton and Frary 1996) harboring putative *R* gene candidates. Agroinfiltration of the lower side of *N. benthamiana* leaves was carried according to Van der Hoorn and associates (2000) using a optical density at 600 nm of 0.1. Two days postinfiltration, a DLA was performed as mentioned above. The phenotyping was done between 4 and 7 days postinoculation.

In ATTA assays, for each construct tested, three leaves of three different *N. benthamiana* plants were infiltrated with either *Tm2*GH-vnt1b (truncated *Rpi-vnt1.1* candidate gene), *Tm2*GH-vnt1.9 (truncated *Rpi-vnt1.3* candidate gene), *Rpi-sto1* (resistant control), or *abpt-RGH-a* (nonfunctional resistance gene). Assays were repeated three times. Infection efficiency was quantified by scoring 36 inoculation spots for each infiltrated constructs (three times one plant with three infiltrated leaves each harboring four inoculated spots).

Marker development.

Candidate gene markers were developed through NBS profiling as described by Van der Linden and associates (2004). Templates were generated by restriction digestion of genomic

DNA using the restriction enzymes *Mse*I, *Hae*III, *Alu*I, *Rsa*I, or *Taq*I. Adapters were ligated to restriction fragments. PCR fragments were generated by radioactive-labeled primers (nbs1, nbs2, nbs3, nbs5a6, or nbs9) designed on conserved domains of the NBS domain (P-loop, Kinase-2 and GLPL motifs) (Calenge et al. 2005; Syed and Srensen 2006). Additional markers from appropriate chromosomal positions were selected from the SGN database and subsequently developed into polymorphic markers in each of the relevant mapping populations.

PCR amplification of candidate *R* genes.

Long-range PCR with Taq-polymerase or *Pfu* Turbo polymerase in a 50-µl reaction mixture was prepared containing 50 ng of genomic DNA, 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 0.8 µl of dNTPs (5 mM each), 5 µl of 10× buffer, 5 units of Taq polymerase (Perkin Elmer, Norwalk, CT, U.S.A.), or 1 µl of *Pfu* Turbo (Invitrogen, San Diego, CA, U.S.A.). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles.

Genome walking.

Marker sequences were extended by cloning flanking DNA fragments with the ClonTech Genome Walker kit (Clontech Labs, Inc., Palo Alto, CA, U.S.A.) according to the manufacturer's instructions using a blunt adapter, comprising the complementary sequences 5′GTAATACGACTCACTATAGGGCA CGCGTGGTCGACGGCCCGGGCTGGA and 5′PO4-TCCA GCCC and the adapter-specific primers AP1 (TAATACGACT CACTATAGGGC) and AP2 (5′ACTATAGGGCACGCGTG GT). A simultaneous restriction ligation was performed followed by two rounds of PCR. A 50-µl restriction-ligation (RL) mixture was prepared containing 250 ng of genomic DNA, 5 units of blunt cutting enzyme (*Bsh*1236I, *Alu*I, *Dpn*I, *Hae*III, *Rsa*I, *Hinc*II, *Dra*I, *Sca*I, *Hpa*I, or *Ssp*I), 1 µl of genome walker adapter (25 µM), 10 mM ATP, 10 µl of 5× RL buffer, and 1 unit of T4 DNA ligase (1 U/µl) (Invitrogen). The digestion mix was incubated at 37°C for 3 h. Samples were diluted 50 times prior to PCR. For the first PCR round, a 20-µl reaction mixture was prepared containing 5 µl of diluted RL DNA, 0.6 µl of specific forward primer 1 (10 µM), 0.6 µl of AP1 (10 µM), 0.8 µl of dNTPs (5 mM each), 2 µl of 10× buffer (Perkin Elmer), and 5 units of Taq polymerase (Perkin Elmer). The first PCR was performed using the following cycle program: 30 s at 94°C as the denaturation step, 30 s at 56°C as the annealing step, and 60 s at 72°C as the extension step; 35 cycles were performed. A second PCR using the same conditions as the first one was performed using specific primer 2 and AP2 and 5 µl of 50× diluted product from the first PCR. The second PCR product (5 µl) was checked on gel (1% agarose) and the largest amplicons were cloned into the pGEM-T Easy Vector from Promega (Madison, WI, U.S.A.) and sequenced.

Gateway cloning of candidate *R* genes into a binary expression vector.

The Gateway cloning technique was used according to the manufacturer's instructions to efficiently clone candidate genes together with appropriate promoter and terminator sequences into the binary Gateway vector pKGW-MGW. In plasmid pKGW, the gateway cassette was exchanged against a multiple gateway cassette amplified from pDESTr4r3, resulting in pKGW-MGW. In this study, we used the promoter and terminator of *Rpi-blb3* (Lokossou et al. in press), which were cloned into the Gateway pDONR vectors pDONRP4P1R and pDONRP2RP3, respectively, generating pENTR-Blb3P and pENTR-Blb3T. PCR amplicons generated with *Pfu* Turbo polymerase were cloned into pDONR221 generating pENTR-RGH clones and, subsequently, cloned together with the *Rpi-blb3* promoter and terminator fragments into pKGW-MGW using the multiple Gateway cloning kit (Invitrogen). The pENTR clones were made by carrying out a BP-Reaction II overnight. DH5α competent cells (Invitrogen) were transformed by heat shock with 5 µl of the BP Reaction II mixture. Cells were selected on Luria-Bertani (LB) medium containing of kanamycin at 50 mg/ml. Colonies were checked for the presence of the relevant inserts by colony PCR. DNA of appropriate pENTR clones was extracted from *Escherichia coli* and used to perform a multiple Gateway LR cloning reaction to generate the final binary expression clones (pVNT1 and pNRS1). DH5α competent cells (Invitrogen) were transformed by heat shock with 5 µl of the LR reaction mixture. Cells were selected on LB medium containing spectinomycin at 100 mg/ml. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with spectinomycin at 100 mg/ml to extract the final expression vector. The final expression vector was transferred to *A. tumefaciens* COR308 through electroporation. Colonies were selected on LB medium supplemented with spectinomycin at 100 mg/ml and tetracycline at 12.5 mg/ml overnight at 30°C.

Stable transformation.

Binary vectors carrying *Tm2*GH-vnt1b, *Tm2*GH-vnt1.9, pSLJ21152, *Tm2*GH-vnt1.1FL, or *Tm2*GH-vnt1.3FL were used for *Agrobacterium*-mediated transformation in strain COR308. Internodia with a size of 2 to 5 mm were cut from explants of cv. Desiree and transferred to R3B-medium plates containing two sterile filter papers. The next day, internodia were incubated for 5 to 10 min in the bacteria solution, dried, transferred back to R3B plates, and placed at 24°C with a light period of 16 h. Two days later, internodia were transferred to selective medium containing kanamycin (100 mg/liter), zeatine (1 mg/liter), claforan (200 mg/liter), and vancomycin (200 mg/liter). Every 2 weeks, internodia were transferred to fresh selective medium. Green shoots emerging from calli were harvested and transferred to MS30 medium supplemented with kanamycin (100 mg/liter) to regenerate plantlets.

5′ RACE.

The GeneRacer Kit from Invitrogen was used according to the manufacturer's instructions to determine the 5′ terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*. Two gene-specific primers (GSP1-5race and GSP2-5race) (Table 3) were designed to perform a nested PCR using cDNA template from relevant parental genotypes and transgenic plants and High Fidelity Platinum *Taq* DNA Polymerase (HT Biotechnology Ltd., Cambridge). PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced.

Sequencing.

Cloned fragments or PCR products generated with either Taq polymerase (Perkin Elmer) or *Pfu* Turbo polymerase (Invitrogen) were sequenced as follows: 10 µl of sequencing reaction mixture was made using 5 µl of PCR product or 5 ng of plasmid, 3 µl of buffer, 1 µl of DETT (Amersham), and 1 µl of forward or reverse primer. The PCR program used was 25 cycles of 94°C for 20 s, 50°C for 15 s, and 60°C for 1 min. The sequences were generated on ABI 3730XL sequencers.

Mapping of *Tm2*GHs in the ultradense genetic map of potato.

The Ultra Dense genetic map of potato, which was previously made with a mapping population consisting of 130 F1 progeny derived from SH × RH (Rouppe van der Voort et al.

1997), was used to map *Tm2*GH. Genomic DNA from the parents (SH and RH) were PCR amplified using the primers ATG-Tm2-F and Rev-C (Tables 2 and 3). Amplification products were digested with restriction enzymes to detect polymorphisms between the parents. A subpopulation of 50 F1 individuals was subsequently analyzed through PCR amplification and appropriate restriction digestion. PCR reaction mixtures (20 µl) contained 50 ng of genomic DNA, 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 0.8 µl of dNTPs (5 mM each), 5 µl of 10× buffer, and 5 units of Taq polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles. PCR product (5 µl) was digested at the required temperature for 3 h with the appropriate restriction enzymes. Segregating polymorphisms were mapped in the ultradense potato map relative to 10,000 amplified fragment length polymorphism markers.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Andreas Untergassen Lab's BP-Reaction protocol webpage: www.untergasser.com/lab/protocols/bp_gateway_reaction_ii_v1_0.htm
European Union project FAIR5-PL97-3565 ultra-dense genetic map: www.plantbreeding.wur.nl/potatomap

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European Coexistence Bureau (ECoB)

Best practice document for the coexistence of genetically modified potato with conventional and organic farming

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Best practice document for the coexistence of genetically modified potato with conventional and organic farming

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Executive summary

The Technical Working Group (TWG) for Potato is the fourth one of the European Coexistence Bureau (ECoB) and is established for elaboration of the coexistence issues between genetically modified (GM) potato cultivation and non-GM potato and honey production in the EU.

The present technical report analysed the possible sources for potential cross-pollination with GM potato and adventitious admixture of GM potato material such as seeds and pollen and presents consensually agreed by TWG for Potato best practices for coexistence. The terms of reference for this review are presented in Section 1. The scope of the Best Practice Document is coexistence in potato production in the EU. It includes the coexistence between GM potato cultivation and honey production.

The ECoB TWG for Potato held two meetings in November 2015 and May 2016 and examined the state-of-the-art from scientific literature, research projects and empirical evidence provided by existing studies for segregation in potato production looking at the factors determining the cross-pollination rates in potato as well as other sources of admixture of GM material in conventional potato harvests and EU-produced honey. The review of this information (coming from a total of 155 references) is presented in a structured manner in Sections 4-6 of this document. Finally, the TWG for Potato reviewed the up to date approaches for the detection and identification of traces of GM potato material in non-GM potato harvests and honey (Section 7).

The TWG for Potato of the ECoB, based on the analysis of the evidence summarised in this document submitted proposals for best management practices, which form the ground for the agreed consensus recommendations presented in Section 8, complemented by an ex-ante view about their economic impact (Section 9).

Table of contents

1. Introduction

1.1. Legal Background

The European legislative framework for coexistence in agriculture was created to ensure that the cultivation of genetically modified (GM) crops is carried out in a way that allows different agricultural systems to co-exist side by side in a sustainable manner, which in turn promotes freedom of choice throughout the food chain. The coexistence rules support market forces to operate freely in compliance with the Community legislation. The legislative basis in the EU for the coexistence of GM and non-GM crops is established by the relevant legislation for the release of genetically modified organisms (GMOs) into the environment, and food and feed legislation for the labelling requirements of GMO presence. Both pieces of legislation provide a harmonised approach for the assessment of all potential environmental and health risks which might potentially be connected to placing of GMOs on the market.

Directive 2001/18/EC[1] on the deliberate release of GMOs into the environment and Regulation No 1829/2003[2] on GM food and feed ensure strict control of placing on the market GMOs in the EU. All GMOs and food and feedstuffs derived from them have to be clearly labelled to ensure freedom of choice for the consumer. In addition to that, and as an exemption of the labelling requirements, the European legislation takes into consideration the presence of technically unavoidable or adventitious traces of GM material. Directive 2008/27/EC[3] which amended Directive 2001/18/EC established the threshold of 0.9% for commodities intended for direct processing, which comprises all crop harvests (excluding the case when they are intended for seed production) below which traces of market-approved GM products do not require labelling. Regulation (EC) No 1829/2003 establishes the same threshold for food and feed. With Directive 2014/63/EU[4] amending Council Directive 2001/110/EC relating to honey the threshold of 0.9% adventitious admixture of GM pollen over total honey was adopted. These labelling rules are also valid for organic products, including food and feed, according to Regulation (EC) No 834/2007[5].

The adopted threshold for labelling exclusion is applicable only for adventitious, technically unavoidable admixtures. For farm-scale activities which are performed in open-space environments, it has always been understood that some admixing will occur. To control adventitious GM presence, adequate technical and organisational measures during cultivation, on-farm storage and transportation are required. Therefore the potential admixing below the threshold for which particular coexistence measures are designed is possible and technically unavoidable and adventitious. Thus the effectiveness of the coexistence measures used to limit the

1 Directive 001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1 Eur.

2 Directive 001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1 Eu.

3 Directive 2008/27/EC of the European Parliament and of the Council of 11 March 2008 amending Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, as regards the implementing powers conferred on the Commission, OJ L 81, 20.3.2008, p. 45-47.

4 Directive 2014/63/EU of the European Parliament and of the Council of 15 May 2014 amending Council Directive 2001/110/EC relating to honey. OJ L 164, 3.6.2014, p. 1–5.

5 Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No 2092/91. OJ L 189, 20.7.2007, p. 1-3.

potential intermixing to below a certain threshold defines what is "adventitious or technically unavoidable" in terms of coexistence for open-space farm activities.

As local environmental conditions and farm structures may have a significant impact on the effectiveness and efficiency of coexistence measures, their development is under the remit of individual Member States (MS).

Recommendation 2010/C 200/01[6] of the EC provides guidelines for development of national coexistence measures to avoid the unintended presence of GMOs in conventional and organic crops, replacing Commission Recommendation 556/2003[7]. Recommendation 2010/C 200/01 recognises that the market demand for particular food crops may result in economic damage to operators who would wish to market them as not containing GMOs, even if GMO traces are present at a level below 0.9%. Therefore MS may establish different thresholds for adventitious and technically unavoidable admixture of GMOs in non-GM harvests, taking into account the demands of the consumers and their market. The Recommendation also takes into consideration the extreme diversity of European farming systems, natural and economic conditions and clarifies that under certain climatic and/or agronomic conditions MS may exclude GMO cultivation from large areas, if other measures are not sufficient to ensure coexistence.

Directive 2015/412[8] amended Directive 2001/18/EC regarding the possibility for MS to restrict or prohibit the cultivation of GMOs in their territory. This Directive reaffirms the existing approach for development of coexistence measures, established by the Commission Recommendation of 13 July 2010. Directive 2015/412 places on MS (in which GMOs are cultivated) the responsibility to take appropriate measures in border areas of their territory with the aim of avoiding possible cross-border contamination into neighbouring MS in which the cultivation of these GMOs is prohibited, unless such measures are unnecessary in light of particular geographical conditions.

6 OJ C 200, 22.7.2010, p. 1-5.

7 Commission Recommendation 556/2003 of 23 July 2003 on guidelines for the development of national strategies and best practices to ensure the co-existence of genetically modified crops with conventional and organic farming. OJ L 189, 29.7.2003, p. 36.

8 Directive 2015/412 of the European Parliament and of the Council of 11 March 2015, amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory OJ L 68, 13.3.2015, p. 1–8.

1.2. The role of the European Coexistence Bureau

The diversity of agricultural practices and legal environments among the MS has led to adoption of the subsidiarity approach in the EU for the implementation of coexistence regulations. Although the development of coexistence measures is under the remit of individual EU MS, the European Commission retains several roles in this process. One important role is the technical advice offered to MS through the European Coexistence Bureau (ECoB).

The mission of the ECoB, created in 2008, is to organise the exchange of technical and scientific information on the best agricultural management practices for coexistence and, on the basis of this process, to develop consensually agreed crop-specific guidelines for technical coexistence measures. The ECoB is managed by and located on the premises of the Joint Research Centre (JRC) of the European Commission.

The work of ECoB is organised into crop-specific Technical Working Groups consisting of experts nominated by EU MS. Their main task is to develop Best Practice Documents (BPDs). The BPDs of ECoB comprise a methodological tool to assist development of national coexistence measures, based on scientific evidence and practical experience.

The ECoB has established TWG for maize, soybean, cotton and potato. The first TWG for maize crop production started its work in 2008. The TWG for maize has developed three BPDs for:

- Coexistence of GM maize crop production with conventional and organic farming (Czarnak-Kłos and Rodríguez-Cerezo, 2010);
- Monitoring efficiency of coexistence measures in maize crop production (Rizov and Rodríguez-Cerezo, 2014); and
- Coexistence of GM maize and honey production (Rizov and Rodríguez-Cerezo, 2013).

The second TWG, for soybean, was established in 2013 and developed a BPD for Coexistence of genetically modified soybean crops with conventional and organic farming (Rizov and Rodríguez-Cerezo, 2015).

The third TWG, for cotton, was established in 2014 and developed a BPD for Coexistence of genetically modified soybean crops with conventional and organic farming (Rizov and Rodríguez-Cerezo, 2016).

The TWG for potato started work on this BPD in 2015.

1.3. Scope of the Best Practice Document

This document focuses on the development, based on current scientific knowledge and agricultural practices, of a set of best agricultural management practices that will ensure coexistence of GM potato with conventional and organic potato while maintaining economic and agronomic efficiency of the farms. The TWG for potato was also asked to examine the issue of coexistence between GM potato cultivation and honey production in the EU. The scope of the BPD is coexistence in the cultivation of potato in the EU.

It is assumed that for the purpose of this document, the coexistence measures should be addressed to GM potato producers. All these measures should be proportionate, technically and economically consistent.

The document considers both the need for compliance with the regulated labelling threshold of 0.9% as well as with lower thresholds of adventitious presence of GM material (0.1%) which may be required by private operators in some markets.

The document exclusively considers GM potato with a single gene transformation event.

2. Potato cultivation in the EU: demand and crop production

2.1. Market and demand

Potato is the fourth most important crop in the world in terms of human consumption, following rice, wheat, and maize (corn) (Arvanitoyannis et al., 2008; Llorente et al., 2011; Zaheer and Akhtar, 2016) and the second most important arable crop in Europe, with 1.7 million hectares under potatoes in the EU-28 in 2016 grown at a value of 9.2 billion Euros in 2014 (Eurostat, 2016a,b). Potato is grown in over 100 countries, with world potato production being 385 million t (Mt) in 2014 (FAOSTAT, 2016). China, India, Russia, the Ukraine and the U.S. are the five largest potato producing countries (FAO, 2013; Zaheer and Akhtar, 2016). Since the early 1960s, the growth in potato production area has rapidly overtaken all other food crops in developing countries. It is a fundamental staple, ensuring food security for millions of people across South America, Africa and Asia, including Central Asia. Presently, more than half of the global potato production comes from developing countries. Potatoes for human consumption also belong to the most competitive sector of EU agriculture, despite the relative and absolute decline in production observed in recent years. Germany, Poland, France, the Netherlands and the United Kingdom are the main potato producing EU Member States (Eurostat, 2016).

The EU potato sector shows a competitive edge in international markets, especially in the sub-sectors of seed potatoes and processed products. Potatoes for human consumption are not covered by the Single Common Market Organisation, except for the standard rules on state aids. Since 2008, all the potato areas in the EU are potentially eligible to receive direct payments. Moreover, potato operators may benefit from the CAP promotion and quality schemes. The legal framework for these actions is laid down in Council Regulation (EC) No 3/2008 of 17 December 2007 on information provision and promotion measures for agricultural products on the internal market and in third countries.

The potato market is complex, and in addition to GM/non-GM/organic separation, consideration must be given to potatoes of specific designated origin. Examples of potatoes that are registered as Protected Designation of Origin (PDO) / Protected Geographical Indication (PGI)[9] comprise, among others, “Patata Kato Nevrokopiu” (PGI - Greece), “Pomme de terre de Merville” (PGI - France), “Pomme de terre de l’Île de Ré” (PDO - France), “Opperdoezer Ronde” (PDO - Netherlands), “Lapin Puikula” (PDO - Finland) and “Jersey Royal potatoes” (PDO - UK).

The potato market is also becoming increasingly segmented as new varieties are created to satisfy particular needs of the value chain. However, as a starting point, early potatoes, main crop potatoes, seed potatoes, and starch potatoes can be identified as some broad categories of potatoes.

Potatoes for human consumption, i.e. early and main crop potatoes (also referred to as ware potatoes), can be used

9 More on PDO/PGI and TSG at http://ec.europa.eu/agriculture/quality/schemes/index_en.htm.

fresh as table potatoes, or as raw material for the food processing industry. The food industry requires potatoes for different types of products:

- pre-cooked products (mostly French fries);
- dehydrated products (i.e. potato flours, potato flakes or potato granules);
- snacks;
- other products (gnocchi, salads, ready prepared meals, etc.).

The extent of potato production varies among different European countries. An overview of potato production for a number of selected countries is outlined in the following paragraphs.

2.1.1. Production in selected Member States

Austria

In Austria potatoes were grown on an area of 20,400 ha in 2015, an 8% reduction within the past 10 years. On 53% of the production area ware potatoes are produced, including 4% early potatoes, and on 7% of the area seed potatoes are propagated. About 40% of the production volume is used for industrial purposes.

Belgium

In Belgium, the potato acreage increased significantly from 60,000 to 81,500 hectares over the last ten years. In 2014 the total Belgian production of consumption potatoes was estimated at 4.58 Mt with yields up to 60 t per hectare. This record production (because of an increased area and higher yields) is almost 30% higher than the average production, which amounted to 3 Mt over the past years. Belgian production consists almost exclusively of consumption potatoes. There is no starch potato production and seed potato production is limited. After strong growth of its potato processing activities Belgium became a world-leader. In 1990, only 500,000 t of potato were processed, increasing to almost 3.5 Mt in 2013, of which 1.87 Mt were exported. Belgium also imports potatoes for processing.

Croatia

The annual production of potato in Croatia is around 160,000 t per year corresponding to a production area of about 10,000 ha. In the last ten years the production area has been reduced from approximately 18,000 ha to 10,000 ha. The production of potatoes takes place in all Croatian regions.

Denmark

In Denmark the potato production area of 46,000 ha represents 1.5% of total agricultural area and has increased in the previous 5 years slightly from 41,500 ha. 20% of this area is used for ware potato production and 57% for starch potato production. The main production region is the Western part of Denmark (middle and west Jutland on sandy soils). There has been an increase in the area of potatoes grown for starch production with an expected future trend towards a further increase, a decline in production of ware potatoes, and a slight increase in production of seed potatoes. Organic potato production represents approximately 3.6% of total potato area.

Estonia

In Estonia, production in 2015 was 117,200 t on an area of 5,800 ha. Although the area sown to potato has decreased by 59% over the past 10 years, the total annual potato yield increased by 34% in the same period.

Finland

Commercial potato production is focused on a narrow strip in the coastal areas of Finland and potato monoculture is very common. The cultivation of the highest seed potato grades is focused on the Northern Ostrobothnia region. Total potato production area in Finland is on average 22,000 ha with a total production volume of 0.65 Mt.

Germany

In Germany, the annual production area of potatoes steadily decreased during the last century and the early part of this century; this trend is likely to continue in the coming years. In 2016, potatoes were grown on an area of approximately

236,000 ha. Prior to 2000, the area used for potato production exceeded 300,000 ha. However, the total tuber yield only decreased slightly from 11.6 Mt in 1999 to 10.2 Mt in 2016 due to significant yield increases. The main production area within Germany is the Federal state of Lower Saxony, followed by the states of North Rhine-Westphalia and Bavaria. About 5% of the total production area is used for the cultivation of early potatoes.

In 2015, about 3.5 Mt of potatoes were processed into food. Whereas the per capita consumption of fresh potatoes is decreasing from year to year, the proportion of processed potato products (French fries, potato chips, mash, cooled and deep frozen potato products, etc.) is increasing. The per capita annual consumption of potatoes has decreased from 285 kg in 1900 to 58 kg in 2015. About 43% of the potato production is used for human nutrition while 20% enters the starch industry. Approximately 30% of this starch is used in non-food applications such as glues, lubricants, paper and corrugated cardboard production, as well as packaging and building material.

The use of potatoes as an animal feedstuff is at present of no significance. Mainly unmarketable potatoes enter this market in addition to being used for the production of energy in biogas plants.

Greece

Approximately 821,500 t of potato are produced annually in Greece with an average yield of 24.7 t /ha (2001-2011 average). Potatoes are produced in all parts of the country with approximately 60% being produced in the southern regions (Sterea Ellada, Peloponissos and Kriti) and 30% in the northern part (Makedonia, Thraki, Ipiros and Thessalia). Production is based on a large number of small production farms with an average area of 1.5 ha in the mainland and 0.1 ha on the islands.

Due to the typical Mediterranean climate, there are three production cycles for potato. There is spring cultivation (planting between December and early April), summer cultivation (planting between late April and early May), and autumn cultivation (planting in August and September). Summer and spring cultivation account for approximately 75% of annual potato production. All potato production is irrigated. Potatoes are produced mainly for direct consumption but also for frozen potato products and for chipping.

Lithuania

Approximately 399,200 t of potato were produced in Lithuania in 2015, with an average yield of 17 t/ha. The total area planted with potatoes in 2015 accounted for 23,500 ha which is a reduction of 13.9% compared to 2014 and 37.7% compared to 2011. Average yield of potatoes over the 2011 to 2015 period was 16.2 t/ha. Over the past five years, potato production in Lithuania has declined by 32.1%. Potatoes are integrated into predominantly cereal based rotation systems and are cultivated every 4-5 years.

The Netherlands

In 2014, 7.1 Mt of potatoes were harvested in the Netherlands on an area of 156,252 ha. Approximately 3.87 Mt of consumption potatoes were produced on an area of 74,068 ha and approximately 1.75 Mt of starch potatoes on 42,310 ha. An area of 39,874 ha was dedicated to seed potato production with a yield of approximately 1.48 Mt. Around 70% of the seed potatoes produced in the Netherlands are exported. In 2013, 1,479 ha were dedicated to organic potato production.

Potatoes for consumption are mainly produced on clay soil in the central part (IJsselmeerpolders) and in the southwest of the country, as well as on sandy soils in the south-eastern part (provinces of Noord-Brabant and Limburg). The main production regions for starch potatoes are the provinces of Groningen and Drenthe in the northeast of the Netherlands, which are characterised by sandy soils. Seed potatoes are produced on clay soils in the north (provinces of Groningen and Friesland) and in the northwestern province of Noord-Holland.

Spain

In Spain, 2.2 Mt of potatoes were produced on 73,000 ha in 2016. Seed potatoes account for 2,300 ha. The main production areas are Castile and León (40%), Galicia (20%), and Andalusia (12%). The area used for potato cultivation has decreased from 95,123 ha in 2005 but production area has not changed to any great extent over the last years.

Sweden

The yearly potato production in Sweden is about 25,000 ha of which 7,000 ha are starch potatoes and 900 ha seed

potatoes. Potatoes are grown in the whole country but with a concentration in the southern part. The average yield for food potatoes is a little more than 30 t/ha and for starch potatoes close to 40 t/ha.

After a long period of slowly decreasing potato cultivation acreage, there was a slight increase in 2016, for both food and seed potatoes.

United Kingdom

The UK is the twelfth largest producer of potatoes globally, harvesting around 6 Mt of the crop each year. Whilst long-term trends show a considerable decrease in the UK planted area, from over 250,000 ha in the 1960s to just over 100,000 ha in 2015, increased yields (from around 23 t/ha in 1960 to around 48 t/ha in 2014) have compensated for this reduction. This yield increase has been driven largely by improved agronomy, crop protection, fertiliser regimes, change in varieties and better irrigation. In 2014 the number of registered growers in the UK stood at 2,160 (down from over 250,000 in 1960), with the average area per grower around 53 hectares. The number of smaller growers is in decline, whilst the number of larger, specialist, growers is increasing. The largest proportion of the area grown, at around 35%, is intended for use in the pre-pack market, with the processing sector, making up the second largest area, at 30% of the total. Seed potatoes are grown predominately in Scotland where the levels of virus-transmitted aphids are low, although there is some seed potato production in England (especially in Yorkshire) and Wales. In terms of ware potatoes, around 57% are produced in the East of England (in Norfolk, Yorkshire and the Humber regions) and around 12% are produced in Scotland, with the remainder spread across England and Wales.

2.2. Growth and cultivation

Potatoes are efficient in using water and therefore produce more food per unit of water than any other major crop (FAO, 2008). They can be grown at altitudes from sea level to up to 4,700 meters above sea level, from southern Chile to Greenland. Although special cultivars have been bred that are adapted to these diverse environmental conditions, extreme low or high temperatures, in particular during the night, can obstruct tuber formation. Tubers of varieties of *S. tuberosum* subsp. *tuberosum* cannot survive temperatures of -3°C or below and potato foliage dies at temperatures of -4°C (van Swaaij et al., 1987; Vayda, 1994). Dale (1992) reported that potato tubers lose viability following a 25 hour-period at -2°C or 5 hours at -10°C. Additionally, the exposure of tubers to low temperatures in the field or during storage can cause low temperature injury, while high soil temperatures and nutrient or water imbalances can cause tuber deformities.

S. tuberosum subsp. *tuberosum* is a daylight neutral crop, which means that tubers are set at a growth stage independent of the day length. But variation for daylight sensitivity can be found among *S. tuberosum* subsp. *tuberosum* cultivars. Short days with less than 14 hours and moderate ground temperatures of 15-18°C enhance tuber formation, while longer days of 14-16 hours and higher day temperatures of 20-25°C enhance flowering and seed formation (Beukema and van der Zaag, 1979; Burton, 1989). The potato is commonly considered a cool season crop, but it also grows at high temperatures if sufficient water is available (Haverkort, 1990).

Potatoes are very sensitive to soil water deficit (Vayda, 1994) and therefore can only be cultivated in areas with adequate rainfall or the ability to irrigate (Bohl and Johnson, 2010; Haverkort, 1990). A wide range of soil pH can be tolerated by potatoes, normally pH 5 and higher is optimal, but even at pH 3.7 good production has been observed, and potatoes can grow well on a wide range of different soil types (Vayda, 1994).

Potato is a perennial crop grown annually from vegetative tubers, known as seed tubers or seed potatoes, which can persist in the soil when the plant dies back each autumn. Under European conditions the tubers persist poorly in cold wet soils and tubers, as well as plants rapidly become infected with a range of fungal and viral diseases, hence the crop is grown as an annual.

Planting time varies considerably from region to region depending not only on local climatic conditions but also on intended market use. This means that potato production can be achieved in many different areas and, indeed, explains why potatoes are grown in all EU countries.

Potato cultivars adapted to different regions within the EU have been bred. Early cultivars mature in less than four months, medium within 4 - 5 months, and late cultivars in up to 7 months, depending on the prevailing weather

conditions. Early potatoes are harvested before being fully mature, have easily removable skin, and are marketed as soon as possible after harvest. Harvest time depends on the climatic conditions and starts in the first semester of the year in the Mediterranean area including Spain, Cyprus, Greece, Malta and Portugal and in late May until August in the Continental and Northern part of Europe. The yields of early potatoes are lower, but as they attract a premium price and farmers usually make a larger profit than with main crop potatoes.

The harvest of main crop (medium and late) potatoes starts later, usually in September, and production costs are lower due to higher yields. The progress in storage techniques allows a prolongation of marketing main crop potatoes until May-June. As a consequence, there is an overlap of the season of main crop potatoes with that of early potatoes from the Mediterranean area.

The geographical distribution of potato production within the European Union is characterised by 5 main aspects:

- The Mediterranean part of the EU is mainly specialised in early potatoes that are commercialised in the first semester of the calendar year;
- Early potatoes cultivated in Northern, Eastern and Central European countries are brought on the market between late May and August. However, these countries focus on marketing main crop potatoes;
- There is a trend towards the concentration of potato production in five Member States: Germany, the Netherlands, France, UK, and Poland (so called EU-5). As a consequence, the potato production of Poland as the former first potato producer in the EU has considerably declined due to the strengthening of the EU-5 countries' position on the EU markets;
- The new Member States' potato production underwent a drastic process of structural change following the end of the former central planning economy;
- The path towards a modern system of market economic relationships is bringing about some developments but the re-organisation of the sector is still not completed.

Usually, seed potatoes normally weigh between 35 and 85 g and seeding rate typically ranges between 1 – 6 t/ha depending on the intended end use (Firman and Allen, 2007). Planting depth is between 10 and 18 cm. Depending on variety, the intended market, soil moisture, planting date, seed potato size and age, in-row spacing ranges from 15 to 46 cm, and rows are typically 75 to 97 cm apart. Potato tubers may be planted before the usual date of the last days with sub-zero temperatures. However, soil temperatures should be at least 8-10°C.

Potatoes draw a lot of nutrients from the soil, and sufficient applications of nitrogen, phosphorous and potassium are generally required to ensure adequate plant growth, tuber yield and quality, and to minimise susceptibility to diseases. Nitrogen is the most likely parameter to limit potato production, but excess nitrogen can have negative impacts as well. Soil and, in some cases, tissue testing is recommended in order to determine the most effective fertilization rates. In areas where the soil is naturally acidic agricultural limestone may be added to maintain pH within the desired range.

Potatoes typically require high levels of soil cultivation (Hopkins et al., 2004) for improved weed control, aeration and bed shaping as well as maintaining proper seed depth and establishing irrigation furrows (Bechinski et al., 2001; Sieczka, 2010). Potato production is generally not conducive to maintaining healthy soil conditions because of intensive tillage, minimal crop residues left on the field, heavy field traffic and long periods of soil being left bare (Hopkins, 2010). In the Northwest of the USA, potato fields are typically tilled both before and after the season (Hopkins et al., 2004).

Irrigation is often applied in potato production, since *S. tuberosum* is a drought-sensitive crop and has a shallow active root zone (Obidiegwu et al., 2015). Water demand is highest during the tuber bulking stage of growth, and an inadequate supply will reduce tuber yield and quality.

There are many serious diseases that may be inherent in seed potatoes, including late blight (*Phytophthora infestans* (Mont.) de Bary), early blight (*Alternaria solani* Sorauer), bacterial wilt (*Ralstonia solanacearum* (Smith) Yabuuchi et al.), bacterial ring rot (*Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann and Kotthoff; Davis et al.)), black leg (*Pectobacterium* spp. and Dickeya ssp.), and black scurf (*Rhizoctonia solani* Kühn), as well as several viral diseases. The best protection against some of these diseases is to use certified disease-free seed potatoes and the use of fungicide sprays. Crop rotations, the use of resistant cultivars, and proper sanitary practices are also important for reducing the incidence of disease (Bohl and Johnson, 2010).

Employing practices to prevent the entry of weeds, such as proper equipment cleaning are common best agricultural practices. Pre-plant incorporated, pre-emergence, and post-emergence herbicide applications as well as pre-emergence burn-off can be used to control weeds in addition to cultural practices such as harrowing and hilling. Integrated pest management is strongly recommended, with a combination of cultural and chemical approaches.

Tubers should ideally be harvested when their skin is ripe, the tubers are chemically mature, and temperatures range between 7 and 15°C to reduce shatter bruises and to avoid frost damage. Chemical maturity of tubers is important for long-term storage and processing and is reached when the amount of free sugars falls below a variety dependent standard minimum level (Western Potato Council, 2003).

3. Potato biology, evolution and breeding

3.1. Biology and taxonomy

Potato, *S. tuberosum* subsp. *tuberosum,* is an herbaceous perennial crop (OECD, 1997). The aerial parts of the plant range from 30 to 80 cm in length, with some cultivars reaching a height of two meters, and with a habit varying from erect to fully prostrate (Spooner and Knapp, 2013). Stems range from nearly hairless to densely hairy and may be green, purple, or mottled green and purple. Leaves are pinnate with a single terminal leaflet and three or four pairs of large, ovoid leaflets with smaller ones in between (Spooner and Knapp, 2013; Struik, 2007). The flowers are white, yellow, purple, blue or variegated, usually with a five-part corolla and exerted stamens with very short filaments. The fruits look like a small cherry tomato and are yellowish or green, globose, and have a diameter of less than 2.5 cm. Some lack seeds, but others may contain several hundred (Linsinka and Leszczynki, 1989). The nomenclature differentiates between potato seed (meaning seeds from fruits, and also known as 'true potato seed') and seed potatoes (meaning tubers for planting).

Cultivated potato (*Solanum tuberosum* L.) and its wild relatives are classified in order Solanales, family Solanaceae, genus *Solanum*. The genus *Solanum* is polymorphous and the largest genus comprising 1,500–2,000 species (PBI Solanum Project, 2014), predominantly found in tropical and sub-tropical regions (Fernald, 1970; Burton, 1989; Spooner and Knapp, 2013). The species *S. tuberosum* is divided into the two subspecies *tuberosum* and *andigena*. The subspecies *tuberosum* is the cultivated potato used worldwide, whereas the subspecies *andigena* is restricted to Central and South America (Hawkes, 1990; OECD, 1997). *S. tuberosum* subspecies *tuberosum* and *andigena* are fully cross-compatible (Plaisted, 1980). Hybrids can occur in nature, although the frequency of occurrence of such crosses is not well documented, as the morphological distinction between the two subspecies is very small. The greatest difference is the short day dependence of the subspecies *andigena* (OECD, 1997). As both subspecies only occur in southern North America and some parts of South America, natural crosses are likely to be found only there.

3.2. Evolution and breeding

A considerable number of highly diverse species exist in the genus *Solanum*, therefore cultivated potato has an extremely large secondary gene pool consisting of related wild species. The evolution of the cultivated potato is quite complex due to introgression, interspecific hybridisation, auto- or allopolyploidy, sexual compatibility among many species, a mixture of sexual and asexual reproduction, recent species divergence, and phenotypic plasticity resulting in a high morphological similarity among species (Spooner, 2009; Spooner and Bamberg, 1994). Wild potatoes are widely distributed in most parts of America, from southwest USA to Mexico and Central America. In South America, they occur in almost every country, mainly in the Andes of Venezuela,

Colombia, Ecuador, Peru, Bolivia and Argentina (Hijmans et al., 2002). The adaptation to a wide range of habitats has made the wild species tolerant to different environmental stresses and resistant to a broad range of pests and diseases (Hawkes, 1994). Wild potato species, however, are not present in Europe.

The value of germplasm of primitive cultivars and wild species in potato breeding is determined by its genetic diversity, availability and utility. In this sense, potato stands out among all other crops (Bamberg and del Rio, 2005). Primitive forms of cultivated potato and their wild relatives provide a rich, unique, and diverse source of genetic variation, which is a source of various traits for potato breeding.

The potential for using these genetic resources in conventional breeding depends on their 'crossability' with the commonly cultivated potato (*S. tuberosum*). Cultivated potato is only sexually compatible with some of the other tuber bearing species in the section *Petota* and rarely with the non-tuber-bearing species in the section *Etuberosum*, and there are very strong barriers to hybridisation with other *Solanum* species (Jackson and Hanneman, 1999; Andersson and de Vicente, 2010), such as differences in the endosperm balance number (EBN) and ploidy level. The EBN concept was first published by Johnston et al. (1980) to explain the success or failure of intraspecific crosses. The EBN is a measure to express the "effective ploidy of a genome in the endosperm". To enable normal development of the endosperm after fertilization, the maternal EBN must be twice that of the paternal EBN (2:1), hence this system forms a strong isolating mechanism present in the section *Petota*. The EBN is independent of ploidy level and is determined based on cross compatibility using standard EBN test crosses. Crosses between species with different EBNs are very often unsuccessful, whereas crosses between species with the same EBN number are frequently successful, even if they have different ploidy levels (Johnston and Hanneman, 1980).

The basic chromosome number in the genus *Solanum* is twelve. *S. tuberosum* subsp. *tuberosum* can be diploids (2n = 2x = 24) or tetraploids (2n = 4x = 48). The diploid form is found primarily in South America, while the tetraploid form is cultivated all over the world. The tetraploidy of cultivated *S. tuberosum* subsp. *tuberosum* originated either from autotetraploid (doubling of the chromosomes of a diploid species) or from allotetraploid (doubling of the chromosomes of a diploid hybrid between two related species) (Hawkes, 1990; Andersson and de Vicente, 2010).

Due to complex chromosome segregation ratios, polyploid crops are inherently more difficult to breed (Hoopes and Plaisted, 1987). Furthermore, vegetatively propagated crops like potato are often poor seed producers due to partial or full sterility. Additionally, continued self-pollination of *S. tuberosum* can lead to large inbreeding depression due to the fact that many traits are determined by non-additive genetic effects (Gopal and Ortiz, 2006).

Potato breeders have developed methods for overcoming this hybridisation barrier, such as ploidy manipulations, bridge crosses, auxin treatments, mentor pollinations, and embryo rescue (Jansky, 2006). Using these effective tools, potato breeders can gain access to the promising traits present in wild potato species.

However, the inherent complexity of genetics makes potato breeding time-consuming. Polygenes are believed to underlie quantitative resistance, which is difficult to maintain intact during the breeding process. Thus, the selection cycle, from initial crosses to variety release, requires approximately 10 or sometimes even more than 30 years (Gebhardt, 2013, Haverkort et al., 2009). Consequently, to overcome these hurdles, marker-assisted selection is applied to reduce breeding time and molecular biology techniques to overcome inter-specific hybridisation barriers (Song et al., 2003; Van der Vossen et al., 2003), both of which significantly speed up the breeding process. By employing cisgenesis, in which genes obtained exclusively from cross-compatible species are used in their native state, efficient stacking of multiple resistance genes can result in potato varieties with a more durable resistance to late blight. Proof of concept has been attained in the DuRPh programme in the Netherlands (Haverkort et al., 2016) as well as in the UK and Belgium (Haesaert et al., 2015), and locally popular varieties are presently being addressed for late blight resistance using cisgenesis. Other novel breeding techniques, such as intragenesis and genome editing, are being used in potato to engineer novel traits such as lower content of asparagines (for lowering amounts of acrylamide produced during heating) and reducing sugars (Cardi, 2016).

The genome sequencing of potato was completed in 2011 based on the DNA from two different diploid genotypes (The Potato Genome Sequencing Consortium, 2011). The sequence information of the potato genome with a size of 844 Mb revealed 39,031 protein-coding genes, suggesting a paleohexaploid duplication event during genome evolution. This genome sequence information, as well as supporting

phylogenetic research on the genus *Solanum*, is also helping to expedite the genetic improvement of potato.

3.3. Reproduction

Potatoes flower under long day conditions, moderate temperatures, high humidity and availability of sufficient soil nutrients (Kumar et al., 2006). The percentage and duration of flowering as well as the influence of environmental conditions on flowering is highly determined by cultivar (Burton, 1989). The flowers can be cross fertilised by insects, but are largely self-pollinated. Wind pollination is of minor importance (White, 1983). The extent of pollen dispersal in potato is related to the pollinating insect species, weather conditions and the fertility of the cultivar (Treu and Emberlin, 2000).

Flowering starts on branches located near to the base of the plant and proceeds upwards. Each flower will typically remain open for two to four days, with the stigma being receptive and pollen being produced for approximately two days (Plaisted, 1980). Fertilization occurs approximately 36 hours after pollination (Clarke, 1940). Viable seeds require a minimum of six weeks to develop.

Flower development does not ensure fruit set, and pollen sterility is frequently encountered under field conditions in parts of Europe (Anonymous, 1996). Very early varieties can complete their vegetation cycle before they start to flower. In some cases, flowers are set but abort early. Some cultivars may also exhibit male sterility, and/or inability to set fruit (Gopal, 1994). The berries are toxic due to the presence of glycoalkaloids (Bailey and Bailey, 1976).

Potato pollen is small and round with little or no ornamentation (Symon, 1981; Mali et al., 2014). Pollen sterility is the most important obstacle to sexual recombination of potato dihaploids (Gorea, 1970; Carroll and Low, 1976; Iwanaga, 1984; Ross, 1986). Pollen sterility and varying levels of pollen fertility can be caused by inbreeding depression as a result of dihaploidization (Carroll and Low, 1976) or by the interaction of nuclear genes and cytoplasm in dihaploids (Howard, 1970).

Many *S. tuberosum* cultivars exhibit reduced fertility, and this may limit their ability to hybridise. Male sterility, premature flower drop and the inability to set fruit are common (Gopal, 1994; Sleper and Poehlman, 2006). Male sterility may result from deformed flowers with anthers that do not dehisce or produce shrivelled microspores. Pollen may not form at all or the pollen may be of poor quality (Sleper and Poehlman, 2006). In a study of 676 tetraploid *S. tuberosum* accessions from 25 countries, it was found that in 20.4% of the accessions, flower buds dropped prematurely and 23% of the accessions were found to be completely male sterile (Gopal, 1994). Pollen sterility occurs frequently in *S. tuberosum* and ovule sterility occasionally; many varieties do not produce any seed.

The cultivated tetraploid *S. tuberosum* subsp. *tuberosum* is self-compatible, although most of the related diploid species are self-incompatible. The S alleles occur in this species, but somehow the incompatibility system is weakened. The mechanism behind this is not known. Plaisted (1980) has shown that under field conditions selfing is most likely for tetraploid *S. tuberosum*, with 80-100% of the seeds formed due to selfing.

Hanneman (1995) reported that the occurrence of unreduced gametes is a common phenomenon in *Solanum* species. In most *Solanum* species, additional to the normal haploid gametes (n), unreduced gametes (2n) can be found that greatly extend the possible number of natural crosses. Also Watanabe and Peloquin (1991) observed the production of 2n pollen in most of the 38 examined tuber-bearing *Solanum* species with a frequency varying from 2 up to 10%. The occurrence of unreduced gametes in *Solanum* spp. provides an exception to the general rule that crosses between species with differing EBN are not successful.

S. tuberosum plants produce rhizomes (often called stolons) that have rudimentary leaves and are typically hooked at the tip. They originate from the basal stem nodes, typically below ground, with up to three rhizomes per node (Struik, 2007). Tubers, spherical to ovoid in shape, are swellings of the rhizome at the end of underground stolons. They maintain the characteristics of the above ground stem, such as nodes, internodes, scale leaves, and lenticel pores. Tubers have two ends – the bud end and stem end, the latter of which is attached to the stolon.

Potatoes are very easily regenerated with the use of *in vitro* tissue culture techniques. This form of vegetative propagation normally leads to genetically identical individuals, but considerable heterogeneity is common after tissue culture in which a callus stage is included. This variation is called somaclonal variation. *S. tuberosum* subsp. *tuberosum* is,

like all potatoes, quite prone to this kind of variation (Cutter, 1992; Hawkes, 1990).

Information on the dispersal of true potato seed is somewhat lacking. Birds are unlikely to distribute the seeds because the berries are green and inconspicuous, although Hawkes (1988) suggests that the distribution of berries by small (or perhaps large) mammals is possible due to their sweet and aromatic nature. However, there is no mention regarding the toxicity of the berries and whether this may impede browsing by animals. Love (1994) reports that true potato seeds can survive and germinate for periods of time in excess of seven years, whilst Lawson (1983) showed that in Scotland true potato seeds could be stored in the ground for up to ten years without losing viability. However, a long dormancy period of true potato seeds makes the resulting plants weak competitors with cultivated crops during a particular cropping year.

In practice, the seed is seldom used in commercial plantings and mostly utilised in breeding programmes. Most common is vegetative propagation using tubers. The major disadvantage of true potato seeds is that they segregate for numerous traits because of high potato heterozygosity, and plants arising from true potato seeds typically take longer to establish than seed tubers, resulting in lower yield than from seed potatoes (Pallais, 1987).

Potato is vegetatively propagated, meaning that a new plant can be grown from a potato tuber or piece of potato tuber. On the surface of the tuber are axillary buds with scars of scale leaves that are called eyes (Struik, 2007). When tubers are planted, the eyes develop into stems to form the next vegetative generation. Thus, tuber formation is a method of reproduction, as each plant produces a multitude of tubers, each of which can theoretically develop into a new plant. The eyes on the tubers are buds that can sprout and develop into new stems. During the growing season tubers are produced continuously leading to the first tubers being the biggest, with smaller fertile tuber as small as one centimetre in diameter. The tuber acts as a source of nutrients for the new plant, and plants grown from tubers tend to have more early vigour than those grown from true potato seeds (Hoopes and Plaisted, 1987). Vegetative propagation may transmit diseases into successive generations.

4. Review of available information on adventitious GM presence in potato crop production

4.1. Seed potato impurities

Potatoes are vegetatively propagated by planting tubers or tuber pieces, while true potato seeds are normally used in breeding programmes. Because of the clonal propagation of commercial potatoes, the risk of affecting seed potato supplies through cross-pollination is negligible. Commercial seed potatoes are certified for purity before distribution to potato producers and contamination with a different cultivar by tubers from volunteer potatoes as well as mixing during sorting and grading can lead to small amounts of admixture. Although this is not critical in the fresh or processing market, it may lead to the rejection of the harvest for seed production (Steiner et al., 2005).

The two important parts of EU legislation covering the purity requirements of seed potatoes are the Council Directive 2014/20/EU determining Union grades of basic and certified seed potatoes and Directive 2001/18/EC on the deliberate release into the environment of GMOs. In annex I and II of the Council Directive 2014/20/EU the conditions which must be satisfied by seed potatoes are laid down. For basic seed potatoes the number of plants not breeding true to the variety and the number of plants of a different variety shall, together, not exceed 0.1%. For certified seed potatoes, the number of plants not breeding true to the variety and the number of plants of a different variety shall, together, not exceed 0.2%.

In terms of adventitious GM presence, there are no tolerance thresholds (for authorised or unauthorised GM events) laid down for the marketing of conventional seed potatoes in the EU. In order to avoid potential admixture of GM seed potato, official controls of conventional seed potatoes are regularly applied by Member States of the EU. However, these controls differ between the countries. In the following paragraphs information is presented for different Member States.

4.1.1. Approach to adventitious GM presence control in selected Member States

Many countries like Denmark, Estonia, Greece and Spain do not apply any controls for the adventitious GM presence in potatoes.

Belgium

In Belgium, about 2,200 ha of seed potatoes were grown. Clear guidelines, taking into account e.g. soil quality, diseases, isolation distances etc., describe how seed potatoes have

to be grown. Before, during and after production the whole process is controlled by the regional inspection services, i.e. the Product Quality Management Division of the Department of Agriculture and Fisheries for the Flemish Government and Direction de la Qualité for the Walloon region. If all quality criteria are met, these authorities provide certification for the seed potatoes. Only certified seed potatoes can be traded. The top varieties of which seed potatoes were produced in Belgium in 2016 were Bintje (487 ha), Spunta (230 ha), Fontane (226 ha), Agria (153 ha) and Royal (81 ha). In particular cases, farmers also have the opportunity to use their own, farm-saved seed potatoes ('hoevepootgoed').

Czech Republic

In the Czech Republic, the GM potato variety "Amflora" was tested on an area of approximately 50 ha and was commercially cultivated by Czech farmers in 2010. After the cultivation of Amflora was stopped, fields on which Amflora potatoes had been cultivated were monitored for several years by the Czech Environmental Inspection together with the company BASF. Volunteers were recorded. In addition, the Czech Food and Feed Inspectorate tested for two years the possible occurrence of GM potato starch in commercial starch, using validated methods of DNA extraction and Amflora potato detection. No GM potato starch was detected.

Finland

GM potatoes were grown in Finland only for research purposes during 2009-2010 by the Potato Research Institute; GM potatoes are not cultivated for commercial use. No coexistence legislation for GM potatoes has been established in Finland, but still farmers have the obligation to notify the cultivation of GM varieties.

Germany

Germany established GM control inspection guidelines for standardised sampling, sample preparation, analysis, and assessment of results in 2006 and subsequently adjusted them in 2010 and again in 2014. Although the focus is on maize and rape seeds, other species including potato are also considered. The results of seed monitoring for GM percentage are available before seeding to avoid post-sowing enforcement activities. In the case of potatoes, fewer samples are taken, but on a regularly basis. The official GM monitoring programme for seed potatoes is only applied to seed potatoes produced within Germany.

The control programme consists of two steps and is based on existing routines and processes of official seed certification and phytosanitary controls. Step 1 is the official field inspection for varietal identity and purity while step 2 is the official investigation of seed potatoes for GM admixture in the laboratory. Samples for GM-analysis are taken from 10% of the seed crops (fields) where admixture has been observed through field inspection. A sample of 200 tubers is taken for every 3 ha either from the field or during storage. The laboratory used for the analysis is accredited for the purposes of PCR analysis for the detection of GM potatoes.

The first year of analyses for the presence of GM was 2011 where 15 samples were taken from the Federal State of Mecklenburg-Vorpommern. No admixture was detected in 2011 or in the years since. Sampling has been continued in 2012 (52 samples), 2013 (51 samples), 2014 (61 samples) and 2015 (19 samples).

Greece

In Greece, basic and certified seed potatoes are imported from other Member States (the Netherlands and Cyprus lead the market) for planting as the domestic seed production is limited with the main production area on the island of Naxos (Kyklades) but also in Tripoli (Peloponissos), Ioannina (Ipiros) and in Thessaloniki (Makedonia). In these regions, private companies and local agricultural co-operatives are responsible for the production of certified seed under the control of the local Departments of the Decentralised Agricultural Development (T.A.A.) of the Ministry of Rural Development and Food.

In the last ten years, potato growers tended to plant basic and certified seed potatoes of high productivity and resistant to pathogens. The main prospective for potato production in Greece is to increase the limited seed potato production in order to reduce the dependence of Greek potato growers on imported seed potatoes.

Lithunia

In Lithuania, nine samples from potato crops were tested for GM admixture in 2015 and showed no presence of GM. No samples were taken from seed potato crops in 2015.

United Kingdom

No GM potatoes are grown commercially in the UK at present, although a number of experimental trials of GM potatoes have been carried out. The Genetic Modification Inspectorate (GMI) for England, based at the Animal and Plant Health Agency, has designated responsibility for ensuring compliance with legislation pertaining to the deliberate release to the environment of genetically modified organisms in England. This includes (where appropriate) carrying out audits of companies that market seed of conventional crops, to assess whether they have appropriate controls in place to minimise the risk of adventitious GM presence (AGMP) in the material they handle. The GMI has assessed the risk of AGMP in potatoes for planting and has concluded that this risk is very low compared to other crops. Consequently the GMI does not currently conduct audits of seed potato producers and/or retailers in England. UK seed potatoes are subject to statutory inspections in terms of varietal purity and freedom from disease.

4.1.2. Registered potato varieties in selected Member States

In the following paragraphs information is presented about the number of registered varieties and their ability to flower for selected EU Member States. Segregation requirements for seed potato production are added if available.

Austria

The Austrian national catalogue contains 48 potato varieties. On about 1,600 ha seed potatoes are produced. The estimated rate of using farm saved seed potatoes in Austria is about 40 – 50%.

Croatia

In the national catalogue of Croatia, 50 potato varieties are registered. In the season 2014/2015, 514,621 kg of seed potatoes were certified for the Croatian market.

Denmark

In Denmark approximately 113 varieties are in the national catalogue. Information on the proportion of male sterile and fertile varieties is difficult to obtain. In 2015, pre basic seed were produced on 239 ha and basic seed on 4,310 ha. In the Danish propagation of seed potatoes the segregation requirements regulate a distance of 50 m for pre-basic seed, of 25m for basic seed, and of 15 m certified seed to potato production fields.

Estonia

In Estonia, 10 varieties are registered in the national catalogue. Certified seed potatoes are produced on an area of about 200 ha. As segregation requirements, a separation distance of 50 m has to be met for pre-basic seed potatoes, whereas for basic seed potatoes 25 m and for certified seed potatoes 10 m are sufficient.

Germany

210 potato varieties are listed in the German national catalogue. However, only a limited number of these are grown in the field. Information in respect to fertility of these varieties is not given in the catalogue. According to the German potato breeders about 30% of the actual potato varieties are sterile. The proportion of sterile varieties is particularly high in starch potatoes.

In 2014, the demand for seed potatoes represented about 0.55 million ha. Around 70 – 75% of these are produced in Germany. For seed potato production, contracts between breeders and farmers are closed. Breeders clearly describe the obligatory production management in annual newsletters. In addition, legal regulation from the German seed marketing act, plant certification, and plant breeders' rights have to be taken into account. It is important to note that only about 50% of seed potatoes in commercial production are certified seed, the other 50% being farm saved seed.

Greece

The current National Catalogue of Greece contains 18 potato varieties which are all fertile. The National production of certified seed potatoes (4-year average, 2012-2015) is approximately 1.191 t.

According to Greek Legislation (Ministerial Decision 276357/29-07-2002, National Gazette 1020/05-08-2002: "Technical Regulation for the certification and control of

potato propagating material for cultivation"), the field requirements for seed potato are:

for basic seed potato production:

- 10m distance for potato cultivation for consumption
- 5m distance from seed potato crop of lower class
- 3m distance from seed potato crop of another variety of the same class
- one skip row to seed potato crop of the same variety and class.

for certified seed potato production:

- 10m distance for potato cultivation for consumption
- 2m distance from seed potato crop of another variety of the same class
- one skip row to seed potato crop of the same variety and class.

In Greece, commercial potato production relies 100% on certified seeds. However, there are also growers who cultivate potatoes for their own use and possibly save seed potatoes for next cultivation. Since these growers are not registered, the kind and quantity of seed potatoes cannot be controlled.

Hungary

There are 60 potato varieties on the Hungarian National Catalogue. While ware potatoes were produced on 18.000 ha in 2015 with a total yield of 412.000 t, 32 varieties are grown for seed multiplication purposes on 181 ha. Approximately only 15% of the ware potato production relies on certified seed potatoes.

There is a 200 m isolation applied between seed potato and ware potato production on the field to protect crops from aphids transmitted virus infections. Each field is inspected at least 4 times a year, and each potato field is tested for quarantine pests.

Lithuania

In 2016, 22 potato varieties were included in the Lithuanian list of plant varieties. The quantity of certified seed potatoes grown in Lithuania during the last five years ranged between about 2,800 t in 2015 and 3,200 t in 2011. In 2015, approximately 2,800 t of seed potatoes were produced. Segregation requirements for propagation of seed potatoes were established in the Order of the Minister for Agriculture ("Concerning Mandatory Requirements on Seed Potatoes Intended for Placing on the Market", 2015 December 18, No. 3D-938).

The Netherlands

In the Netherlands, there were 511 consumption and 77 starch varieties on the national list in 2016. No information is available about the fertility of the registered varieties. Some information is presented in cultivation manuals pointing out profuse berry production e.g. by the varieties Désirée, Hansa, Morene, Saturna, and Van Gogh, whereas poor berry formation is described in, for example, the variety Bintje. In 2014, 1,475,000 t of seed potatoes were produced in the Netherlands (~70% for export) on an area of 39,874 ha. 38,626 ha were inspected and 1,083,000 t certified by the inspection service NAK (The Dutch General Inspection Service). As segregation requirement for propagation of seed potatoes, a separation distance of 3 m to other potato cultivations has to be met. About 10% of the starch potatoes are grown from farm-saved seeds (one round of multiplication).

Spain

Most of the potato varieties grown in Spain are registered in the European Common Catalogue, but not in the Spanish Catalogue. Around 40,000 t of seed potatoes are produced on an area of 2,300 ha. Around 75% of seed potatoes are certified seed and 25% farm saved seed.

United Kingdom

In 2015 there were 183 varieties on the UK National List. The most popular variety in terms of production is Maris Piper (a main crop multipurpose variety), accounting for around 15% of planted area in 2015. This is followed by Markies (a popular variety for chipping) at around 6%.

4.2. Potential admixing during cultivation

4.2.1. Outcrossing to wild relatives

Numerous biological and geographical obstacles make gene flow from cultivated potato varieties to the two wild relatives in Europe, *S. nigrum* and *S. dulcamara,* a highly unlikely occurrence, and there have been no reports that such crosses have ever occurred naturally (Love, 1994; Spooner et al., 2004). In most parts of the world, no *Solanum* species from the section *Petota* with a ploidy level and an endosperm balance number (EBN) of 2 or 4 will occur next to cultivated tetraploid *S. tuberosum* subsp. *tuberosum*. Crosses are therefore not likely, due to geographical isolation. Only in the southern United States and South America do potential crossing partners with a suitable EBN occur next to cultivated tetraploid *S. tuberosum* subsp. *tuberosum*. (OECD, 1997; Celis et al., 2004; Scurrah et al., 2008; Capurro et al., 2013).

Within the family *Solanaceae*, potatoes have a number of crop species as relatives, the closest being tomato (*Solanum lycopersicum*), as well as tobacco (*Nicotiana tabacum*), sweet pepper (*Capsicum annuum*) and petunia (*Petunia hybrida*). However *S. tuberosum* is not able to hybridise with any of the non-tuber bearing *Solanum* species outside of the section *Petota* (Conner, 1994; Love, 1994). There is also no evidence to suggest that intergeneric hybridisation can occur between potato and its related crop species (Treu and Emberlin, 2000). Other than potato, there are around 13 species within the genus *Solanum* found in various parts of Europe. Most of these species are introduced casuals, although some, including *S. dulcamara* (bittersweet nightshade) and *S. nigrum* (black nightshade) are native and common. Eijlander and Stiekema (1994) and McPartlan and Dale (1994) found that the cross of tetraploid *S. tuberosum* subsp. *tuberosum* with *S. dulcamara* did not result in any viable seeds and plants. For the cross of *S. nigrum* with *S. tuberosum* the same is valid. Therefore, the natural gene flow from potato to its wild relatives *S. nigrum* and *S. dulcamara* is highly unlikely. Eastham and Sweet (2002) concluded that naturally occurring cross-pollination and subsequent gene flow between potato and its related wild species in Europe is unlikely. Without the help of sophisticated embryo rescue techniques no viable hybrids between cultivated potato and its related wild species in Europe have been obtained. Also, it is likely, given the breeding barriers known within the genus that even if cross-pollination was successful, strong post-zygotic barriers would prevent the formation of a viable hybrid (DoE, 1994).

4.2.2. Outcrossing between GM and non-GM potato

Cross-pollination between fields of potatoes may be less significant than in some other crops since the potato tuber as a harvest product is not affected by the fertilisation of the plant with foreign pollen. Furthermore, the crop is almost exclusively sown with seed tubers rather than true seeds (Treu and Emberlin, 2000).

Outcrossing has primarily been observed to occur between adjacent plants and the rate of outcrossing decreases rapidly thereafter, with only small rates observed beyond 4.5 m (Conner, 1993; Dale et al., 1992; McPartlan and Dale, 1994; Tynan et al., 1990).

Tynan et al. (1990) measured outcrossing using a gene encoding a chlorsulfuron-insensitive form of acetolactate synthase as a selectable marker. They found that within the plot with marked potatoes, 1.14% of seedlings were resistant to chlorsulfuron, while between 0-1.5 m from the trial, only 0.03% of seedlings were resistant. At a distance of 1.5-3 m and 3-4.5 m, 0.05% of seedlings were resistant. No resistance was detected beyond 4.5 m.

McPartlan and Dale (1994) carried out a similar field experiment using the variety 'Désirée' transformed for herbicide tolerance. A central 20 m x 20 m plot of the transgenic potato plants was established, with non-transgenic sub-plots planted in four directions from the central plot at distances of 0 to 20 m. The frequencies of herbicide tolerant seedlings obtained from the non-transgenic potato plants were 2% in a distance of 3 m, 0.017% in case of 10 m distance, and 0% in a distance of 20 m.

In a study by Skogsmyr (1994) much higher rates of outcrossing using the variety Désirée transformed with the *npt*II and GUS marker genes as the pollen donor and Stina as the pollen receptor were observed. Rates were 72% at a distance of 0-1 m and 31% at 1,000 m. The authors attributed the high rates of outcrossing observed in this study to the behaviour of the predominant pollinator species found in the plots, the pollen beetle *Meligethes aeneus*, which tends to move together in large numbers and fly over large distances (Skogsmyr, 1994). This research was scrutinised by Conner

and Dale (1996) who concluded that there had been a large number of false positives during the PCR analysis of the *npt*II marker gene, giving the impression of high levels of gene dispersal. They collected outcrossing data from several field experiments with genetically modified potatoes, performed in New Zealand, the United Kingdom, and Sweden. In none of these studies was outcrossing detected when the pollen-receiving plants were separated by more than 20 metres from the genetically modified plants.

Another study from seven field-test sites over six seasons screened a total of over 1.3 million progenies from non-transgenic pollen-trap buffer rows (Erasmuson et al., 2005; Conner, 2006). The accuracy of this phenotypic screening was verified by PCR. In the buffer row immediately adjacent to the donor plot, the frequency of transgenic progeny ranged from 0.007 to 0.059% and declined to between 0 and 0.005% at the third buffer row from the field trial, representing a distance of 2.25 m.

Petti et al. (2007) also found higher rates of outcrossing between the varieties Désirée and British Queen, due to the latter's male sterility. Using a microsatellite marker system, they found evidence of out-crossing at the maximum distance studied of 21 m, but with very low frequency (23 germinating seeds from 140 berries).

Capurro et al. (2013) examined pollen mediated gene flow from a commercial potato cultivar to the compatible cloned genotype of the related wild potato *S. chacoense* Bitter in a field experiment in Argentina. Berry formation with hybrid seeds occurred at 30 m from the pollen source (1 out of 69 harvested berries contained 3 hybrid seeds). In another study outcrossing was investigated using a male fertile commercial potato cultivar as pollen donor and a male sterile cultivar as pollen recipient (Capurro et al., 2014). Three berries with seeds were collected from plants at a distance of 40 m from the pollen source; these contained 21, 22 and 70 seeds/berry, respectively. However, again a quantification of the results is difficult.

The extent of pollen dispersal undoubtedly varies with cultivar, climatic conditions during flowering and presence and frequency of pollination vectors. The majority of field studies have detected potato pollen at a maximum distance of about 20 m from the source (Eastham and Sweet, 2002).

Because potato is planted with seed tubers rather than true seed, any GM contaminant would not be transmitted to progeny crops (Eastham and Sweet, 2002).

4.2.3. Insect impact on cross-pollination

Cross-breeding and selfing is enhanced by some insects. In particular bumblebees (e.g. *Bombus funebris* Smith and *B. impatiens* Cresson) are good pollinators for potatoes (White, 1983). Potatoes possess apically dehiscent anthers that only disperse pollen to bees that vibrate the anthers to collect it (Roulston et al., 2000). This specific plant-bee mechanism is called "buzz pollination" (Buchmann and Hurley, 1978; Buchmann, 1983), meaning bees use their thoracic muscles to produce very high frequency vibrations that expels pollen from the anthers. Moreover, bumblebees preferentially visit the flowers of potato cultivars that produce viable pollen grains instead of cultivars that produce primarily unviable, shrunken pollen grains (Batra, 1993). Since potato flowers do not produce nectar, honeybees (*Apis mellifera* L.) and *Bombus fervidus* Fabricius are not pollinators of potato (Sanford and Hanneman, 1981). Moreover, honeybees do not practice buzz pollination and it is likely for this reason that they are uninterested in *S. tuberosum* flowers (Sanford and Hanneman, 1981).

It was observed that bumblebees are more likely to visit plants at the edges of plots as opposed to their centres, allowing them to stay closer to their nests (Batra, 1993; Free and Butler, 1959; McPartlan and Dale, 1994). Highest levels of berry formation were also recorded at the edges of plots, compared with the centre, suggesting that bumblebee activity was a contributing factor to pollination. Bumblebees will selectively visit different potato cultivars, preferring those with fertile pollen (Arndt et al., 1990; Batra, 1993; Sanford and Hanneman, 1981).

Besides Hymenoptera, the pollen beetle species *Meligethes aeneus* Fabricius has also been observed to transfer potato pollen in Europe (Petti et al., 2007; Skogsmyr, 1994).

4.2.4. Volunteers

The presence of volunteer potatoes and the resultant problems in crop rotations have been recognised for almost 80 years (Bonde, 1942; Fernow, 1959) and are the subject of continual research efforts. Volunteer potatoes appear to occur in virtually all crops to a greater or lesser extent on all farms where potatoes have been grown in the rotation (Askew, 1993).

S. tuberosum volunteers may develop either from true potato seed or from tubers that are left behind following harvest

(Andersson and de Vicente, 2010). While many potato cultivars are partially or fully sterile and rarely produce fruits, some cultivars are capable of prolific fruit and seed production. The amount of true potato seed produced in a given crop will depend on the cultivar as well as environmental conditions, particularly photoperiod, temperature, plant density, and nitrogen supply (Askew, 1993; Struik, 2007). However, the early growth of seedlings from true potato seed is slow compared to that of plants growing from tubers, and daughter tubers are generally smaller as well (Pérombelon, 1975; Rowell et al., 1986). Therefore, the majority of volunteer potato plants originate from tubers (Bond et al., 2007).

Most potato volunteers are the result of harvesting methods of commercial potato production, and the fact that potatoes keep on producing a magnitude of small tubers which are not picked up by commercial harvesters or are lost in the process of loading and transport of the harvest. The number of potato tubers left on top of the soil or up to 20 cm underground following harvest varies greatly and ranges approximately between 20,000 and 460,000 tubers/ha (Lutman, 1977; Kempen et al., 2005, Pérombelon, 1975; Steiner et al., 2005), with most of these tubers being small in size. Due to this great variability, the exact effect of this phenomenon on different following crops is unpredictable. Moreover, the small size of some of the viable tubers results in an underestimation of tuber volunteers as they may not have been identified (Askew and Struik, 2007). Rahman (1980) reported 367,000 tubers per hectare; this corresponds to 10% of the potential yield or 1 – 4 t/ha and represents a total potential population of 2 to 30 volunteer potato plants per m^2 (20,000 to 300,000 plants per ha). Phelan et al. (2015) reported an average post-harvest tuber loss of 141,758±911 tubers per ha, with a maximum of 210,513±973 and a minimum of 39,082±669. As a consequence, volunteer establishment in the following crop ranged from 400±59 plants per ha to 55,698±47 plants per ha. These data correlate quite well with previously reported values by Andersson and de Vicente (2010) of up to 20% of tubers left in the soil being able to sprout in the next season. The persistence of viable daughter tubers as small as one centimetre in diameter is an exacerbating factor in volunteer management.

In areas with mild winters, it is estimated that it may take up to 4 – 5 years to get rid of *S. tuberosum* volunteers grown from daughter tubers in most arable crops (Makepeace and Holroyd, 1978).

Tubers on top of the soil and up to 10 cm below the surface are often exposed to low temperatures during winter and are killed by temperatures below -2°C, but the deeper tubers may be insulated from the cold by the soil. These findings are further influenced by snowfall, stubble and soil cover crops which all serve as insulation. Under Finnish conditions all potato tubers planted at soil depths of 10 and 20 cm were killed by frost during two out of three winters. However, in one winter when the field was covered with 30-40 cm of snow and the soil temperature ranged between -0.4 and -0.9°C up to 3.5% of tubers survived (Mustonen et al., 2009).

Under Canadian conditions potato plants will not proliferate and become established as weeds; volunteers were detected just periodically near animal feed lots, waste disposal sites or in the vicinity of production sites (Anonymous, 1996). The restriction to such habitats in Europe would seem consistent with the findings of Evenhuis and Zadoks (1991), who assert that this is caused by the limited competitiveness of *S. tuberosum*. *S. tuberosum* is not a primary coloniser in unmanaged ecosystems, and seedlings do not tend to compete successfully with plants of a similar type for space (Anonymous, 1996). However, research on the subject should continue to ensure new varieties do not lead to an increase in feralisation (Treu and Emberlin, 2000). Therefore, although *S. tuberosum* can be cultivated throughout Europe, it is unlikely to grow outside of cultivation areas (Holm et al, 1979; Muenscher, 1980; Love, 1994; OECD, 1997). Potatoes are not known to escape from fields (become feral) or show weedy potential.

4.2.5. Volunteer management

Volunteer potatoes affect crop production in four ways (Petti et al., 2007; Kim et al., 2010):

- competition with the following crop;
- transmission of pests and diseases to the next crop;
- the contamination of the succeeding crop during crop rotation; and
- possible spread of transgenic material to other potato plants through pollen or seed.

The potato tuber is a living organism and can thus protect spores or eggs of pathogens and their vectors until the next season. Even if disease is not a problem for the follow-on crop, the persistence with which volunteer potato tubers can reproduce year after year in the soil can lead to the next

potato crop and in some cases also neighbouring potato crops to be seriously infected with a pest or disease (e.g. *Phytophtora*). These pests and diseases can not only have a direct effect on yield, but cause problems during storage after harvest as well. Like volunteer potato tubers, volunteer potato plants can also act as hosts for insect vectors, especially aphids that commonly carry plant pathogenic viruses (Thomas and Smith, 1983). For these reasons, farmers usually grow potatoes only every third or fourth year within a crop rotation.

Although this practice may prevent the carryover of potato diseases to healthy plants in the following season, there is still the competition of volunteer potatoes with the following crop for water, nutrients and light leading to lower yields. The more volunteers appear, the larger is the effect on the yield of the successive crop. Therefore, it is imperative that volunteers are controlled as quickly as possible within a crop rotation. Additionally, if volunteer potato plants are not controlled, they can regenerate within the rotation crops so that they ultimately carry over to contaminate the following potato crop. Therefore, controlling these plants is very important, but also difficult, and can only be achieved successfully using integrated management methods.

Potato tubers have a fairly low frost tolerance; shallow tubers and those exposed to the surface are often destroyed by frost. In regions with subzero temperatures during winter, delayed or no ploughing during the preparation for the next crop contributes to volunteer reduction. By ploughing deeper, buried tubers can be lifted up and be exposed to lethal frost temperatures (Thomas and Smith, 1983). Soil cultivation like ploughing can also transfer tubers deeper into the soil, and thereby protecting them against freezing (Boydston et al., 2006). In temperate climates up to 20% of tubers left in the soil show no dormancy and will sprout in the next season (Andersson and de Vicente, 2010). Soil temperatures below -2.8°C have been shown to result in significant tuber mortality (Boydston et al., 2006). Thus, in areas where *S. tuberosum* is grown commercially, the measures required to control *S. tuberosum* volunteers do not differ from the cultural and pest management practices that are usually applied in a crop rotation.

However, several methods of volunteer management have been developed and it has to be decided on a regional scale which ones are most appropriate. In general, it is better to follow a holistic, multi-pronged, management approach to face this problem.

There are basically five approaches that can be used to manage volunteer potato plants: preventative, cultural, mechanical, biological, and chemical. Preventative management is used to avoid the introduction of volunteer tubers to a field, so focussing on the cause of the problem. Cultural management relies on the use of cropping practices to either reduce the occurrence of the problem, or to create an environment that is less suitable for the survival of the volunteer potato plants. For example, plants that are very competitive with potatoes can be used in the rotation system, planning the rotation system in such a way that suitable herbicides can be applied without damage to subsequent crops. Mechanical management relies on the use of farming equipment to either remove or destroy the tubers or volunteer potato plants mechanically before they can create a major problem. An example of such a mechanical management is shallow tillage following harvest. Biological management relies on living organisms, such as natural enemies in order to suppress volunteer potato plants. Most commonly, chemical management methods are used to control weeds of any type. In order to control volunteer potatoes, this might include the use of suitable herbicides and soil fumigants within the rotation crops to kill the potato plants, as well as sprout inhibitors to prevent tubers from sprouting.

All of these methods should be considered in a collaborative approach in order to be able to successfully address the problem of managing volunteer potatoes (Steiner et al., 2005). It should be kept in mind that agronomic practices and pest control measures have to be site specific and adapted to potato cultivation and crop rotation.

Some varieties of potato are capable of producing large numbers of true seed as well as tubers if not controlled, and although the main volunteer problems are caused by the tubers, germinating seeds can also cause problems if plants are allowed to form seed. Since no single method is fully effective for controlling *S. tuberosum* volunteers, an integrated weed management approach is recommended.

4.2.5.1. *Preventative management*

Preventative management is one of the most cost effective measures for controlling volunteer potatoes. These strategies consist of any measure that reduces the number of tubers that remain behind in the field following harvest, and can easily be incorporated into a holistic approach to volunteer potato management. According to Steiner et al. (2005) the management procedures that are applicable to prevent volunteer potato plants emergence are harvester management, proper harvest time (e.g. plants have to be completely

dead rather than still green), and the use of a sprout inhibitor. In some cases these procedures need to be coupled with the agronomic management of the crop.

During mechanical harvest smaller tubers stay in the soil or on the surface, medium tubers are mainly lost during the harvesting process and even large tubers can fall from the harvester and transport vehicles. Hand weeding during or after harvesting is an effective, although time-consuming, method for controlling *S. tuberosum* volunteers, and grazing has also been applied in some countries (Rahman, 1980; Steiner et al., 2005).

Phelan et al. (2015) pointed out that reducing tuber loss at harvest plays a central role for the reduction of volunteer quantity. They further stipulated that reducing the level of harvest loss would require either a re-engineering of the harvester to include an additional mechanism to collect tuber pieces/unsalable tubers or a reversal of current practises towards the removal of all harvested material from the field for processing and grading. As both options have cost implications for the grower, any motivation to pursue either option will only occur in the presence of a financial benefit and/or due to a regulatory decree.

4.2.5.2. *Mechanical control*

During soil preparation for the succeeding crop the soil is disturbed and tubers and tuber pieces will start to germinate and be well established by the time the following crop is planted. Favourable weather conditions in terms of rain and temperature will enhance potato growth (Steiner et al., 2005).

Improving the efficiency of the harvesters at separating tubers from soil would reduce the number of tubers left behind as potential volunteers. Some harvesters have been developed for *S. tuberosum* that retain or crush tubers that would normally be lost during harvest (Rahman, 1980; Steiner et al., 2005). Crushers can be used to destroy tubers, although their efficiency varies with soil type and environmental conditions, and they are not effective for small tubers with a size of 1 cm or less (Rahman, 1980).

Ploughing tends to bury tubers deeper, which will protect them from frost, allowing them to survive longer in times with unfavourable conditions (Lumkes and Beukema, 1973; Rahman, 1980). Tubers at the surface may also be more prone to rotting and their earlier germination allows them to be controlled with pre-planting herbicides. Non-turning soil cultivation or shallow harrowing is therefore recommended (Lumkes and Beukema, 1973; Phelan et al., 2015).

Proper management of the harvesting process reduces the number of lost tubers, which not only results in a reduction of volunteer potatoes in the following season, but also increases yields. According to Steiner et al. (2005) the following steps help to minimise the number of tubers that are lost during harvest:

- The blade depth should be managed in a way to ensure that all tubers are removed from the soil. If the blade is too shallow not all the tubers will be lifted and some will be sliced, so leaving a portion of these tubers behind in the soil. This should be coupled with the agronomic practices to ensure that the earthing up is sufficiently high so that all tubers will develop within the ridge;
- Tubers should be removed from the haulms by the harvester so that they are not carried off of the harvester;
- The trucks that receive the tubers from the harvester should be positioned in a way to prevent spillage;
- Harvesters should be operated in a way to avoid pushing tubers out around the throat of the harvester;
- Soil separation and tuber transport should be maximised by using the optimal ratio of forward speed to chain speed;
- The gaps between the links in the primary chain should be set in a way to reduce the number of tubers that fall through the chain, but this must be compatible with the intended market.

The condition of the potato vines at harvest has been found to play an important role as both premature senescence of vines and green versus dead plants affect the number of tubers that are left in the soil after harvest as well as the depth at which tubers are formed in the soil (Steiner et al., 2005). Agronomic factors such as soil fertility and soil moisture management as well as pest and disease control can contribute to premature vine senescence. Plants that senesce early produce a greater percentage of small tubers than those plants that mature later, and therefore more tubers will remain behind on the field at harvest. Additionally, plants that are still green at harvest and must be defoliated prior to harvest, produce more large tubers than dead plants at harvest. Steiner et al. (2005) state that in the Washington

state area of the USA the numbers and sizes of tubers from green plants are double that harvested from dead plants.

In a study carried out in Washington it was found that 75% of the tubers were within 10 cm of the soil surface if plants were still green at harvest in contrast to only 34.2% if plants were already dead (Steiner et al., 2005).

As sprouts from tubers buried as deeply as 20 cm below the soil surface still can emerge, it is crucial that the harvester is able to reach this depth to catch all those tubers. This should be combined with ridge planting.

In the northern climatic conditions mid-winter and early spring ploughing can bring buried tubers to the surface and expose them to low temperatures. This may be combined with fumigation and sprout inhibiting hormone treatment. In some cases animals were released into the fields to graze, but this has to be handled with care (Thomas and Smith, 1983).

Mechanical control has proven to be far more effective when it followed the application of herbicides (Allemann and Allemann, 2013). The efficiency of all herbicide treatments can be improved by combining them with a tillage operation (Boydston and Seymore, 2002).

4.2.5.3. *Chemical control*

A seed potato usually provides enough nutrients for 30 days of growth. The smaller the tuber and the deeper it is buried in the soil, the smaller the chance that the stem will emerge. It normally takes between 10 and 20 days for the above-ground parts to produce enough photosynthates to become independent of the tuber. At this stage the plant is most sensitive to herbicides as few if any daughter tubers will have already been formed (Colquhoun, 2006).

Volunteer potato plants are very difficult to eradicate using herbicides, with most products tested proving to be either ineffective or only partially effective at best (Rahman, 1980). The greatest problem is caused by the biology of the potato tuber, as large food reserves available in the parent tuber, coupled with a number of adventitious buds that can sprout after the death of the apical sprout, enable recovery from damage that would be lethal to most other plants. The problem is further compounded by the variation in the time of emergence of volunteer potato plants. Potato volunteer emergence usually takes place long after many crops have been planted, which makes application of many post-emergence (foliage-applied or contact) products very difficult to time correctly to obtain good control (Lutman, 1977). As contact herbicides will only affect the plant parts they come into contact with, the parent and/or daughter tuber is able to produce new sprouts which then emerge long after the primary plants have been killed (Rahman, 1980).

The only way to prevent tuber production is through complete shoot removal prior to the shoots initiating tubers.

Use of single conventional herbicides has proven to be unsuccessful in the control of volunteer potatoes. Due to the devastating effect these plants have on succeeding crops such as carrots and onions as well as grains such as maize, various regimes of herbicide combinations have been investigated and limited and varied success has been achieved (Koepke-Hill et al., 2010).

Soil application of herbicide treatment allows tubers to be exposed to the herbicide for a longer period of time, and soil-applied herbicides are readily available for absorption by the roots of developing potato sprouts, so making this an attractive option for control of volunteer potatoes.

One of the biggest advantages of foliage herbicide application (post-emergence) is that the extent of the weed problem is already evident, and spot treatments can be used rather than applying herbicide over the entire field. Generally, post-emergence applications should be considered when the potato plants are starting to initiate tubers on the stolons.

Volunteer potato control of between 80 and 90% was demonstrated in research trials conducted at Michigan State University using 92 g/ha tembotrione or 5 g/ha topramezone (Everman et al., 2010). Tembotrione belongs to the same family as mesotrione but is not persistent in the environment except when present in loamy sands. It has a high mobility in the soil and the potential to leach into ground water, but the relatively rapid rate of biodegradation may alleviate this process (EPA, 2007).

Since 1974, evidence about the efficacy of glyphosate as a post-emergence herbicide on potato has been amassed (Rahman, 1980). This product can be applied prior to planting or after harvest (Steiner et al, 2005). The greatest advantage of this herbicide is that it does not only kill the aerial parts of the plant, but is also translocated to the underground parts, including the early-formed tubers. Field trials demonstrated excellent control of potatoes with application rates in excess of 1 kg/ha, if applied sprouts had fully emerged at the time of treatment.

Boydston (2001) reported that applications of 2, 4-dichlorophenoxyacetic acid (2, 4-D or fluroxypr) can significantly reduce volunteer numbers in follow-on maize crops. However, the study of Phelan et al. (2015) revealed that multiple factors (e.g. machinery performance, timing of application relative to volunteer growth stage, appropriate chemical mix preparation, environmental conditions at time of application) influence herbicide efficacy. In one of their field surveys, fluroxypr applications were found to significantly reduce the number of volunteers in follow-on crops by up to 96%. In another survey the observed reduction of more than 65% in tuber weight in the second year was not found to be related to herbicide usage across the fields.

Considering the information provided on single herbicide applications in conjunction with the biology of volunteer potato plants, it is not surprising that a great deal of research has been conducted on the use of more than one herbicide to control these plants.

Sprout inhibitors are applied mainly to prevent sprouting of tubers during storage of harvested potatoes, but can also be applied to plants at the end of the growing season. This prevents the formation of small unusable tubers, which are often the source of volunteer potato plants. These chemicals inhibit cell division, and should therefore not be applied to seed potato fields or where spray drift can contaminate seed potato fields (Anonymous, 2011). According to Rahman (1980), three chemicals are available that effectively inhibit sprouting in potato tubers: maleic hydrazide (MH), chlorpropham (CIPC [isopropyl N-(3-chlorophenyl) carbamate]) and TCNB (tetrachloro-nitrobenzene). Newberry & Thornton (2007) studied the suppression of volunteer potato emergence with MH and concluded that success is cultivar and tuber-size dependent. Suppression was least in the smallest tuber category. MH treatment reduced emergence of treated tubers in all size categories and all cultivars tested and should be considered for use in integrated weed management plans Phelan et al. (2015) also reported that the application of a sprout suppressant prior to harvesting of potato crops proved a very effective method of volunteer control, with the suppressant eliminating volunteer emergence through two succeeding rotational crops.

Soil fumigation consists of the introduction of a volatile compound into the soil, primarily to suppress nematodes and other soil pathogens in crop rotations (Thomas and Smith, 1983; Boydston and Williams, 2003). A number of products have been tested in potato producing countries for their efficacy against volunteer potatoes, with varying degrees of success. One of the biggest problems is finding a suitable product that fits into the rotation programme used by producers, as many products are capable of controlling volunteer potato plants, but can be phytotoxic to other plants in the rotation system.

It is very important to bear the next crop in the rotation system in mind when choosing a chemical for the control of volunteer potatoes. The reason is that certain products that control volunteer potatoes can have fairly long periods of residual activity in the soil, and have a negative impact on sensitive crops if these are planted while the residual activity of the herbicide is still sufficiently high to cause damage.

4.2.5.4. *Crop rotation*

Crop rotation is mainly used to reduce the pest load from diseases, nematodes and insects (Wright and Bishop, 1981; Thomas, 1983; Steiner et al., 2005) by planting crops not susceptible to those affecting the previous crop. Crop rotation has to be implemented together with cultivation and an integrated weed control programme (Rahman, 1980).

S. tuberosum volunteers do not compete well in cereals and perennial ryegrass, but are a greater problem in vegetable crops, silage maize, sugar beet and subsequent potato crops (Lumkes and Beukema, 1973). A proper rotation can therefore also contribute to minimizing the number of *S. tuberosum* volunteers in subsequent crops. Frequent rotation of other crops with potatoes is recommended in order to increase potato yield and reduce insect and disease pressure, as well as to reduce the population density of weeds (Hopkins, 2010; Seaman, 2013). Farmers are also advised to avoid planting potatoes near fields where potatoes were planted the previous year.

4.3. Extent of mechanical admixture during planting, harvesting, transportation and storage

Management and phytosanitary practices must be in place to minimise the spread of diseases by contact with machinery, tools or with surfaces encountered during planting, harvesting, transport and storage. In addition to the problem

of volunteer plants, the risk of accidental admixture exists, which is mainly related to the cleanliness of equipment (counter-rotating planter, calibrator, lorries, etc.) and may be cumulative across production steps.

4.3.1. Planting

Given the size of potatoes, the risk of significant numbers of potatoes remaining in the planter or passing out unnoticed, is very low. Usually, farmers empty planters, leaving the last rows more or less bare. Manual cleaning of the planter can remove the tubers that have been stuck in the machine; this can be done simply and quickly, the planters are relatively small and all parts are accessible.

4.3.2. Harvesting

The risk of admixture during harvesting is higher than at planting due to immediate and delayed consequences. First, the tubers from the previous plot can stay in the harvester; therefore it is necessary to always ensure the cleanliness of the harvester at the end of harvesting a field. The circuit is generally visible and somewhat streamlined, allowing the control and maintenance of the chain. Second, the harvester is equipped with a main grid with mesh sizes of 30-32 mm for potato crops and optionally with grids with a variable mesh dimension according to the particular production requirements (but typically greater). Therefore, tubers with a diameter less than 30 mm are not collected and thus remain in the field. These small tubers and those left in the soil by the harvester are the main source of regrowth. The choice of a suitable calibre mesh can limit these losses.

The collecting, cleaning and initial sorting of the harvested potato is done either simultaneously on the plot or cleaning and/or sorting is carried out on the farm. The chosen practice has different consequences on the risks of admixtures and volunteer appearance.

With a combined harvester (equipped with a hopper), the potato crop is collected, cleaned and sorted simultaneously. Debris and defective tubers in this case are immediately returned to the field.

With a simplified harvester, the collected potatoes are directly discharged into the trailer and sorting takes place on the farm. This second scenario is the one encountered in seed potato production because it minimises health risks and varietal contamination. The total waste (non-marketable tare; composed of earth, stones, vines, stem scraps, defective or damaged tubers and foreign varieties of tubers) is mixed and usually stored as a heap on the edge of land. To promote the destruction of the included tubers, the piles are covered with a tarpaulin, preventing the sprouting of potatoes in the spring. Piles usually end up being spread on land.

Harvested potatoes are continuously sorted at farm, warehouse and processors. The harvesters are commonly equipped with a receiving hopper in which a moving carpet backs up the tubers for the subsequent unearthing and sorting. The sorted tubers are then calibrated by a large table equipped with a series of square mesh grids decreasing in diameter. At each gate, tubers with a diameter greater than the mesh are retained and crated or packed in bins.

4.3.3. Storage, packaging and transportation

Harvested potatoes are first dried for about 15 days. After this intermediate stage, the dried tubers are packaged in bags or boxes and then stored in two different ways:

- The storage can take place in a fridge. After verifying that the lot has not changed and has not degraded during the conservation, tubers are packaged for delivery to the final consumer.
- The storage for shorter time takes place in ventilated stores before bagging, certification and distribution.

The type of packaging depends on the considered market: Jute bag 25 or 50 kg; big bag sealed (from 500 to 1,200 kg) or crate (wooden bins) when the goods are sold from one producer to another. In all cases, the packaging carries a certificate, required for the declaration of goods to control.

Up to 85% of potato crop storage is on farm. Afterwards, storage is undertaken by the industrial site processor, wholesaler or, rarely, by a cooperative.

Required storage conditions depend on the market designation:

- For the fresh market potatoes are put in refrigerated pallet boxes of one to two t.
- For processing and starch production potatoes are stored

in bulk in ventilated stores equipped with partition walls for managing multiple lots.

A final sorting is done before stocking and transport to the place of use (processing, packaging). During storage, which can last up to 6-8 months, the tubers are regularly visited and eventually sorted to remove tubers that have turned green or are rotting. Defective tubers are placed on the waste pile already established at harvest.

In processing plants, waste is mainly controlled after the initial preparation stage, for example sorting is done after washing or peeling. Co-products and waste are especially valued in animal feed and bio-energy production. Preservation comprises a drying or cooling phase and usually is combined with application of a sprout inhibitor (maleic hydrazide); such treatment can be avoided by maintaining a sufficiently low temperature.

Calibration of potatoes can be done at different stages. For the fresh market, a pre-calibration is conducted on the farm. For processing, calibration is less common due to the use of specific varieties for a particular purpose and the associated difficulty to change the intended use.

Unlike storage and bulk transport, the use of boxes (bins), which are small packaging units, can effectively ensure the traceability of production identification with variety-by-variety, plot-by-plot and even intra-plot segmentation. This facilitates maintenance of the local storage and transport trailers and avoids admixture between batches. In some production plants and consumer production manifolds (conservation treated batches), labelling of boxes includes a conspicuous colour code to minimise orientation errors in the handling steps.

Transportation of potatoes encompasses risks of admixtures between products from different fields or different farms, unless the cleanliness of the trailers is ensured. Precaution should be taken by the farmer for transportation from the field to the farm and by the wholesaler for transport outside the farm. The management of potato transportation to the place of use/processing is 95% provided by the wholesaler.

Finally, potato producers are diversifying their markets and therefore the number of varieties simultaneously grown on the farm. However, the tuber size (compared to that of most seeds) and the fact that farmers have their own equipment are likely to facilitate the cleaning of equipment and premises.

In general, it can be concluded that the potato chain is well organised in order to ensure qualitative and pure end-products and also to ensure traceability in case of food safety problems.

5. Existing systems for segregation and identity preservation in potato production in selected EU Member States

The following information was provided by the representatives of the EU Member States in the Technical Working Group (TWG) on potato, and is presented in alphabetical order.

Belgium

Belgium adopted legislation on co-existence of genetically modified crops with conventional and organic farming in 2009. In Belgium, the competence lies at the regional level. The Flemish government issued a general coexistence decree, next to crop-specific regulations for maize (2010), potato (2011) and sugar beet (2011). Besides some administrative regulations, the following crop-specific technical regulations were defined:

- 5 m minimum isolation distance from the border of the GM plot
- A mandatory volunteer control in the three years following the GM potato crop. No tilling allowed for the installation of a crop the same year or the year after
- Separate storage of GM seed potatoes. Unused GM seed potatoes can only be sold or given to registered professional growers. Leftovers of GM seed potatoes that will not be used have to be destroyed, avoiding germination of the seed potatoes
- Traders of GM seed potatoes have to make a register containing data about buyer, amount and selling date of GM seed potatoes
- Mandatory cleaning of machinery after GM potato sowing and harvesting on the plot where GM potatoes were planted and harvested
- Transport and storage of GM potatoes physically separated from non-GM potatoes, with a clear labelling of the GM variety at any time
- Specific regulations for the production of GM seed potatoes can be put in place
- Material derived during cleaning of harvested GM potatoes can only be brought back to a field where during the same production season GM potatoes were grown

In Belgium, no commercial GM potato cultivation took place. In 2011-2012 a single field trial with GM potatoes was carried out to evaluate the resistance of the susceptible

potato variety Désirée transformed with single or multiple late blight (R) resistance genes (*Rpi-sto1*, *Rpi-vnt1.1* and a stack of *Rpi-sto1:Rpi-vnt1.1:Rpi-blb3*).

Czech Republic

Based on extensive research and field trials with various GM potato cultivars, coexistence rules were established before GM potato cultivation in the Czech Republic was launched. The Czech Republic coexistence rules are defined by Act on Agriculture no. 252/1997 amended by Act no. 441/2005 and Act 291/2009. Specific rules for the coexistence of GM crops are regulated by Decree no. 89/2006 Coll. on detailed conditions for the cultivation of genetically modified crops amended by Decree no. 58/2010 Coll. An amendment is foreseen to come into force in 2017. All farmers cultivating GM potatoes have to take measures against the mixing of potato tubers. The isolation distance between GM and conventional potatoes is 3 m and 10 m between and along the rows, respectively, considering the width of the planting machine. A minimum of 20 m isolation distance is necessary in case of organic potato production. The Decree imposes an obligation to notify the owner of a neighbouring field if the GM potato field is located at a distance of less than 20 m (conventional potato production) and 40 m (organic potato production).

Denmark

No GM seed potatoes are grown in Denmark. In the Danish regulation "Bekendtgørelse 1559 of 11/12/2015 the following measures are included for potatoes:

- isolation distance of 20 m to seed potato (15 m if not flowering or male sterile)
- isolation distance of 10 m to commercial potato production (2 m if not flowering or male sterile)
- a minimum of 4 years without potato production after GM potato production (seed potatoes)
- a minimum of 3 years without potato production after GM potato production (commercial potatoes)
- requirements for control of volunteers and cleaning of machinery

Additionally, general requirements include:

- a special GM course/education and a license/approval (includes also contractors working on the field and in transport until 1. stage)
- distance to non GM field can be reduced/neglected in agreement with the non-GM neighbour
- information of neighbours about GM cultivation
- information in case of sale or rent of an area where GM crops have been grown
- new owner or leaseholder of an area where GM crops have been grown takes the responsibilities for volunteer control and crop rotation regulations
- cultivation of GM must be reported and a fee (100 kr/ha) has to be paid

Estonia

In Estonia, no GM potatoes are grown or have been grown. However, a coexistence provision is available (legislation, scientific reports etc.; https://www.riigiteataja.ee/en/eli/ee/PÕM/reg/522122014013/consolide).

Germany

In Germany, 72 field trials with transgenic potatoes were carried out in the period 1992 – 2008. In 2010 and 2011, the GM potato variety Amflora was commercially cultivated for seed potato production on an area of 15 and 2 ha, respectively.

Until now, only general coexistence regulations and crop-specific regulations for maize have been adopted but no special coexistence regulations for potato. However, in 2007 an expert hearing was held at the Federal Ministry of Food and Agriculture and recommendations for good farming practice of GM potato cultivation were given based on the available literature and the knowledge about potato biology. These recommendations included:

- an isolation distance of 2 m,

- a reasonable crop rotation for optimal volunteer control (e.g. tillage, herbicide application, but no recommendation for a single specific measure) with at least 2 years potato cultivation break after GM potato production,
- the thorough cleaning of all machines, storage places, and containers and
- the obligation to inform neighbours (not a scientific but a political decision).

Lithuania

In Lithuania, no field trials or commercial cultivation of GM potatoes have been carried out. According to the Order of Minister for Agriculture of the Republic of Lithuania and Minister for the Environment of the Republic of Lithuania of 16 November 2007 (No. 3D-504/D1-608 concerning the Approval of the Rules on Co-existence of Genetically Modified Crops with Conventional and Organic Crops), key elements for potatoes coexistence are:

- 50 m minimum isolation distance between GM potatoes and other Solanaceae family crops
- a 2 years minimum period for conventional or organic potato in crop rotation after GM potatoes
- a mandatory 3 m wide buffer zone around GM potato crops
- a mandatory 2 years volunteer control in crop rotation
- the use of separate machinery or a mandatory cleaning of machinery after GM potato sowing, harvesting and transportation
- the storage of the harvest of GM potatoes separately from conventional and organic potatoes
- a minimum distance of 5 km from GM plants to apiaries

The Netherlands

In the Netherlands, no commercial GM potato production took place and no data review on field trials with relevance to coexistence is available. A cultivation regulation (WJZ/14148909) exists and includes (1) the announcement of plans for GM cultivation by the GM grower to neighbours before February 1st, (2) a minimum isolation distance of 3 m from conventional and 10 m from GM-free potato fields, and (3) all growers to take measures for separating GM at all stages of cultivation, in particular including control of volunteers. These measures are based on the proposal of the Dutch Coexistence Committee in 2004, based on a literature review and summarised in Van de Wiel and Lotz (2006). Recently a proposal for monitoring coexistence in GM potato cultivation was published (Van de Wiel et al., 2015, in Dutch).

Sweden

The production of seed potatoes is regulated according to the EU regulation. Coexistence measures for potato are regulated in the Swedish ordinance 2007:273 and regulation 2008:34. There are several general rules applicable for all GM crops including administrative measures, care during transport and cleaning of equipment. Specific measures for cultivation of GM potato are:

- isolation distance of 3 m to non-GMO potato
- a grower who has cultivated a GM potato variety in a field has, during the two following growing seasons, to inform another grower of the same field about the GM cultivation in the field.

6. Occurrence of potato pollen in honey

van Droogenbroeck et al. (2013) carried out a two-year field trial with late blight-resistant GM potatoes and conducted PCR analysis of honey samples produced within a distance of 5 km from these experimental fields. In all four of the samples no potato pollen was detected. After this initial finding, an additional experiment was set up with five beehives which were placed in different locations of a conventional potato field (in the middle of the field, at the border and at different distances from the potato field), forcing honeybees to overfly the potatoes. The experiment was carried out when the potato variety was in full bloom. As part of the experimental design, visual checks on honeybee visits to potato flowers were carried out as well as mellissopalynological observation by microscopy and PCR analysis of pollen collected by honeybees. Honeybees were not observed on potato flowers at any of the observational inspections. The observed insects on potato flowers were mainly hoverflies and to a lesser extent butterflies, beetles, bugs and bumblebees. The mellissopalynological analysis of the pollen collected by honeybees placed inside potato fields revealed that it was from the families *Asteraceae*, *Fabaceae*, *Castaneae*, *Geranicaceae*, *Malvaceae*, *Brassicaceae*, *Poaceae* and a limited number of other plant families, but not from potatoes. The potato-specific DNA analysis led to the same conclusion as the visual observations and microscopic pollen analysis. No evidence could be found that honeybees visit potato flowers and collect the pollen.

Jørgensen et al. (2012) studied the pollen availability for honeybees in an agricultural landscape. Denmark has the world's most intensive agricultural landscape. More than 60% of Denmark is arable land of which 92% is under crop rotation. This agricultural landscape, for some periods of the season, provides an abundant nectar and pollen source, but at other periods the landscape is a virtual desert for honeybees and other beneficial insects. The nectar flow stops normally mid-July as the main crops are winter wheat, maize, sugar beets and potatoes. In these conditions it has been shown that potatoes are an important pollen source in some areas with intensive production of potatoes for industry, and that potato pollen could comprise up to 29% of the pollen collected by honey bees.

The differences in findings of these two studies (Van Droogenbroeck et al., 2013, Jørgensen et al., 2012) are in fact in line with pre-existing knowledge about the interaction between honeybees and potatoes. Under natural conditions with different pollen sources available, honeybees are not interested in collecting potato pollen (Van Droogenbroeck et al., 2013). However, as a starvation response, in conditions lacking a pollen supply, honeybees can collect potato pollen as a source for colony survival (Jørgensen et al., 2012). Logically, this stimulus is very powerful and has long-lasting effects (Sanford and Hanneman, 1981). However, in such extreme conditions it is likely that honeybee colonies produce honey that contains potato pollen.

7. Detection of GM events in potato harvest and honey

The European Union Reference Laboratory for GM food and feed (EU-RL GMFF) validated a quantitative PCR method for the detection of potato event EH92-527-1 (starch potato Amflora). For potato event AM04-1020 (starch potato Amadea) a method was validated but not published due to withdrawal of application, and for the potato events PH048 and AV 43-6-G7 the validation was ongoing but not completed due to the withdrawal of the applications.

More PCR methods for identification and quantification of several other GM potato events can be found in the EU Database of Reference Methods[10] maintained by the Joint Research Centre in collaboration with the European Network of GMO Laboratories (ENGL).

When the results are primarily expressed as GM-DNA copy numbers, in most cases they need to be converted into mass fraction or vice versa. This ratio may depend on the number of copies of the transgene that were inserted in the GM crop's genome during transformation, and on the relative amounts of embryo, endosperm and maternal tissue in the case of true seeds (Holst-Jensen et al., 2003; Miraglia et al., 2004; Van De Wiel and Lotz, 2006 and Le Ny et al., 2011). The endosperm in most cases is derived from a fusion of two maternal nuclei and one sperm nucleus, and therefore contains two maternal genomes for each paternal genome. Using the real-time PCR method with the tetraploid potato, outcrossing results will be multiplied by a conversion factor of 0.25 from a number of tubers or plants with a quantity of DNA, since a single chromosome of quadruplet chromosome counterparts will cause the sequence established in the case of a simple transformation event. This factor needs to be adapted on a case-by-case basis, depending on the number of copies or the number of the transgenes inserted in the case of transgenes of stacked genes (Le Ny et al., 2011).

At the current state of the art of the technology, a practical and robust PCR protocol able to quantify GM pollen relative to total pollen in honey is not available. The reason is that in all honeys, even if classified as unifloral, the pollen fraction consists of pollen from several species (for details please refer to Rizov and Rodríguez-Cerezo, 2013).

10 http://gmo-crl.jrc.ec.europa.eu/gmomethods/.

8. Best practices for coexistence in potato crop production

8.1. Scientific background

The adventitious admixture of GM potato in non-GM harvests can only be efficiently managed if the whole production chain is covered. Also, because in some EU Member States fields are often rented by different farmers, the practice of 'coexistence within a given field' (meaning the cultivation of non-GM potatoes after growing GM potatoes in a given field) must be taken into account. Summarizing the aforementioned information in the preceding chapters, the following aspects are most important for coexistence considerations and the proposal of coexistence regulations.

(1) The use of good quality seed potatoes is important to successfully grow potatoes. Therefore the presence of GM seed potatoes in conventional seed potato lots is a critical factor and must be appropriately managed to achieve coexistence. The best approach to manage this is the use of certified seed potatoes that comply with EU regulations. Seed production, whilst being a very important factor, is not included in this work as it is already covered by EU requirements to ensure varietal purity.

(2) Due to the clonal propagation and low pollen transmission distances in potato, the potential for pollen-mediated gene flow from potato production systems to challenge the coexistence threshold in adjacent potato fields is regarded as negligible (Petti et al., 2007).

Isolation distances (buffer zones) are not only required to limit cross-pollination, but also to avoid the spread of potato volunteers caused by field work, machinery utilization, and probably animal and bird activities. The efficiency of the isolation distances (buffer zones) in potatoes is mainly determined by existing agricultural practices and differences in flower abundance among the cultivars. The available information from literature and current practices (e.g. in potato breeding) shows that, in order to limit adventitious GM presence caused by spatial dispersal of GM reproductive material (including pollen, tubers, and true potato seed) to 0.9%, 5 m between the fields is enough; in order to limit adventitious GM presence to 0.1%, 10 m isolation is sufficient.

(3) The rare occurrence of feral potatoes in the EU, the infrequency of potato seed production and high percentage of self-pollination probably mean that feral plants present little or no risk of acting either as a GM pollen source or as recipient.

(4) The field-to-field coexistence, where GM and non-GM potatoes are grown in adjacent fields at the same time has, no direct impact on the harvested crop during one cultivation cycle, since cross- pollination does not affect the harvested parts (tubers) of the potato plant. How-

ever, where the consecutive cultivation of GM and non-GM potatoes is carried out in the same field, an effective volunteer control strategy is important for coexistence (Phelan et al., 2015; Turley, 2001). For quantification of this recommendation, the authors used the Irish potato production figures from 2007 to 2010, which indicated a mean number of 339,533 (±30,721) tubers harvested per hectare. Imposing the labelling coexistence threshold of 0.9% would imply that the number of volunteer-derived tubers should not exceed 3,058 per hectare. While the study of Phelan et al. (2015) did not go beyond examining the fecundity of second generation volunteer-derived tubers, they refer to a previous report of McGill et al. (2005) recommending a minimum of three different crops in rotation before a conventional potato cultivar could be sown on a field that was previously used for GM potato cultivation. Due to short growing seasons and hard winters, the number of required rotations might be lower in northernmost EU Member States.

(5) The replacement of isolation distances by temporal isolation, meaning planting GM and non-GM potato varieties of different maturity classes, may be an effective measure in the case of appropriate climatic conditions; although scientific data proving this assumption could not be found. However, farmers in some Member States, as well as certain regions within a given Member State, are often specialised in cultivation of early or late potatoes, hence this within-year type of temporal isolation may not always be feasible.

(6) Since seed potatoes are bigger than seeds of other crops, cleaning of machines and transport bins as well as storage places is usually easier. Harvesting is the most critical step in potato cultivation, since harvesters are in general a primary source of on-farm comingling. Additionally, lost tubers and tuber pieces may act as volunteers in following years mainly within a given field.

(7) The current practices in honey production and marketing in Europe are sufficient to ensure that adventitious presence of GM potato pollen in honey is far below the legal labelling thresholds and even below 0.1%, as was concluded in the Best Practice Document for coexistence of GM maize and honey production (Rizov and Rodríguez-Cerezo, 2013). Therefore, there is no need for additional spatial segregation between GM potato fields and beehives.

Based on this scientific information, the TWG on potato analysed the possible sources for potential GM admixture in potato crop production and agreed on the following best practices for the coexistence of GM and non-GM potato cultivation as well as honey production. The thresholds for coexistence which were considered are the legal labelling threshold (of 0.9%) and the limit of quantification (generally accepted to be about 0.1% for routine analysis using PCR-based testing), which is required by operators in some markets. These two different coexistence thresholds are in line with the Commission Recommendation of 13 July 2010 on guidelines for the development of national coexistence measures.

It is suggested that the current practice of potato production allows respecting the 0.9% labelling threshold for adventitious GM admixture (Le Ny et al., 2011). Conversely, it is suggested that maintenance of an adventitious presence of GM below 0.1% instead requires the implementation of specific coexistence measures for potato production and distribution, even if low varietal purity thresholds are obeyed.

8.2. Best practices for ensuring seed potato purity

The use of certified seed potatoes that comply with EU legislation is considered best practice since according to EU legislation any seed lot containing traces of GM material needs to be labelled and therefore can be easily identified.

In the case of cultivation of both GM and non-GM varieties on the same farm, the seed potatoes of GM varieties should be transported to the farm and stored upon arrival in their original packaging, and separately from non-GM varieties. Label information should be retained with the seed potatoes.

8.3. Best practices for coexistence

8.3.1. Isolation distances

Isolation distances are feasible and effective coexistence measures to reduce adventitious presence of GM potato in conventionally and organically produced potato even if they are the only measure applied. All available information from

the literature and pre-existing segregation systems shows that to limit adventitious GM presence caused by cross-pollination to 0.9%, a 5 m isolation distance is required. To achieve a threshold of 0.1%, a 10 m isolation distance is sufficient.

8.3.2. Sowing, harvesting, drying and storage on farm

To achieve the 0.9% GM threshold, separate treatment and storage of GM potatoes (including seed potatoes) are required; planting and harvesting machines should be properly cleaned before and after use, preferably on the plot where GM potatoes are handled. The storage space must be thoroughly cleaned and inspected after emptying of GM tubers and prior to storing of non-GM tubers. Ancillary plant material collected during cleaning of harvested GM potatoes should be properly destroyed. For achieving a GM threshold of 0.1%, in addition to the requirements for 0.9%, machinery should be dedicated to planting and harvesting either GM or non-GM potatoes.

The definition of specific recommendations for cleanout depends on type of the equipment and its construction. Additionally, choosing the appropriate technique for equipment cleaning should be based on the desired level of purity. In general, the use of dedicated equipment for different production systems (GM or non-GM) or its use for non-GM crops prior to GM crops is recommended.

8.3.3. Volunteer control

For a GM threshold of 0.9%, a cultivation break of three years in rotation is recommended, followed by monitoring of GM potato presence during the third year. If the amount of volunteers does not fall below the expected threshold, this period should be prolonged by another year of cultivation break followed by a further inspection of GM potato presence. This step may be substituted or complemented by a sprout inhibitor application followed by monitoring of its efficacy. This field inspection should be repeated until the required volunteer level is achieved to meet the threshold of 0.9%.

For a threshold of 0.1%, a cultivation break of four years in rotation is recommended, followed by a control check of GM potato presence during the fourth year. The optimization of the crop rotation shall follow the same systematics as for achieving a threshold of 0.9%, again with the option of the complementary use of a sprout inhibitor. This approach for crop rotation optimization has been chosen since the required cultivation break between GM and non-GM potato is highly dependent on the climatic conditions, and which can vary significantly between Member States.

8.3.4. Coexistence with honey production

There is no available empirical data to establish a statistical relationship between potato pollen content in honey and distance of beehives to potato crops. Potato pollen is not a major fraction of total pollen in polyfloral honey. In any case, considering the maximum pollen content (number of grains) in commercial honey and the average weight of potato pollen grains, the weight fraction of potato pollen in honey will definitely be below 0.1%.

In conclusion, the current practices in honey production and marketing in Europe in line with quality legislation are sufficient to ensure that the adventitious presence of GM potato pollen in honey is far below the legal labelling threshold and even below 0.1%.

8.3.5. GM detection and quantification

For detection and quantification of GM potato presence including GM potato pollen in honey, only quantitative PCR-based approaches such as EU-RL GMFF validated methods should be used.

9. Cost analysis of the management practices

In contrast to the crop species covered in the preceding Best Practice Documents of genetically modified maize, soybean, and cotton with conventional and organic farming (Czarnak-Kłos and Rodríguez-Cerezo, 2010; Rizov and Rodríguez-Cerezo, 2015), GM potato has not been grown commercially worldwide since 2001. Until that time, Monsanto sold insect resistant GM potato varieties, but consumer rejection has kept GM potatoes off the global market since then. Nowadays, the focus lies on late blight resistance, resistance to bruising and reduction of asparagine, an amino acid in potato that reacts with some sugars to oxidise into acrylamide, a possible carcinogen, especially during high-temperature frying (CBAN, 2016). However, with the exception of 160 ha in the US in 2015 these new GM potatoes called the "Innate" potato from the company Simplot were not planted anywhere in the world (ISAAA, 2016). For this reason, information about economic consequences of coexistence in potatoes along the whole value chain is extremely scarce.

Additional costs may result from minimizing unintended mixing during planting, harvest, on-farm storage, transportation, storage, processing and other activities beyond the farm gate such as shipment testing and labelling costs (Greene et al., 2016). However, USDA has not collected data on the cost of separation practices, but the environmental non-profit and organic grain cooperative Food and Water Watch estimated these costs by a survey of 1,500 U.S. organic grain producers representing about 19% of the farmers mainly from the Midwest (Food & Water Watch and OFARM, 2014). For grain production, the total median annual cost of practices to avoid GM material in their crops was $6,532 to $8,500 per farm, including the cost of buffer strips ($2,500), delayed planting ($3,312 to $5,280), testing ($200), and other measures ($520). However, only additional costs per farm are indicated and not the costs for a special field, field size or crop species. Since GM potatoes are only recently being grown commercially in the US on a small scale, the transferability to potato is not given. Tolstrup et al. (2003) evaluated the extra costs of complying with a given threshold value for adventitious presence of GM material in conventional or organic potatoes under Danish production conditions. Calculated extra costs amounted to 1-2% of average growing costs per ha for both conventional and organic production and arose from volunteer control as well as cleaning of soil treatment, sowing and harvest machinery and cleaning of storage facilities.

No empirical data is available to estimate the costs of implementing the above-mentioned best practices for coexistence by EU farmers intending to grow GM potatoes. However, the necessary isolation distances between GM and non-GM fields to limit outcrossing to GM contents below the regulated labelling threshold are small due to the low cross-pollination potential of potatoes in combination with the fact that potatoes are planted and harvested as tubers. Therefore, resulting additional costs for implementing distances should also be low. This is supported by the suggestions of Schenkelaars and Wesseler (2016) mentioning that the minimum distance requirements are lower for potatoes, followed by sugar beet, maize and oilseed rape. In general, isolation distance cost can be defined as the lost profit on the area bordering a crop plot on which farmers are not able to raise a crop (Gustafson,

2002). The total value of the lost area can be divided by the amount of crop yield sold to determine the value on a per unit basis. At a regional level, depending on the position of the farm and whether the potato variety grown by neighbours is GM or non-GM, the economic effect will depend on the physical landscape of the affected area (Messean, 2006). Moreover, increasing the cultivation of different crops than potato in crop rotation forces the farmers to cultivate potatoes in fields further away from farmhouses, which increases transportation costs. Potato production is characterised by the transportation of more bulky harvests than the cultivation of other crops, and by several pesticide sprayings during the growth season. This is one of the reasons contributing to the concentration of potato cultivation in the proximity of farmhouses (Tuomisto and Huiti, 2006).

Bullock and Desquilbet (2002) estimated the on-farm costs for non-GM soybean segregation and Identity Preservation to be 1 and 0.5 working hours per t, respectively. In contrast to soybean (and maize) seed, potato tubers are considerably bigger and therefore cleaning of machines for planting and harvesting (physical removal of soil, remaining seed potatoes and debris) should be easier to manage. Consequently, additional costs for cleaning potato equipment should be lower than the costs for cleaning of planters and combines in soybean. Furthermore, to minimise spread or recurrence of a pest, a good sanitation programme for equipment and storage facilities is necessary anyway for potatoes (Olsen and Nolte, 2011). Therefore, any additional costs for thorough cleaning of agricultural machines for GM segregation purposes should be low.

A thorough cleaning is also always important for potato storage facilities on-farm and transportation containers from the field to farm and from farm to processing, again from a phytosanitary point of view. As even small infected tuber pieces left behind can transfer diseases effectively to the next storage bulk, a further effort in cleaning for coexistence reasons is not considered necessary, even for complying with a threshold of 0.1%.

A cost calculation must also take the GM trait into account. As far as for example insect tolerance or late-blight resistance reduce costs for pesticides and, therefore, stabilise yields, costs for coexistence measures may thus be compensated or even overcompensated. In case of reduced asparagine or resistance to bruising, a price premium might be necessary. However, due to the absence of GM potato cultivation, a precise calculation is not possible.

With regard to within-field coexistence, there may be additional costs associated with the regulation of crop rotation where the land is rented to different growers on a yearly basis.

Additionally, the GM testing of a given potato harvest lot needs a considerable amount of tubers, and therefore of weight, to accurately estimate the GM content.

In conclusion, more research is needed to examine the cost and effectiveness of various coexistence strategies in potatoes.

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Miljømæssig risikovurdering af ansøgning om udsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (*Phytophthora infestans*)

Rådgivningsnotat fra DCA – Nationalt Center for Fødevarer og Jordbrug, Aarhus Universitet

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Institut for Ecoscience

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Datablad

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Baggrund

Styrelsen for Fødevarer, Landbrug og Fiskeri beder AU om at foretage en miljømæssige risikovurdering af den foreslåede forsøgsudsætning (jf. udsætningsdirektivet), herunder en vurdering af de introducerede genetiske ændringer. Risikovurderingen skal tage udgangspunkt i det vedlagte materiale fra ansøgeren, herunder ansøgers egen vurdering af virkningen på miljøet.

Ansøgningen om forsøgsudsætning er indsendt af KMC AmbA, og forsøgsarbejdet vil blive udført i samarbejde med Ytteborg Field Trials, 7560 Hjerm.

Formålet med forsøgsudsætning er at undersøge muligheden for at reducere anvendelse af kemiske plantebeskyttelsesmidler imod kartoffelskimmel (*Phytophthora infestans*) i kartoffel.

Besvarelse

Miljømæssig risikovurdering

Vurderingen omhandler forsøgsudsætning af 8 linjer af genetisk modificeret kartofler af sorten YDUN, som ved Agrobacterium-transformation har fået indsat ekstra resistensgener mod kartoffelskimmel forårsaget af *Phytophthora infestans.* Tre af linjerne har fået indsat to ekstra resistensgener, og disser linjer er identiske med linjer godkendt til forsøgsudsætning i 2025. Yderligerer fem linjer har fået indsat tre resistensgener, og fire af disse linjer indeholder også fragmenter af potato spacer-sekvensen.

AU's miljømæssige risikovurdering omfatter følgende punkter, som alle har til formål at identificere uønskede effekter af forsøgsudsætningen. Vurderingen er gældende for den ansøgte forsøgsudsætning.

1. Risiko for spredning af den genetisk modificerede kartoffel til omgivelserne
2. Risiko for spredning af den genetisk modificerede kartoffel til naturen
3. Risiko for spredning af de indsatte gener fra den genetisk modificerede kartoffel til vilde slægtninge i Danmark
4. Risiko for spredning af de indsatte gener fra den genetisk modificerede kartoffel til konventionelt dyrkede kartofler
5. Risiko for effekter på naturen og miljøet i øvrigt
6. Behov for overvågning
7. Vurdering af, om ansøgers risikohåndtering er fyldestgørende

AU's vurdering:

1. Knolde fra kartofler, *Solanum tuberosum*, kan afhængigt af vinterens kuldegrader og varighed i varierende grad overleve milde danske vintre (Kudsk 2012, Boelt & Kudsk 2021, OECD 1997). Udsættes knolde for frostgrader lavere end -3 ℃, er deres overlevelse dog ringe og afhænger af, hvor dybt i jorden de ligger, og hvor tør jorden er (bedre overlevelse i tør end våd jord) (Boydsten

et al. 2006, Mustonen et al. 2009, Phelan et al. 2015). Knolde, der overlever milde vintre, ses som spirende kartofler i efterfølgende afgrøder, og selv efter fem år kan der forekomme kartofler i efterfølgende afgrøder (Schnipper 2019). Dette forventes ikke at være anderledes for den genetisk modificerede kartoffel, og der er således en sandsynlighed for, at der sporadisk vil forekomme overlevende genetisk modificerede kartofler på det pågældende forsøgsareal i årene efter dyrkningen. De genetiske ændringer i kartoflen, som udsætter angreb af skimmelsvamp, forventes ikke at ændre på dette. De af ansøger nævnte forholdsregler (håndopgravning, knolde pakket i dobbeltsække og placeret i sikrede kasser transporteret med ledsagelse af person med GMO-kørekort, gentagne harvninger og manuel opsamling af spildkartofler efter hver høst, overvågning af arealet efter forsøgetsafslutning i minimum 4 år) forventes at være tilstrækkelige for at forhindre spredning af den genetisk modificerede kartoffel til omgivelserne via spildkartofler.

Kartoffelfrø kan overleve i jorden, også ved lavere temperaturer end knoldene (Mustonen et al. 2009), og ses som nye kimplanter det efterfølgende år. De af ansøger nævnte forholdsregler om afklipning af kartoflernes blomster ved blomstring vil forhindre, at den genetisk modificerede kartoffel producerer frø. En eventiel spreddning af den genetisk modificerede kartoffel til omgivelserne via frøspredning må derfor forventes at være forhindret.

2. Der er ikke fundet oplysninger om permanente selvreproducerende bestande af kartoffelplanter i dansk natur. Kartoffelplanter optræder sporadisk flere steder som følge af tilfældig spredning fra spildkartofler (langs vejkanter, markkanter, jordbunker, tangvolde og strandbredder mv. (Hartvig 2015). En sådan spredning til naturen sker typisk ved tab i forbindelse med transport og bortskaffelse af haveaffald. De af ansøger anførte forholdsregler om håndtering af knolde ved høst forventes at være tilstrækkelige til at undgå en sådan spredning.

3. I Europa er de nærmeste vilde slægtninge til kartoffel, *Solanum tuberosum*, arter i slægten natskygge, *Solanum*, som kartoffel også tilhører. I Danmark findes der to hovedarter af slægten, som er naturligt forekommende: sort natskygge og bittersød natskygge samt nogle underarter af bittersød natskygge (Hartvig 2015). Krydsninger mellem kartoffel og de europæiske natskyggearter forekommer ikke i naturen. I praksis må kartoffel således betragtes som en art, der ikke kan krydse med vilde europæiske arter af natskygge, *Solanum*, og selv hvis dette skulle ske, vil hændelsen resultere i sterilt afkom. De indsatte resistensgener mod kartoffleskimmel forventes ikke at ændre på dette. Desuden vil afklipning af blomster ved blomstring fjerne risiko for pollen-spredning af de indsatte gener fra den genetisk modificerede kartoffel til vilde slægtninge i Danmark. Risikoen for natur og miljø forbundet med en sådan spredning vurderes på den baggrund at være negligerbar.

4. Spredning af gener fra de genetisk modificerede kartofler til konventionelle dyrkede kartofler kan ske ved spredning af pollen. Pollenspredning fra kartoffel kan ske ved insekters pollenindsamling, hvor nogle arter af humlebier er effektive fremmedbestøvere af kartofler (Batra 1993). Hybridisering mellem kartoffelsorter aftager hurtigt med afstanden mellem planterne. McPartlan og Dale (1994) fandt således, at nærtstående planter havde en hybridiseringsrate på 24%, mens der ved en afstand på 10 m var en hybridiseringsrate på 0,017%. Hvis afstanden var 20 m, blev der ikke fundet hybridfrø. Ansøger beskriver, at vil være mindst 10 m til nærmeste kartoffelmark, og mindst 20 m til konventionelle eller økologiske læggekartofler, samt at de genetisk modificerede kartofler vil blive omgivet af et værn af ikke-GM-kartofler. Et sådant værn vil kunne fungere som pollenfanger og mindske spredning af GM-pollen til omgivende marker. Da blomsterne af de genetisk modificerede kartofler desuden vil blive afklippet ved blomstring,

vurderes risikoen for natur og miljø forbundet med genspredning fra de genetisk modificerede kartofler til dyrkede kartofler at være negligerbar.

5. Påvirkningen af naturindholdet forventes ikke at adskille sig fra den påvirkning, der finder sted ved dyrkning af konventionelle kartofler. En reduceret anvendelse af kemiske bekæmpelsesmidler mod kartoffelskimmel forventes at være gavnlig for natur og miljø på markfladen og omgivelser. Risikoen for forøgede negative effekter på markfladens natur som følge af dyrkning af den genetisk modificerede kartoffel vurderes derfor at være negligerbar. Der er ikke fundet eksempler på studier, som viser horisontal genoverførsel fra kartofler til mikoroorgansimer. Risikoen for denne teoretisk mulige, men højst usandsynlige, hændelse vurderes ligeledes negligerbar. Sandsynligheden for effekter på miljøet i øvrigt som følge af forsøgsudsætningen forventes således ikke at adskille sig fra dyrkning af konventionelle stivelseskartofler på et tilsvarende areal. Risikoen for en forøget negativ påvirkning af natur og miljø vurderes derfor at være negligerbar.

6. Overvågning i forbindelse med forsøgsudsætningen, som foreslået af ansøger, vurderes at være tilstrækkeligt. Den foreslåede efterbehandling og overvågning i årene efter forsøgsudsætningen efterlader en meget lille sandsynlighed for, at enkelte genetisk modificerede kartofler overlever på arealet ud over det første år. Det vurderes, at langt de fleste genetisk modificerede kartofler vil forsvinde i løbet af en kort årrække.

7. AU vurderer samlet, at ansøgers risikohåndtering for uønskede effekter på natur og miljø er fyldestgørende.

Konklusion

I forbindelse med den specifikke forsøgsudsætning vurderes det, at de af ansøger foreslåede tiltag for at hindre spredning af materiale fra den genetisk modificerede kartoffel sikrer en meget lille sandsynlighed for, at der sker spredning til omgivelserne. De indsatte gener i den genetisk modificerede kartoffel, som kan medføre en udsættelse af angreb af skimmelsvamp, forventes ikke at ændre den modificerede kartoffels påvirkning på natur og miljø i forhold til andre konventionelt dyrkede kartofler.

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DTU, Food Institute
The research group for Chemical Risk Assessment and GMO

The Danish Agency for Agriculture
Nyropsgade 30
1780 Copenhagen V
Att. Morten Storgaard and Christine Gundelach Rannes

Dato: 15-04-2026
J.nr. 26/1002522
RK-JP/KMC 2026 cisgenic potato Ydun_LBST.docx

Application for release of cisgenic starch potatoes containing multiple complementary resistance genes with increased resistance to late blight (*Phytophthora infestans*) by KMC[1]

Order from the Danish Agency for Agriculture and Fisheries (LBST) for Field trial release of GM-potatoes.

In connection with above mentioned case, DTU, National Food Institute has received a request from the Danish Agency for Agriculture and Fisheries (LBST) concerning an application from KMC for experimental field release. The application relates to several lines of cisgenic starch potatoes of the Ydun variety, containing multiple complementary resistance genes conferring increased resistance to potato late blight, intended for use in field trials in 2026 - 28.

DTU, Food is requested to deliver the following (translated from Danish):
A health risk assessment of the proposed experimental release (cf. the release directive), including an assessment of the introduced genetic changes of the potato varieties. The risk assessment must be based on the material provided by the applicant, including the applicant's own assessment of the effect on human and animal health.

The following is the assessment of the application that relates to potatoes based on the Ydun variety with increased resistance to potato blight caused by *Phytophthora infestans.*

Brief description of the project.

Cultivation of genetically modified potatoes for an experimental field trial release which takes place from 1st April until 30th October in the year 2026/2027/2028. The final two months of the trial will be used for harvesting. The field is located in 7560 Hjermand the area for cultivation is set at 100-500 m^2 gross with 50-400 m^2 used for the cultivation of GM-lines.

[1]Ansøgning om udsætning af cisgene stivelseskartofler med flere komplementære resistensgener med øget resistens imod kartoffelskimmel (*Phytophthora infestans*)"

The application from KMC concerns the experimental trial release of the potato variety Ydun with improved resistance to potato blight (*Phytophthora infestans*), achieved through insertion of multiple complementary resistance genes. The purpose of the genetic modification is to reduce yield losses due to late blight and to avoid or reduce spraying with fungicides. The purpose of the experimental release is to investigate the performance of the improved resistance to late blight under field conditions. The genetically modified potato plants included in the release will consist of 3 lines (YSF5, YSF12 and YSF13) that are identical to those used in the 2025 field trial, as well as five new lines (YA10_51, YA_mfp_1, YA_mfp_2, YA_mfp_3, YA_mfp_5).

Construction

The applicant describes that the genetically modified potato described as cisgenic starch potatoes with multiple complementary resistance genes (called R-genes), were created with the intention to insert three genes from *Solanum* species that are cross-compatible with *Solanum tuberosum.* One gene *vnt1.1* is from *Solanum venturii* and the two genes *blb1* and *blb2* are from *Solanum bulbocastanum.* Along with the structural genes also the part of their flanking region (~1 kb upstream) ak nd 400- 600 bp downstream) was part of the three genes attempted to be inserted as one continuous insertion into the potato variety Ydun. The three lines in this application YSF5, YSF12 and YSF13 only contain the entire genes for *blb1* and *blb2,* whereas truncation of the gene for vnt1.1 has occurred leading to loss of the entire promoter sequence for vnt1.1 in all the three lines. The five lines YA10_51, YA_mfp_1, YA_mfp_2, YA_mfp_3 and YA_mfp_5 all contain the three genes *vnt1.1, blb1* and *blb2.* The two lines YA_mfp_1 and YA_mfp_5 have two copies of the genes. For all the lines the insertion is only at one genomic locus.

Due to the insertion of the two or three of the functional genes, *vnt1.1, blb1* and *blb2* in full length, it is expected that the lines will acquire increased resistance towards potato blight. Genes are expected to be expressed throughout the plant. Detailed molecular characterization, which includes whole genome sequence analysis, confirms that the genes were integrated, consisting of one copy of *blb1* and *blb2* for three lines, one copy of *vnt1.1, blb1* and *blb2* for three lines and two copies of *vnt1.1, blb1* and *blb2* for two lines. Furthermore, the analysis found no unwanted parts (backbone) of the plasmid in genome of the GM potato lines, which includes no antibiotic resistance genes. The line YA10_51 was created by a second step where CRISPR/Cas9 mediated genome editing was used to remove the inserted kanamycin resistance (KanR) gene.

DTU, National Food Institute considers the description of the genetic modification sufficient to allow a preliminary risk assessment of the eight GM potato lines proposed for release. The genetic material used to create GM potato comes from closely related/wild potato varieties (cisgenesis) that are unlikely to raise concerns in terms of human health or food safety. It cannot be ruled out that during genetic engineering, unintended mutations have occurred elsewhere in the genome, but this must be considered to constitute an insignificant risk in relation to traditional breeding and DTU, National Food Institute does not assess that the intended mutations will alter the health status of the potatoes.

The application concerns release of eight different lines that only have the two or three of the expected functional genes for *vnt1.1, blb1 and blb2* that have been selected for closer examination under cultivation conditions.

Overall, it is DTU, National Food Institute´s assessment that the genetically engineered GM potatoes have not acquired new genes of concern and may be considered as healthy as other traditional potatoes.

Containment:

The biological containment is assessed as high for potatoes due to their natural biology of the crop. Potatoes do not normally reproduce via pollen-mediated outcrossing, rely primarily on vegetative propagation via tubers, sexual reproduction and cross-pollination only rarely occur under field conditions (also limited by self-pollination). In addition, potatoes are sensitive to frost as in Denmark and rarely overwinter.

The physical containment does not seem particularly high for the current release in relation to access restrictions. For example, there is no fence around the experimental area. However, the applicant will grow a wide belt of non-genetically modified potatoes around the field. The distance to the nearest potato field is 10 m.

Harvesting is done manually (manual hand digging and collection is chosen, which is considered an effective protection against tubers that are not harvested), and procedures for transport, as described in the application, ensure good containment.

Cutting off flowers (beginning of July until the end of the flowering in early August) will largely prevent unintentional pollen dispersal and seed formation.

Overall, DTU, the National Food Institute assesses that the containment of the potatoes during cultivation is high and that spread to other fields or potato plants is highly unlikely.

The subsequent monitoring of the area for the year(s) after release and removal/destruction of any potatoes on the area is assessed to be able to ensure that temporal spread is avoided.

Overall assessment.

DTU, National Food Institute considers the description of the genetic modification sufficient to allow a preliminary risk assessment of the eight GM-potato lines for proposed release. Based on the information, it is the assessment that the GM potatoes used in the field trial are unlikely to raise concerns in terms of human health or food safety.

DTU, National Food Institute assesses that the combination of the physical and biological containment of the potatoes during cultivation largely ensures that spread of GM material (potatoes/pollen/seeds) outside the growing area would be highly unlikely.

It is the assessment of DTU, National Food Institute, that a "worst-case scenario" where the genetically modified potatoes are spread to potato fields via tubers or pollen (e.g. for consumption or propagation) will not constitute a health problem based on knowledge of the design. The expected new properties are not associated with a health risk of potatoes and do not cause the formation of new constituents other than intended.

Best regards,

Radhakrishna Shetty and Jan Pedersen

KOMMISSIONEN

KOMMISSIONENS BESLUTNING

af 29. september 2003

om fastlæggelse, i henhold til Europa-Parlamentets og Rådets direktiv 2001/18/EF af en model for fremlæggelse af resultatet af udsætning i miljøet af genetisk modificerede højerestående planter i andet øjemed end markedsføring

(meddelt under nummer K(2003) 3405)

(EØS-relevant tekst)

(2003/701/EF)

KOMMISSIONEN FOR DE EUROPÆISKE FÆLLESSKABER HAR —

under henvisning til traktaten om oprettelse af Det Europæiske Fællesskab,

under henvisning til Europa-Parlamentets og Rådets direktiv 2001/18/EF af 12. marts 2001 om udsætning i miljøet af genetisk modificerede organismer og om ophævelse af Rådets direktiv 90/220/EØF [1], særlig artikel 10, andet punktum, og

ud fra følgende betragtninger:

(1) I forbindelse med udsætning af genetisk modificerede organismer (GMO'er) i andet øjemed end markedsføring kræves det efter artikel 10 i direktiv 2001/18/EF, at anmelderen, når en udsætning er fuldført og derefter med de intervaller, der er fastsat i tilladelsen på grundlag af resultaterne af miljørisikovurderingen, meddeler den kompetente myndighed resultatet af udsætningen, hvad angår enhver risiko for menneskers sundhed eller miljøet, i givet fald med særlig henvisning til de typer produkter, som anmelderen agter at anmelde på et senere tidspunkt.

(2) Hidtil har de fleste GMO'er, som er udsat i Fællesskabet i overensstemmelse med del B i direktiv 2001/18/EF, været genetisk modificerede højerestående planter (GMHP). Det er derfor nødvendigt at fastsætte en model, som anmelderen af en sådan udsættelse skal anvende i forbindelse med fremlæggelsen af resultaterne af udsætningen af disse planter til den kompetente myndighed. Modellen bør afspejle behovet for at gøre udvekslingen af relevante oplysninger så fyldestgørende som muligt på en standardiseret og letforståelig måde. Modellen bør gøres så generel som muligt, så den kan omfatte udsætninger i flere områder eller udsætninger over flere år og flere GMO'er i én og samme rapport.

(3) Da genteknologi ikke er begrænset til højerestående planter, er der behov for yderligere bestemmelser med henblik på at udarbejde modeller for andre typer af GMO'er, som f.eks. genetisk modificerede (GM) dyr (herunder GM insekter), veterinære og medicinske produkter (der indeholder eller består af GMO'er) eller for andre GM planter, som kan benyttes til at producere farmaceutiske produkter. Derudover kan det som følge af den fremtidige udvikling blive nødvendigt at tilpasse de allerede udarbejdede rapporteringsmodeller.

(4) De i denne beslutning fastsatte foranstaltninger er i overensstemmelse med udtalelse fra det i medfør af artikel 30 i direktiv 2001/18/EF nedsatte udvalg —

VEDTAGET FØLGENDE BESLUTNING:

Artikel 1

Med henblik på at fremlægge resultaterne af udsætning i miljøet af genetisk modificerede højerestående planter (GMHP) for den kompetente myndighed i henhold til artikel 10 i direktiv 2001/18/EF skal anmelderen benytte modellen i bilaget til denne beslutning (i det følgende: »rapporteringsmodellen«).

Artikel 2

En rapporteringsmodel kan højest være knyttet til én tilladelse udstedt af den kompetente myndighed, og den skal være identificeret med et enkelt anmeldelsesnummer.

[1] EFT L 106 af 17.4.2001, s. 1.

Artikel 3

1. Anmelderen indgiver en endelig rapport for hvert anmeldelsesnummer, og der indgives en endelig samt en eller flere delrapporter om overvågningen efter udsætningen, hvor dette er relevant. Begge typer af rapporter skal udfærdiges i overensstemmelse med rapporteringsmodellen.

2. Den endelige rapport indgives efter den sidste høst af de GMHP. Hvis der ikke kræves overvågning efter udsætningen i forbindelse med denne anmeldelse, skal der ikke indgives yderligere rapporter.

3. Den endelige rapport om overvågningen efter udsætningen indgives, når overvågningen efter udsætningen er afsluttet.

I de relevante tilfælde specificerer den kompetente myndighed i sin tilladelse varigheden af overvågningen efter udsætningen samt et tidsskema for, hvornår delrapporter over overvågningen efter udsætningen indgives.

4. Den kompetente myndighed opfordrer anmelderne til at fremlægge rapporten i elektronisk form.

Artikel 4

Den kompetente myndighed kan anmode anmelderen om yderligere oplysninger, navnlig i form af en logbog eller delrapporter, som skal indgives i løbet af forskningsprogrammet, før udsætningen er afsluttet.

Artikel 5

Denne beslutning er rettet til medlemsstaterne.

Udfærdiget i Bruxelles, den 29. september 2003.

På Kommissionens vegne

Margot WALLSTRÖM

Medlem af Kommissionen

BILAG

MODEL TIL FREMLÆGGELSE AF RESULTATET AF UDSÆTNING I MILJØET AF GENETISK MODIFICEREDE HØJERESTÅENDE PLANTER I HENHOLD TIL ARTIKEL 10 I DIREKTIV 2001/18/EF

VIRKSOMHEDENS ELLER FORSKNINGSINSTITUTTETS LOGO (VALGFRIT)

Rapporteringsmodellen udfyldes af anmelderen.

Anmelderen udfylder rapporteringsmodellen i henhold til angivelserne (afkryds bokse og/eller, så vidt muligt, specifikke stikord, der benyttes i tekstfelterne).

Anmelderen illustrerer så vidt muligt de forelagte data med diagrammer, figurer og tabeller. Der kan også fremlægges statistiske data, hvor dette er relevant.

I forbindelse med udsætninger i flere områder, udsætning af flere genetisk modificerede organismer og/eller udsætninger over flere år fremlægger anmelderen en generel oversigt over de trufne foranstaltninger og de konstaterede virkninger i tilladelsens gyldighedsperiode.

Den plads, der er afsat til hvert punkt, er ikke udtryk for den detaljeringsgrad, der er påkrævet i forbindelse med oplysningerne i denne rapport.

1. ***Generelle oplysninger***

1.1. **Europæisk anmeldelsesnummer: B/XX/YY/ZZ**

1.2. **Anmeldelsesmedlemsstat:** .....

1.3. --- **Dato og nummer for tilladelse**: .....

2. ***Rapportstatus***

2.1. **Angiv venligst, om den foreliggende rapport i henhold til denne beslutnings artikel 3 er:**

— den endelige rapport

— en rapport om overvågningen efter udsætningen

— ○ Endelig rapport ○ Interimsrapport

3. ***Nærmere oplysninger om udsætningen***

3.1. **Recipientorganismens videnskabelige navn:** .....

3.2. **Transformationshændelse(r) (akronym(er)) eller anvendte vektorer (1) (hvis transformationshændelsens identitet ikke er kendt)**.....

3.3. **Entydig identifikator, hvis den er kendt:** .....

3.4. **Angiv venligst følgende oplysninger sammen med en skitse over området/områderne:**

Geografisk beliggenhed (administrativ enhed og i givet fald angivelse af kvadratnetsreference)	**Arealet af udsætnings-området eller -områderne** (1) (m^2)	**For hver hændelse angiv identifikation** (2) **og giv et overslag over antallet af genmodificerede højerestående planter, der udsættes** (antal frø/planter pr. m^2)	**Udsætningens varighed**: (fra…(dag/måned/år)……til… …(d/m/å)……)

(1) Angiv størrelsen af GM-området og, hvis det er relevant, størrelsen af det GM-fri område (f.eks. GM-fri randområder).
(2) Benyttede vektorer.

(1) I forbindelse med små markforsøg, hvor der kan udføres forsøg med flere sorter, anføres de benyttede vektorer, som giver indsigt i de indførte egenskaber og/eller genetiske elementer. I forbindelse med større markforsøg begrænses antallet af anmeldte hændelser til én eller enkelte hændelser.

4. ***Alle typer af produkter, som anmelderen påtænker at anmelde på et senere tidspunkt***

4.1. **Har anmelderen til hensigt på et senere tidspunkt at anmelde den eller de udsatte transformationshændelse(r) som et eller flere produkter til markedsføring under fællesskabslovgivningen**

☐ Ja ☐ Nej ☐ Vides ikke p.t.

Hvis ja, angives anmeldelseslandet eller -landene: .....

Hvis ja, specificeres til hvilke(t) anvendelsesformål:

— Import

— Avl (f.eks. produktion af frø/plantemateriale)

— Fødevarer

— Føder

— Farmaceutisk anvendelse (eller forarbejdning med henblik på farmaceutisk anvendelse)

— Forarbejdning med henblik på

  — videre anvendelse i fødevarer

  — videre anvendelse i foder

  — videre anvendelse i industrien

— Andre (anfør hvilke)

.....

5. ***Type(r) af udsætning(er)***

Afkryds venligst den eller de væsentligste type(r) (i boksene) og undertype(r) i forbindelse med udsætningen eller udsætningerne. I forbindelse med udsætninger i flere områder, udsætning af flere genetisk modificerede organismer og/eller udsætninger over flere år fremlægges en generel oversigt over den eller de type(r) udsætning(er), der er gennemført i tilladelsens gyldighedsperiode. Afkryds venligst den eller de relevante type(r):

5.1. **Udsætning(er) med henblik på forskning** ☐

5.2. **Udsætning(er) med henblik på udvikling** ☐

— Screening af indsætningsbegivenheder

— Afprøvning af koncept (2)

— Agronomiske egenskaber (f.eks. plantebeskyttelsesmidlets effektivitet/selektivitet, udbytte, spireevne, afgrødens etableringsevne, plantens levedygtighed, plantens højde, følsomhed over for klimafaktorer/sygdomme osv.) (specificeres nærmere)

— Ændrede agronomiske egenskaber (f.eks. resistens over for sygdomme, skadedyr, tørke eller frost osv.) (specificeres nærmere)

— Ændrede kvalitative egenskaber (forlænget holdbarhed, forbedret næringsværdi, ændret sammensætning osv.) (specificeres nærmere)

— Ekspressionens stabilitet

— Opformering af linier

— Undersøgelse af hybriders levedygtighed

— Anvendelse af planten som en fabrik (»molecular farming«) (3)

— Phytoremediering

— Andre: ..... (beskriv nærmere): .....

5.3. **Officiel prøvning** ☐

— Registrering af sorten på en national sortsliste

  — SES (= Selvstændighed, Ensartethed og Stabilitet)

  — Værdiafprøvning (= værdi af dyrkning og anvendelse)

— Andre (anfør hvilke): .....

(2) F.eks. afprøvning af den nye egenskab under miljøforhold.

(3) »*Molecular farming*« står for produktion af stoffer (f.eks. proteiner og lægemidler) på grundlag af planter, som er genetisk modificeret med henblik på en særlig egenskab. »*Molecular farming*« kunne defineres som produktion af lægemidler ved syntetisering af planter, som lægemiddelproducerende planter, eller som plantebaseret produktion af proteiner o.s.v.

5.4. **Herbicidtilladelse** ☐

5.5. **Udsætning(er) med henblik på demonstration** ☐

5.6. **Opformering af frø** ☐

5.7. **Udsætning(er) med henblik på forskning i biosikkerhed/risikovurdering** ☐

— Undersøgelser af vertikal genoverførsel

  — Krydsbestøvning med konventionelle afgrøder

  — Krydsbestøvning med vilde slægtninge

— Undersøgelser af horisontal genoverførsel (overførsel af gener til mikroorganismer)

— Håndtering af selvsåede planter

— Potentielle ændringer i persistens eller spredning

— Potentiel invasionsevne

— Potentielle virkninger på målorganismer

— Potentielle virkninger på ikke-målorganismer

— Observation af resistente slægtninge

— Observation af resistente insekter

— Andre (beskriv nærmere): .....

5.8. **Anden/andre type(r) udsætning(er):** ☐

(Beskriv nærmere): .....

6. ***Udsætningens metode(r) og resultat(er) samt forvaltnings- og overvågningsforanstaltning(er) i relation til enhver risiko for menneskers sundhed eller miljøet***

6.1. **Risikostyringsforanstaltning(er)**

Anfør venligst de risikostyringsforanstaltninger, der er anvendt for at undgå eller minimere spredningen af GMO'er uden for udsætningsområdet eller -områderne, og navnlig de foranstaltninger

— som ikke er anmeldt i den oprindelige ansøgning

— som er anvendt ud over de i tilladelsen fastsatte betingelser

— som ifølge tilladelsen kun er krævet under særlige forhold (f.eks. tørkeperioder eller ved oversvømmelse)

— hvor anmelderen ifølge tilladelsen kunne vælge mellem flere foranstaltninger.

Afkryds de relevante eksempler:

6.1.1. *Før såning/plantning:*

— De genetisk modificerede frø/plante partier mærkes tydeligt (så disse kan skelnes fra andre frø, rodknolde osv.) (beskriv nærmere).

— Adskilt forarbejdning og transport af frø og planter (beskriv den anvendte metode og giv et eller flere eksempler på indeslutningen med henblik på at undgå spild i forbindelse med forarbejdning og transport).

— Overflødige frø/planter destrueres (beskriv den anvendte metode).

— Midlertidig indeslutning (anfør nærmere).

— Sædskifte (angiv den eller de tidligere afgrøde(r)).

— Andre (anfør hvilke): .....

6.1.2. *I forbindelse med såningen/plantningen:*

— Metode anvendt til såning/plantning.

— Tømning og rensning af så- eller plantemaskiner i udsætningsområdet.

— Adskilt såning/plantning (Giv et eller flere eksempler på indeslutningsforanstaltninger for at undgå udslip under såning/plantning).

— Andre (anfør hvilke): .....

6.1.3. *I løbet af udsætningsperioden:*

— Isolationsafstand(e) (× meter)

  — fra krydsningskompatible kommercielt dyrkede plantearter

  — fra krydsningskompatible beslægtede vilde plantearter

— Beplantning i randområder (med samme eller en anden afgrøde, med en ikke-transgen afgrøde, × meter osv.)

— Bur/net/hegn/skiltning (angiv nærmere)

— Pollenfælder (angiv nærmere)

— Fjernelse af GM blomsterstandene før blomstringen (angiv med hvilken hyppighed de fjernes)

— Fjernelse af stokløbere/slægtninge/hybridiseringspartnere (angiv med hvilken hyppighed de fjernes, × meter rundt om GM-området osv.)

— Andre (anfør hvilke): .....

6.1.4. *Ved udløbet af udsætningsperioden:*

— Høst/destruktionsmetoder (af afgrøden eller dele af denne)/andre foranstaltninger (f.eks. indsamling og analyse af sukkerroepulp) (beskriv nærmere)

— Høst/destruktion inden frømodning

— Effektiv fjernelse af plantedele

— Adskilt oplagring og transport af afgrøder/affald (angiv et eller flere eksempler på indeslutning for at forebygge spild af indsamlede frø/afgrøder/affald)

— Rensning af maskiner i udsætningsområdet

— Affaldets bestemmelse, håndtering af affald/overskudsudbytte/planterester (beskriv nærmere)

— Håndtering og dyrkningsforanstaltninger på udsætningsområdet efter høsten (beskriv metoden eller metoderne, som benyttes til at forberede og forvalte udsætningsområdet efter udsætningen, herunder dyrkningspraksis)

— Andre (beskriv nærmere): .....……..

6.1.5. *Foranstaltninger efter høsten*

Anfør, hvilke foranstaltninger der er truffet på udsætningsområdet efter høsten:

hyppighed af besøg (gennemsnit): .....

— efterfølgende afgrøde (angiv nærmere)

— sædskifte (angiv nærmere)

— brakjord/ingen afgrøde (angiv nærmere)

— overfladisk jordbehandling/pløjefri dyrkning

— falsk såbed

— bekæmpelse af selvsåede planter (angiv intervaller og varighed)

— passende kemisk(e) behandling(er) (angiv nærmere)

— passende jordbehandling(er) (angiv nærmere)

— andre (anfør hvilke).

6.1.6. *Andre foranstaltninger (beskriv nærmere)*

6.1.7. *Beredskabsplan(er)*

Angiv:

a) om udsætningen forløb som planlagt

  — Ja

  — Nej (beskriv årsagerne til dette, f.eks. vandalisme, klimaforhold osv.): ……..

b) om det var nødvendigt at træffe foranstaltninger i henhold til en eller flere beredskabsplaner (artikel 6, stk. 2, litra a), nummer vi), og bilag III B i direktiv 2001/18/EF)

  — Nej

  — Ja (beskriv nærmere): .....

6.2. **Overvågningsforanstaltninger efter udsætningen**

Da den nuværende rapporteringsmodel kan anvendes til den endelige rapport og en eller flere rapporter om overvågningen efter udsætningen, anmodes anmelderen om tydeligt at skelne mellem disse rapporttyper ved udfyldelsen af dette afsnit 6.2. Anfør venligst, om

— **planen for overvågningen efter udsætningen vil blive iværksat** (hvis det drejer sig om en endelig rapport udarbejdet efter den sidste høst af de genetisk modificerede højerestående planter

— **planen for overvågningen efter udsætningen er iværksat** (hvis det drejer sig om en delrapport om overvågningen efter udsætningen)

— **planen for overvågningen efter udsætningen er afsluttet** (hvis det drejer sig om en endelig rapport om overvågningen efter udsætningen)

— **der ikke er krav om gennemførelse af en plan for overvågning efter udsætningen.**

Hensigten med resultaterne af denne overvågning er at få be- eller afkræftet tidligere antagelser i forbindelse med risikovurderingen.

Angiv venligst i henhold til ovennævnte tilfælde, hvilken eller hvilke overvågningsforanstaltning(er) der vil blive truffet, der træffes eller er blevet truffet (i udsætningsområdet/i nærheden af udsætningsområdet (f.eks. i udsætningsområdets randområder)). Bemærk venligst, at alle overvågningsforanstaltninger, der er truffet i hele perioden efter udsætningen, skal anføres her.

Angiv:

— overvågningsforanstaltninger inden for området

  varighed:

  hyppighed af besøg (gennemsnit):

  — observation af resistente slægtninge

  — observation af resistente insekter

  — bekæmpelse af selvsåede planter (angiv intervaller og varighed)

  — overvågning af genflow (angiv nærmere oplysninger)

  — passende kemisk(e) behandling(er) og/eller jordbehandling(er)

  — andre (anfør hvilke):

— overvågningsforanstaltninger i tilstødende områder

  varighed:

  hyppighed af besøg (gennemsnit):

  overvåget område:

  — observation af resistente slægtninge

  — observation af resistente insekter

  — bekæmpelse af selvsåede planter og/eller overvågning af forvildede populationer (angiv intervaller og varighed)

  — overvågning af genflow (angiv nærmere oplysninger)

  — passende kemisk(e) behandling(er) og/eller jordbehandling(er)

  — andre (anfør hvilke).

6.3. **Plan og metode(r) for overvågningen**

I dette afsnit redegøres for overvågningsplanen og de metoder, der er anvendt til at fastslå de påvirkninger, som skal rapporteres i det næste afsnit (afsnit 6.4). Der redegøres udførligt for eventuelle omformuleringer eller ændringer i planen, som blev fremlagt i ansøgningen og i SNIF ([4]) B.

I tidsrummet mellem anmeldelsen og fremlæggelsen af den endelige rapport kan der være udviklet ny videnskabelig viden eller videnskabelige metoder, hvilket medfører en ændring i de anvendte metoder. Det er særligt ændringer af den art, som skal anføres i dette afsnit.

6.4. **Konstaterede påvirkninger**

6.4.1. *Forklarende note*

Alle resultater af udsætningen eller udsætningerne i relation til enhver risiko for menneskers sundhed eller miljøet skal anføres, uanset om det af resultaterne fremgår, at en risiko er øget, mindsket eller uændret.

Hovedmålene med oplysningerne, der afgives i dette afsnit, er:

— at be- eller afkræfte antagelser i miljørisikovurderingen om forekomsten og konsekvenserne af potentielle påvirkninger fra GMO'erne

— at identificere påvirkninger fra GMO'erne, som ikke blev forudset i miljørisikovurderingen.

([4]) Model til resumé af anmeldelser — Summary Notification Information Format (=SNIF).

GMO'ernes konstaterede **påvirkning(er)/vekselvirkning(er)**

— med hensyn til enhver risiko for menneskers sundhed

— med hensyn til enhver risiko for miljøet

skal rapporteres i dette afsnit

Der skal ofres særlig opmærksomhed på uventede og utilsigtede påvirkninger.

Nedenfor redegøres der for de påvirkninger, som anmelderen i givet fald må rapportere. Det er indlysende, at påvirkningerne skal betragtes på baggrund af afgrøden, den nye egenskab, recipientmiljøet samt konklusionerne i miljørisikovurderingen, der udføres i hver enkelt sag. For at strukturere oplysningerne og lette en effektiv søgning i de afgivne oplysninger skal anmelderen så vidt muligt anvende specifikke stikord ved udfyldelsen af tekstfelterne i kapitel 6, navnlig i afsnit 6.4.2, 6.4.3 og 6.4.4. Den nyeste ajourførte liste over disse stikord findes på følgende internet-adresse: http://gmoinfo.jrc.it

6.4.2. *Forventede påvirkninger*

Nærværende afsnit vedrører »forventede påvirkninger« dvs. potentielle påvirkninger, som blev identificeret allerede i anmeldelsens miljørisikovurdering, og som derfor kunne forudses.

Anmelderne bør fremlægge data fra den eller de udsætning(er), som bekræfter antagelserne i miljørisikovurderingen.

6.4.3. *Uventede påvirkninger* ([5])

»Uventede påvirkninger« henviser til påvirkninger af menneskers sundhed eller miljøet, **som ikke blev forudset eller identificeret i anmeldelsens miljørisikovurdering**. Denne del af rapporten bør indeholde eventuelle oplysninger med hensyn til uventede påvirkninger eller observationer af relevans for den oprindelige miljørisikovurdering. Hvis der er konstateret uventede påvirkninger eller observationer, bør oplysningerne i dette afsnit være så udførlige som muligt for at muliggøre en korrekt fortolkning af dataene.

6.4.4. *Andre oplysninger*

Anmelderne opfordres til at fremlægge oplysninger, som kunne være relevante for de pågældende markforsøg, også selv om de ikke er omfattet af anmeldelsen. Dette kunne også omfatte konstaterede gavnlige påvirkninger.

7. ***Konklusion***

I dette kapitel redegør anmelderen for sine konklusioner og de foranstaltninger, der er truffet eller bør træffes på grundlag af resultaterne af udsætningen, hvad angår yderligere udsætninger, og i givet fald anfører anmelderen oplysninger om ethvert produkt, som vedkommende har til hensigt at anmelde på et senere tidspunkt.

Oplysninger, der er fremlagt i denne rapport, betragtes ikke som fortrolige i henhold til artikel 25 i direktiv 2001/18/EF.

Dette hindrer ikke den kompetente myndighed i at kræve yderligere fortrolige og ikke-fortrolige oplysninger fra anmelderen.

Fortrolige data bør fremlægges i et bilag til rapporteringsmodellen sammen med et ikke-fortroligt resumé eller generel beskrivelse af disse data, som vil blive stillet til rådighed for offentligheden.

DATO:

([5]) Medmindre andet fremgår af artikel 8 i direktiv 2001/18/EF hvad angår behandling af ændringer eller nye oplysninger.