The importance of cis- and intragenesis for (classical) plant breeding

Evert Jacobsen,
Plant Breeding, Plant Sciences Group, Wageningen UR

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www.cisgenesis.com

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Overview

- Introduction
- Classical plant breeding
- The role of GMO by cisgenesis/intragenesis
- Examples: Potato late blight resistance with *R* and/or silenced *S* genes
- Developments in EU GM regulations

History of modern plant breeding

Three main steps

- 1. **Crop domestication** out of, uncultivated, wild plants: low levels of toxic compounds; adaptation to modernized agricultural techniques

- 2. **Classical plant breeding**:

  selection methods

  genetic variation within breeders' gene pool: crop species itself or crossable species, including mutations, trait domestication by introgression/translocation, marker assisted and whole genome based selection

- 3. **GM and new breeding techniques**:

  New - and classical breeders' gene pool with domestication of trans- , intra- and cisgenes; targeted mutations; reverse breeding; etc..

Classical breeding with modern tools is safe

- Breeding and domestication of plants is possible without the need of general biosafety rules
- **Unintended** negative effects on food are seldomly observed
- **Unintende effects** on other traits such as yield, morphology, lateness, etc.. has been found regularly
- **Example**: Specific safety rules in potato tubers for glycoalkaloid content (NL and Sweden)

Newest source of domestication: cloned genes

a. **cisgenes**: natural genes (not engineered) from the breeders' gene pool (with a lot of experience)

b.**intragenes**: hybrid genes (RNAi) with functional gene parts only from the breeders' gene pool

c. **transgenes** (new gene pool)

Hybrid genes (partly) based on bacteria, viruses and/or non-crossable species. In 2013 > 174 million ha transgenic GM crops.

At the beginning, new gene pool combined with transformation process asked for **biosafety regulations** (2018/EC)

Why is GM potato highly needed?

- Too many **old susceptible** (Russet Burbank, Bintje) varieties are still grown and ontaminating environment
- Existing varieties with a safe consumption history can e improved easily
- **Improvement of existing varieties** for quality traits and disease resistance
- **Cis- and intra** genes are preferred because of higher level of acceptance

What kind of GM traits in potato?

To speed up conventional plant breeding

- 1. **Loss of function:** mimicking mutations with **RNAi**

Examples: starch composition (*Amf*); bruising, acrylamide, disease resistances (*S* genes)

- 2. **Insertion** of existing traits from the breeders gene pool. Examples: disease resistances (*R* genes) from different wild species

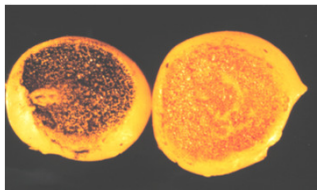
Three types of Genes available

- A **transgene** is a natural gene from a non-crossable species or it is a hybrid gene. It represents the new gene pool
- An **intragene** is coding for a trait with functional parts of genes from the crop plants itself or crossable species. It represents new genes with functional parts of only the breeders' gene pool. Example: RNAi
- A **cisgene** is a natural gene, coding for a trait, from the crop plant itself or from a crossable species which is normally used in classical breeding. It represents functional parts of the breeders' gene pool

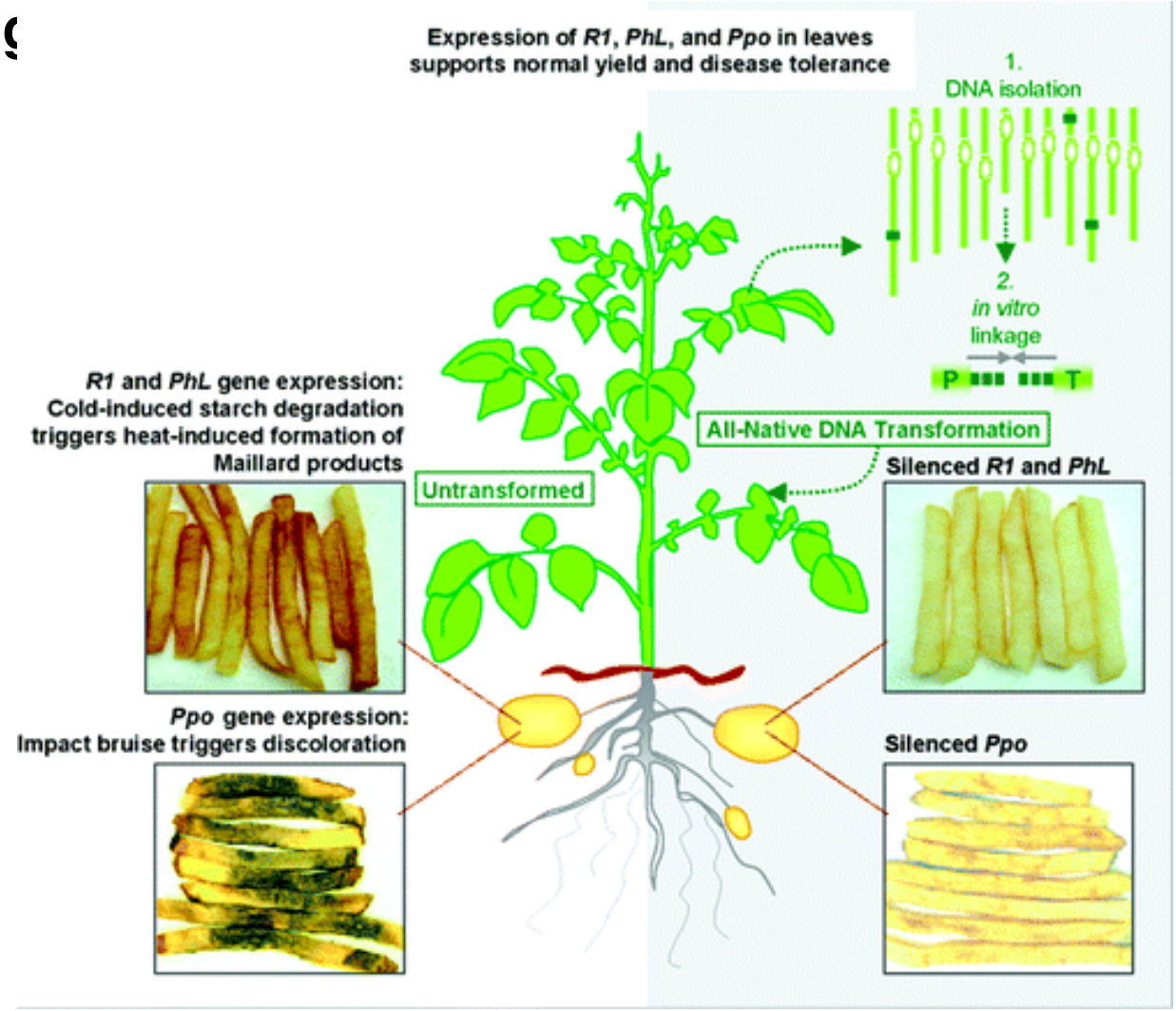
RNAi: Intragenics for quality enhanced potatoes without amylose,

with reduced bruising

Amylose free potato
starch by RNAi of *GBSS*
First RNAi varieties obtained in 1997



gbss mutation, 1 bp
First *amfamf* classical variety in 2005. Now thousands of hectares



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Rommens, 2007
J. of Agricultural and Food Chemistry

RNAi based potato varieties

- **Amylose free potato** in 1997 from wildtype cv Karnico, 2500 ha
  Withdrawn in 1998 by our ministry of Government. Reason:
  presence of *NptIII* gene as backbone

- **Russet Burbank Innate™ variety**: non bruising and reduced acrylamide formation. Selection marker free (Simplot).

Resistance breeding strategies

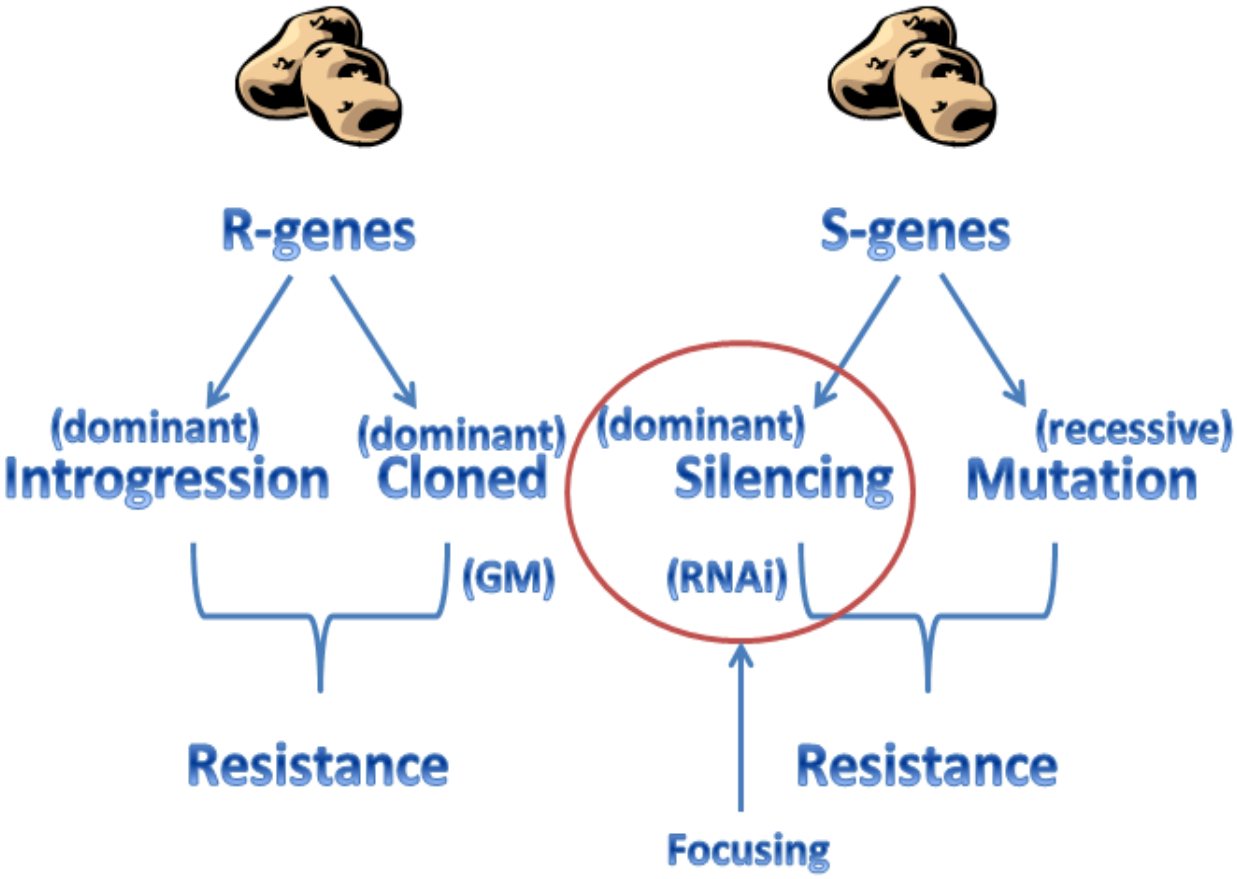
- In all breeding programs a main item
- Depends on the genetic variability of the pathogen
- Use of different *R*-genes
- Pyramiding with MAS: (bottle neck: linkage drag during introgression of *R*-genes from wild species
- Pyramiding of *R*-genes by cisgenesis (no linkage drag)
- Susceptibility genes: recessive *mlo*-gene or dominant by RNAi

Methods to protect potato against late blight

Chemicals

Environment unfriendly

Resistance breeding



Environment friendly

by Liu Jingyi

Interspecific/bridge crosses an additional bottle neck in introgression breeding (>50 years)

>50 years ago – **Bridge crosses** for introduction of *Phytophthora* resistance

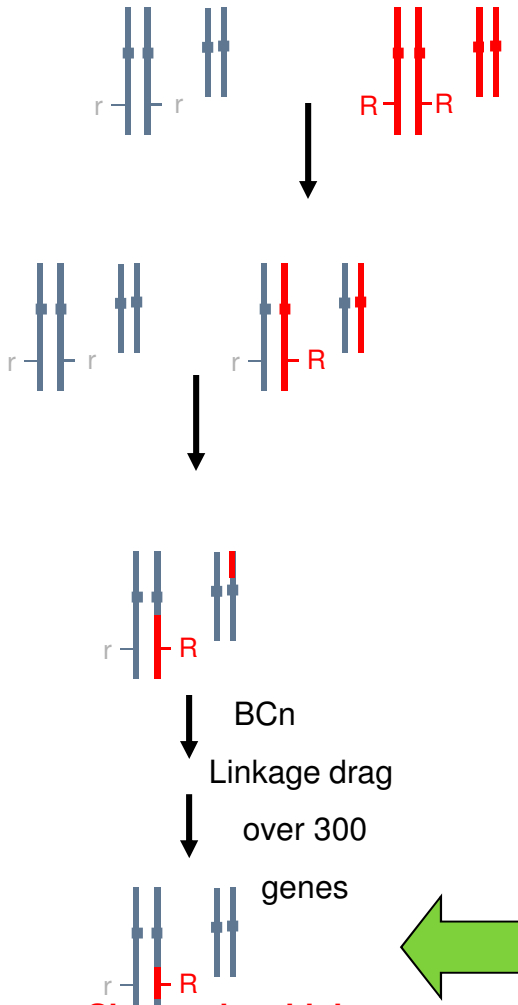
S. acaule 4x × ***S. bulbocastanum*** 2x (*R genes*)
↓
AB hybrid 3x
↓ colchicine doubling
AB hybrid 6x × *S. phureja* 2x
↓
ABP hybrid 4x × *S. tuberosum* 2x
↓
ABPT material 4x *R*-gene + linkage-drag

First resistant varieties came out, like cvs Toluca and Bionica, all with **only 1 *R*-gene**
Stacking of *R*-genes for **sustainable resistance** in this way is **difficult** and always accompanied with a lot of **linkage-drag. *Rpi-blb2*** has already **been broken. Stacking is next step**

Very slow: multiple steps approach; GMO approach more efficient and effective

The big differences between cisgenesis and conventional breeding

Introgression breeding

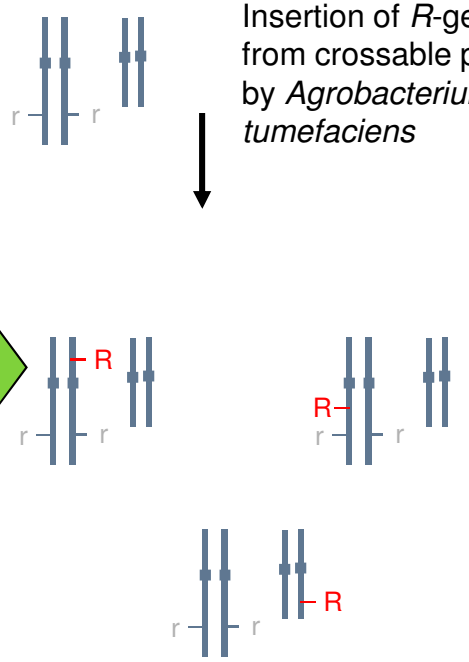


Slow and multiple steps

Introgression of *R*-gene with piece of donor DNA with hundreds of new genes/ alleles in multiple steps

Insertion of cisgenic *R*-gene in 1 step without linkage drag can be made in existing varieties. Insertion of cisgen is random

Cisgenesis



Quick and single step

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Cisgenic resistance breeding

Conditions

- Availability of sufficient number of cloned cisgenic *R*-genes
- Marker free transformation
- No transgenes in the final product
- Backbone free

Potato Late blight

- Late blight pathogen
  - *Phytophthora infestans*
  - Oomycete
  - Hemi-biotrophic

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Jo, PhD thesis 2013

Late blight costs in the Netherlands

- 12 - 15 sprays per season
- 1424 ton active ingredients per year on 150.000 ha
- > 50% of all biocides in the Netherlands
- Environmental toll
- Economical costs
  - Fungicides: 60 M€/yr
  - Spraying: 60 M€/yr
  - Losses: 30 M€/yr
  - **Total: 150 M€/yr**

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Resistance (*Rpi*) genes from related wild species

Host resistance - New sources of resistance

- **Source**: 1000 accessions of 200 wild species
- **Screening for resistance**:
  - *In vitro* inoculation
  - Detached leaf essay
  - Field trial
- **Genetics**: mapping, cloning of R genes
- ***P. infestans***: complex isolate 90128

Groups of cloned *Rpi*-genes in potato

- 27 *Rpi*-genes isolated
- They belong to 9 linkage groups (in different species). Per group, they have the same specificity with *Phytophthora* isolates

1. *Rpi-R1*
2. *Rpi-R2*; *-R2-like*; *-abpt* ;*–blb3; -mcd1-1; -edn1.1; -snk1.1 and 1.2; -hjt1.1; -hjt1.2; -hjt1.3*
3. *Rpi-R3a and –R3b*
4. *Rpi-blb1*; *-sto1* and *-pta1*
5. *Rpi-blb2*
6. *Rpi-vnt1*; *-nrs1*
7. *Rpi-mcq1*; *-phu1*
8. *Rpi-chc*
9. *Rpi-edn2; R9*
10. *R8*

Message:
Resistance found in different species does nc mean different class of *R*-genes with other resistance spectrum.
Important examples are: *R2, Rpi-blb1* and *Rpi-edn2*

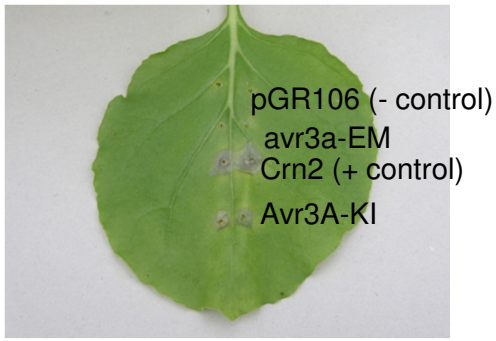
Interaction between *R* and *Avr's*

Gene for Gene hypothesis from Flor (1942)

Interaction		Plant	
		R	*r*
Pathogen	*Avr*	resistance	susceptibility
	avr	susceptibility	susceptibility

How to achieve more durable resistance in the field?

See nature: multiple *R* genes



Frequently, single *Rpi*-genes are easily broken.

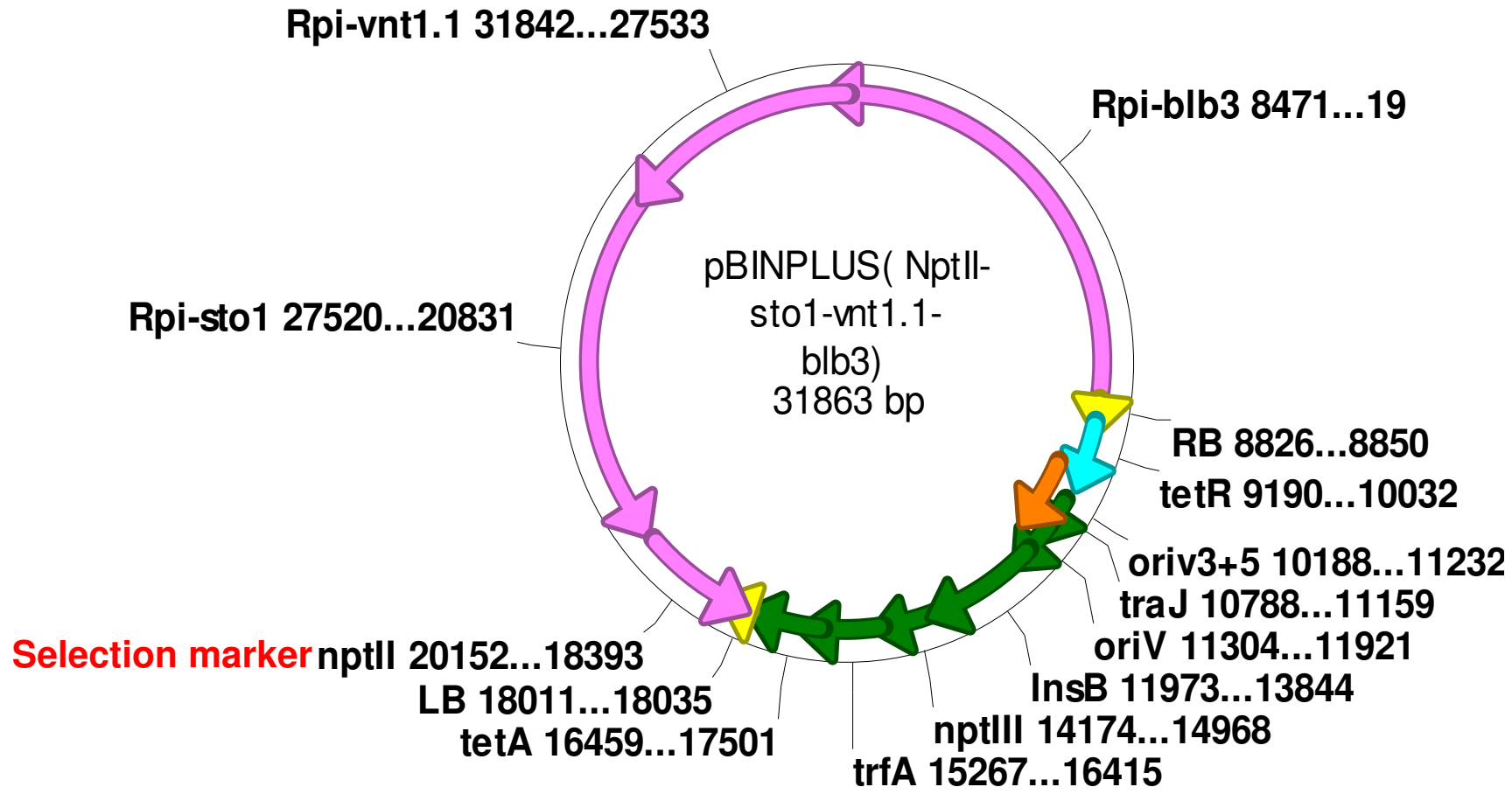
8 *Avr* genes isolated (R3a, R3b, R4, Rpi-blb1, -blb2, -blb3, -vnt1.1, -R8),

R-gene stacking for durable resistance

Durable resistance can be improved by:

- 1. selection of more durable *R*-genes
- 2. broad spectrum *R*-genes
- 3. functional stacking of durable *R*-genes with help of *Avr* genes

Map of a triple *R*-gene vector with *Rpi-sto1,-vnt1.1* and-*blb3*



Molecular analysis of 128 regenerated plants

PCR analysis transformants using construct pBINPLUS: *sto1-vnt1.1-blb3*

nptII	*Rpi-sto1*	*Rpi-vnt1.1*	*Rpi-blb3*	Presence of backbone sequence	# of plants	Percentage (%)
+	+	+	+	-	73	57.0
+	+	+	+	+	25	19.5
+	-	-	-	-	23	18.0
+	-	-	-	+	5	3.9
+	+	-	-	-	2	1.6

Representative of *nptIII* to the presence of backbone sequence			
Backbone sequence without *nptIII*	*nptIII*	# of plants	Percentage (%)
+	+	26	86.7
+	-	4	13.3

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Triple *R*-genes against *Phytophthora infestans*

Field test

before after

Tuber test

Message: Several *R* genes did show
after transformation resistance in tubers

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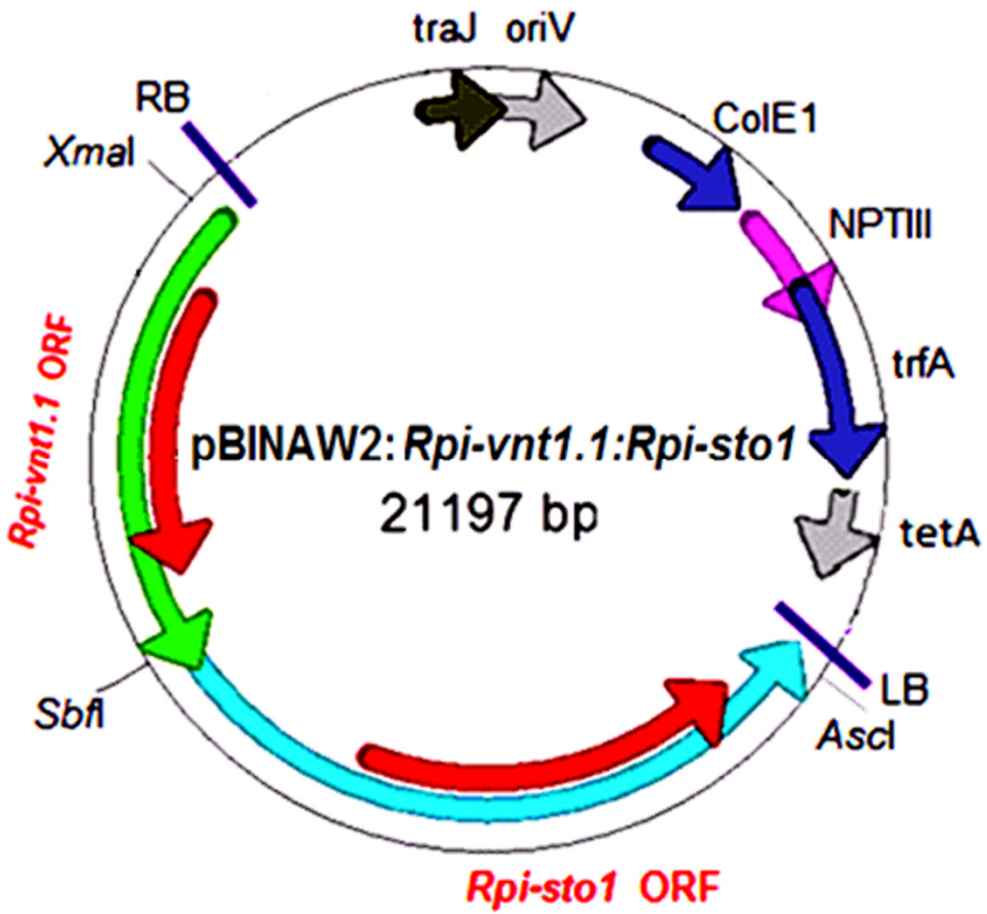
DuRPh duurzame resistentie tegen *Phytophthora* in dappel door cisgene merkervrije modificatie

Cisgenic stacking with marker free double *R*-gene construct pBINAW2: Rpi-vnt1.1:Rpi-sto1



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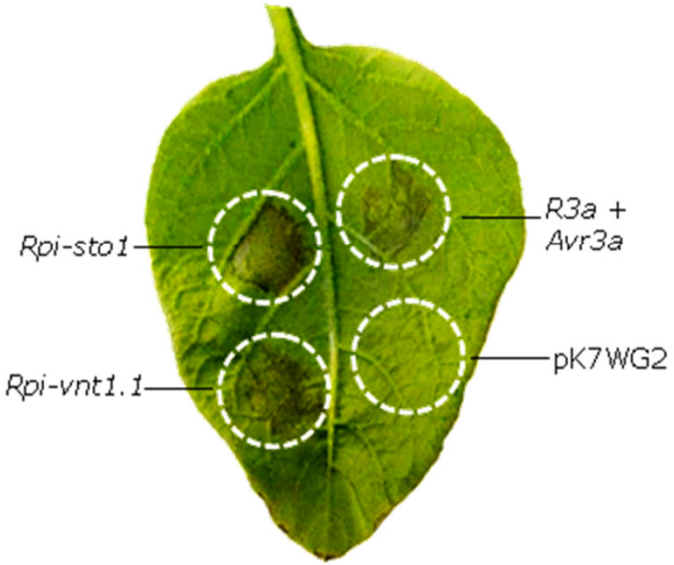
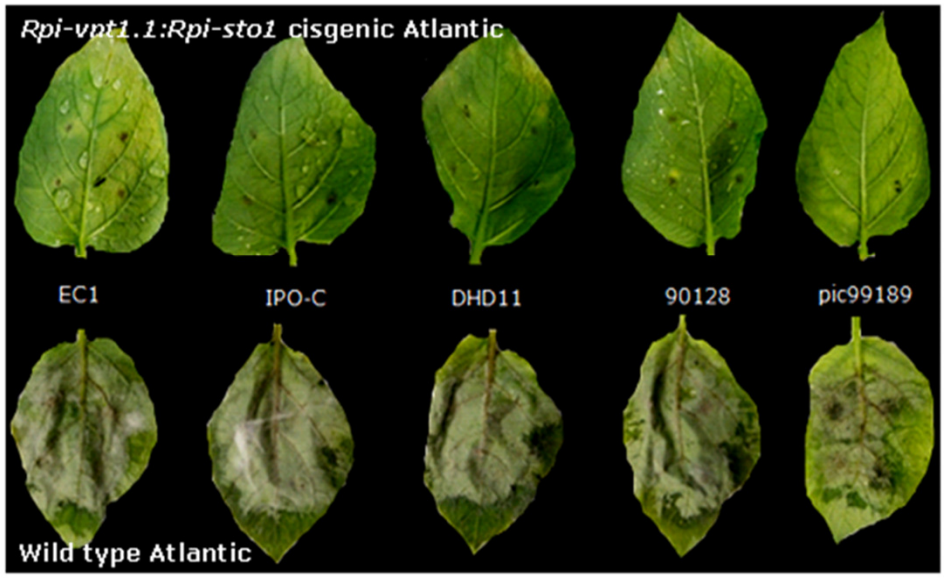
Validation of cisgenic transformants by avirulence test and resistance assays in H43-7 (*Rpi-vnt1.1::Rpi-sto1* of cv Atlantic)

A Agroinfiltration



B Detached leaf assay



Characterization of cisgenic transformants carrying two *Rpi* genes in different potato varieties

No	Plant ID	Host plant	PCR				Greenhouse	Agroinfiltration		DLA				
			Rpi-vnt1.1	*Rpi-sto1*	copy number[a]	back bone	abnormal type	*Rpi-vnt1.1*	*Rpi-sto1*	EC1	IPO-C	DHD11	90128	pic99189
1	H43-1	Atlantic	+	+	1/0	no		+	-	S	R	R	R	R
2	H43-2	Atlantic	+	+	1/1	no	curly leaf	n	n	n	n	n	n	n
3	H43-3	Atlantic	+	+	1/0	no	curly leaf	n	n	n	n	n	n	n
4	H43-4	Atlantic	+	+	1/0	no		+	-	S	S	S	S	S
5	H43-7	Atlantic	+	+	4/2	no		+	+	R	R	R	R	R
6	H43-8	Atlantic	+	+	4/1	no		+	+	R	R	R	R	R
7	H43-10	Atlantic	+	+	1/0	no		+	-	S	R	R	R	R
8	H43-11	Atlantic	+	+	3/1	no	curly leaf	n	n	n	n	n	n	n
9	H43-12	Atlantic	+	+	1/0	no		+	-	S	S	S	S	S
10	S43-2	Doip1	+	+	3/1	no		-	-	R	S	S	R	S
11	F43-1	Bintje	+	+	2/0	no		+	-	S	R	R	R	R
12	F43-2	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
13	F43-3	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
14	F43-4	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
15	F43-5	Bintje	+	+	2/2	no		+	+	R	R	R	R	R
16	W43-1	Potae9	+	+	3/1	no		+	+	R	R	R	R	R
17	W43-2	Potae9	+	+	3/1	no	curly leaf	n	n	n	n	n	n	n
18	W43-3	Potae9	+	+	3/0	no		+	-	S	R	R	R	R
19	W43-4	Potae9	+	+	3/0	no	dwarf	n	n	n	n	n	n	n
20	W43-5	Potae9	+	+	3/1	no		+	+	R	R	R	R	R

a: copy number for Rpi-vnt1.1 or Rpi-sto1, respectively. “0” means that no estimates were made

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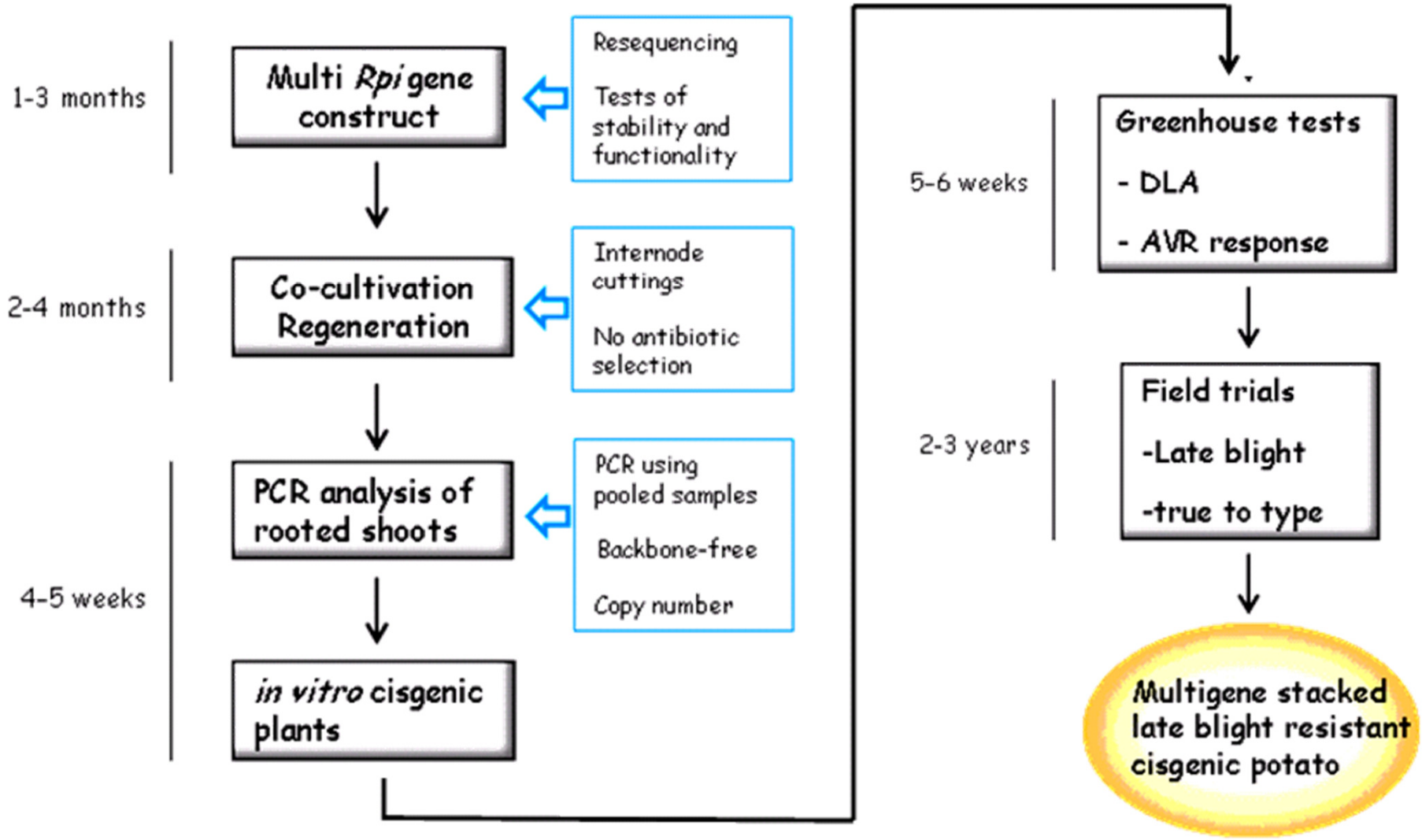
Breeding time scheme for potato with cisgenic, multiple *R*-gene resistance to late blight



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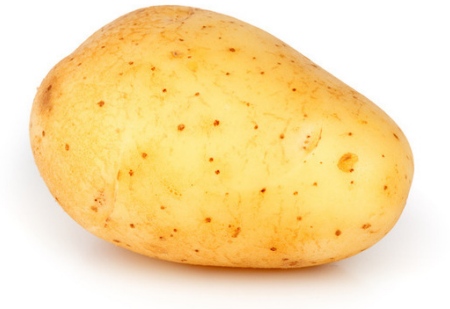
Cisgenic products in development



Potato (DURPh) and other initiatives: resistance to *P. infest*

Apple (Malnoy et al., 2007)
Non-browning apple

Barley (Holme et al., 2012)
Improved phosphate uptake by animals

Other examples are: 1. Wheat with multiple powdery mildew resistance alleles;

2.Rice with bacterial blight resistance; 3. Poplar with altered morphology

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S-gene approach by RNAi

Loss of function of *S*-genes by loss of function mutation or RNAi can lead to durable, broad-spectrum, and multiple pathogens resistance

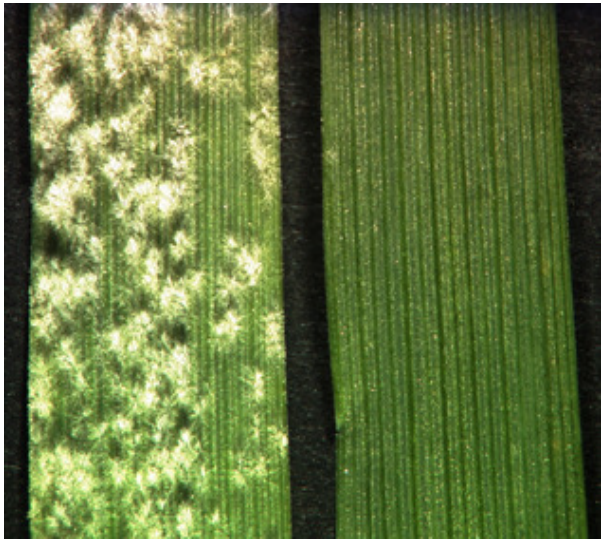
A *S* gene is a plant encoded gene helping pathogenesis after induction by the pathogen

Such *S* genes can have also other functions in the plant. Loss of function can lead to pleiotropic effects

S-gene based resistance started with a barley *mlo* mutant against powdery mildew

- **Barley *mlo* mutants** show non-race specific resistance to powdery mildew and have been successfully used in European agriculture for more than 30 years

- In **pea, tomato, grape,** mutants from the same *Mlo* gene found, causing resistance to powdery mildew



Phenotype of a susceptible (*Mlo* , left) and a *mlo* -resistant (right) barley genotype infected by powdery mildew.

TALENs and CRISPR-Cas9 systems based mutation induction of *MLO* in A, B and D genome of wheat resulted in mildew resistance

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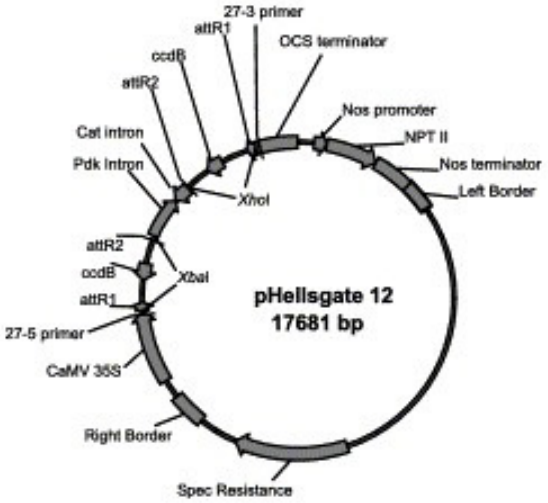
For quality of life

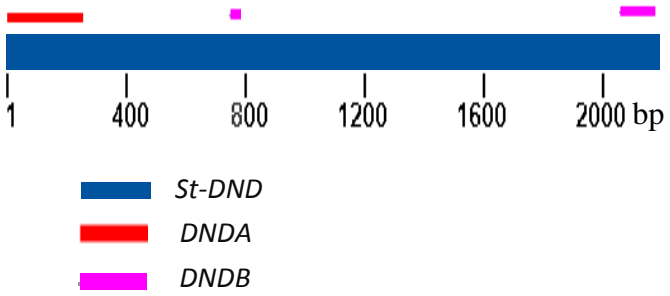
Eleven possible *S*-genes identified in *Arabidopsis* were tested in potato

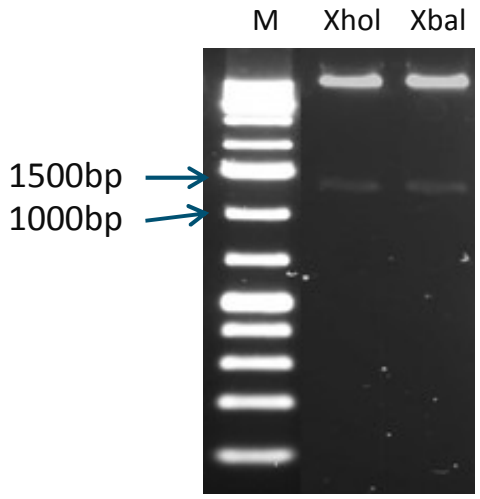
gene name	Function (*Arabidopsis*)
BIK1	membrane-anchored protein kinase
CESA	cellulose synthase
CPR5	transmembrane protein
DMR1	homoserine kinase
DMR6	2-oxoglutarate-Fe oxygenase
DND	**cyclic nucleotide-gated ion channel**
PMR4	callose synthase
PMR5	unknown
PMR6	pectate lyase-like protein
SR1	Ca/calmoduline-binding transcription factor

StDND RNAi constructs in potato



StDND :*DNDA and DNDB constructs*





pHellsgate12-*StDND2*

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Late blight tuber assay after *StDND* silencing

Pic99189 7 dpi

Desiree *A13-013* *DNDA-8(+)* *DNDB-11(+)*

Transformants DNDA-8 and DNDB-11 showed resistance to late blight isolate Pic99189 on leaves and tubers like transformant A13-013 containing the major *R* gene *Rpi-vnt1.1*

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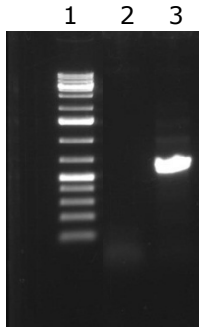
Powdery mildew resistance after *StDND* silencing

resistant

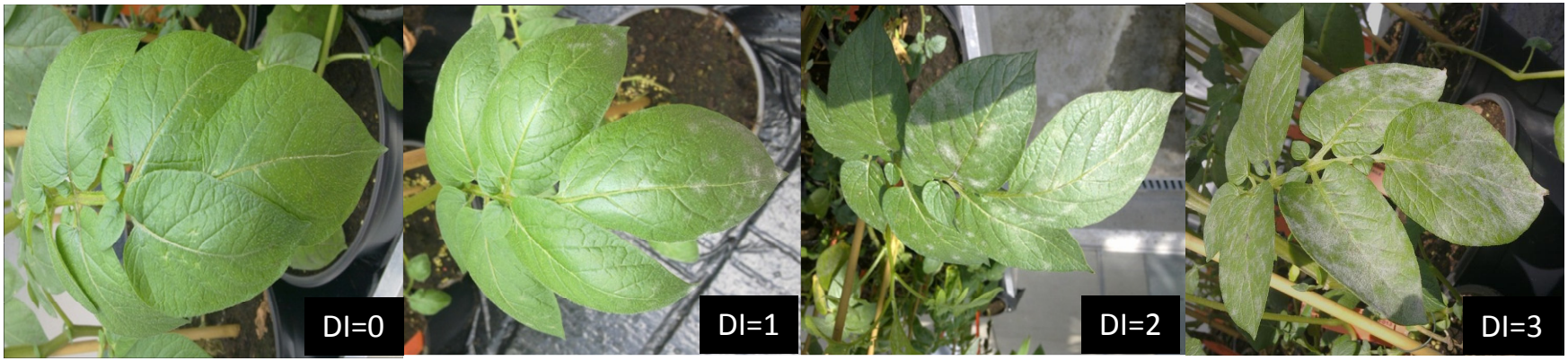
the PCR product from ITS4/ITS5



1:1KB ladder; 2:water; 3: infected potato leaf DNA

Disease score system

Potato plants grown in greenhouse are naturally infected with the powdery mildew pathogen



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Effects of RNAi of *StDND* in potato

Resistance to: *Phytophthora infestans (* leaves and tubers)

Powdery mildew species *Golovinomyces orontii*

Susceptibility to: Potato virus Y

Golden cyst nematode *Globodera rostochiensis*

Potato early dying by *Verticilium dahliae*

Pleiotropy: auto-necrosis; early senescense, low degree of dwarfing

Intragenic gene constructs with own promoter provide the same resistances but with less pleiotropic effects

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Impact of cis- and intragenesis for potato breeding

- **Intragenesis**: mimicking loss of function mutations of *S* genes by RNAi. This is important for many traits in existing varieties

- **Cisgenesis**: important for dominant traits, partly replacing introgression breeding

- **Combination of cis- and intragenesis** provides opportunities for durable resistance based on *R*-gene stacking and loss of function of *S* genes in one genotype

Actual situation around regulation of cisgenesis in EU

Today: Cisgenesis is treated like transgenesis

Six reports:

- 1. RIKILT: for food safety. Definition of cisgenesis needed
- 2. EFSA: as safe as conventional breeding
- 3. New Techniques Working Group: provides a definition. Majority is in favour of exemption or non-GMO
- 4. JRC State of the art on new plant breeding techniques
- 5. USA 2004: Safety of GMO foods: assessing unintended effects. Plants with own genes have not to be considered to be a GMO
- Eurobarometer: Majority of consumers in favour of cisgenesis

Future: cisgenesis will be treated as non-GMO?

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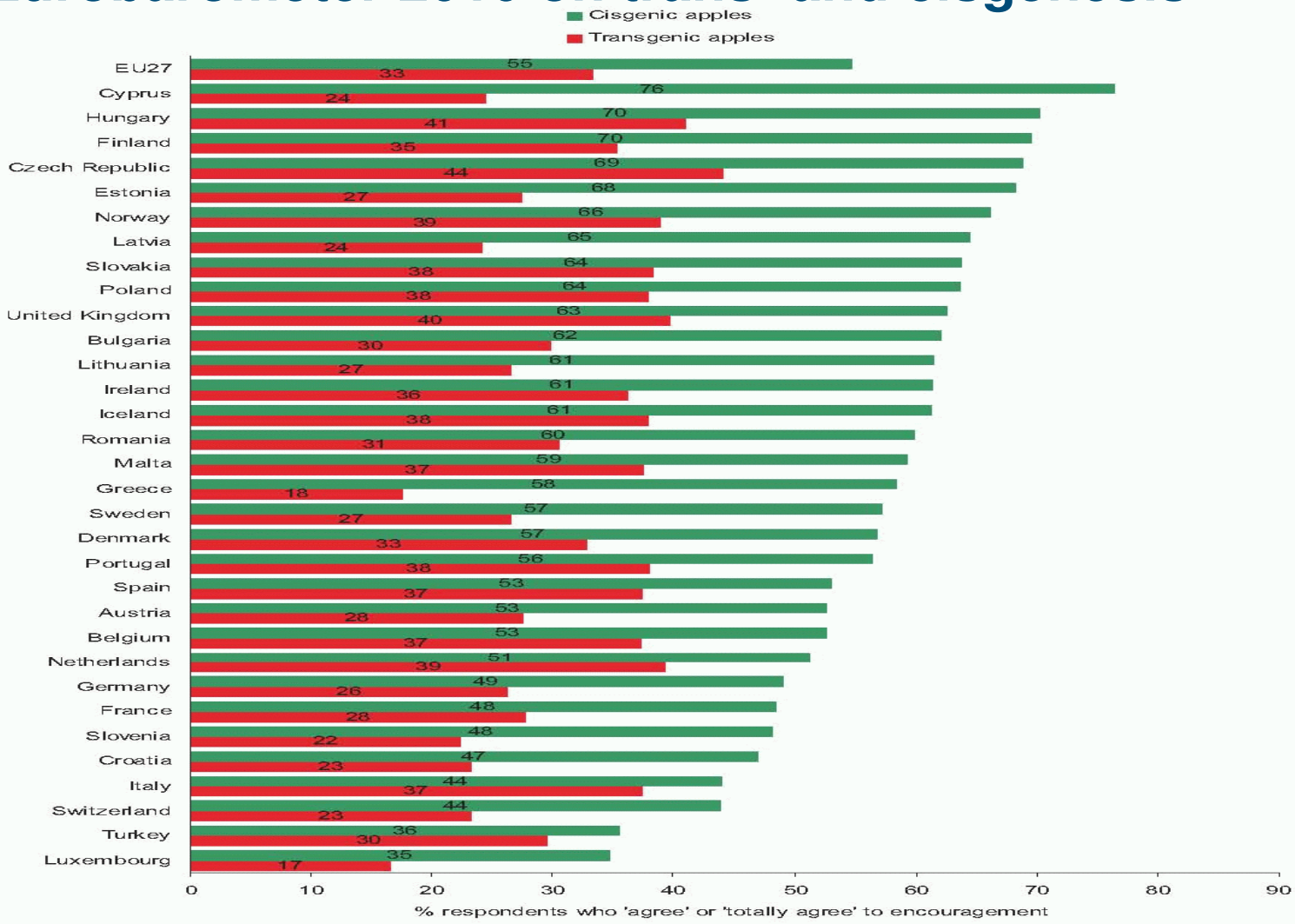
Comparison: unintended effects in cisgenic and conventionally bred plants

- **Conventional breeding**
- Likeliness unintended effects: rel. low- rel. high
- Self-monitoring system prevents against unintended health effects (potato: glyco-alkaloids)
- **Cisgenesis**
- Likeliness unintended effects: low
- Upto 20-24bp LB considered as same gene pool
- No other type of unintended effects expected

Proposal: Use the same self-monitoring system for cisgenesis as used in conventional plant breeding to come to cisgenic varieties

Eurobarometer 2010 on trans- and cisgenesis



24 out of 32 countries >50% in favour of cisgenesis

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Gaskeel et al., 2010

Future of Cisgenesis and Intragenesis

- EU authorities don't like to change regulation in the short run

- Instead of a cisgene, an **intragene is not found in nature** causing additional safety questions to be answered

- More and more **legal arguments** are found for cisgenesis to be treated as non-GMO.

Are cisgenesis and later intragenesis next steps in classical plant breeding?

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THANK YOU