Slide 1

New Plant Breeding Techniques (NPBTs)

Promising applications

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Slide 2

New Plant Breeding Techniques (NPBTs)

New techniques for more precise and/or faster breeding

- Use of genetic modification, but final plant product usually does not contain foreign DNA

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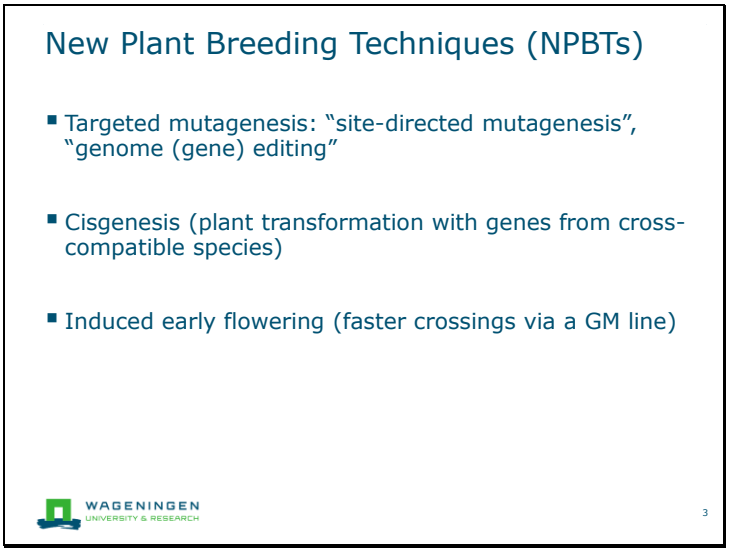
No foreign DNA means that no genes, genetic material or mutations present that could also not be introduced through conventional breeding methods.

Slide 3



Targeted mutagenesis is performed with SDNs (site-directed nucleases, also called SSNs, sequence-specific nucleases, e.g. CRISPR-Cas, TALENs, see further in following slides). "Site-directed mutagenesis" is actually the most precise description as most applications up to now use the cell's imprecise repair mechanism for the cuts made by the SDN. The cut itself is precise, i.e. at a specific site in the sequence, but the repair is more or less random in the sense that some nucleotides may be lost or some gained at the cut site. For that reason, the term "genome editing" may be somewhat euphemistic as the sequence is not changed as one would do in editing using a word processing software; still the exact genomic positioning of mutagenesis (in this case mostly gene knock-outs) is a huge improvement on classical mutagenesis. Furthermore, with homology-directed repair (explained in later slides), exact sequence changes can be introduced, but this is at an earlier stage of development so less efficient yet. Some use "gene editing" instead of "genome editing", but this may be too limited as one could also change all sorts of regulatory sequences or longer stretches of genomic DNA. See further in later slides.

Slide 4

Targeted mutagenesis (genome editing)

- Changing the genome at a specific site

- Changes: two possibilities

  1. Small deletion — Targeted mutagenesis

  Aim: changing effects of specific gene, usually knocking it out

  2. Exchange of DNA with another piece of DNA, e.g. desirable gene variant (allele)

  Targeted recombination (homologous)

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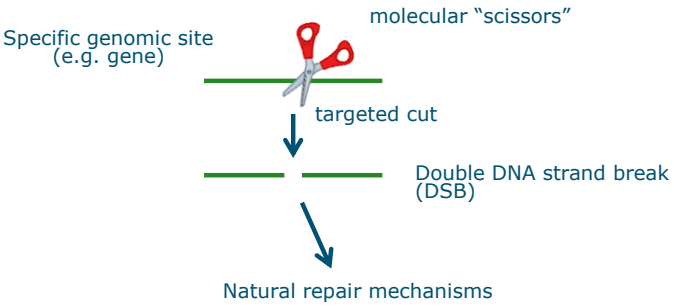
Slide 5

How does it work?



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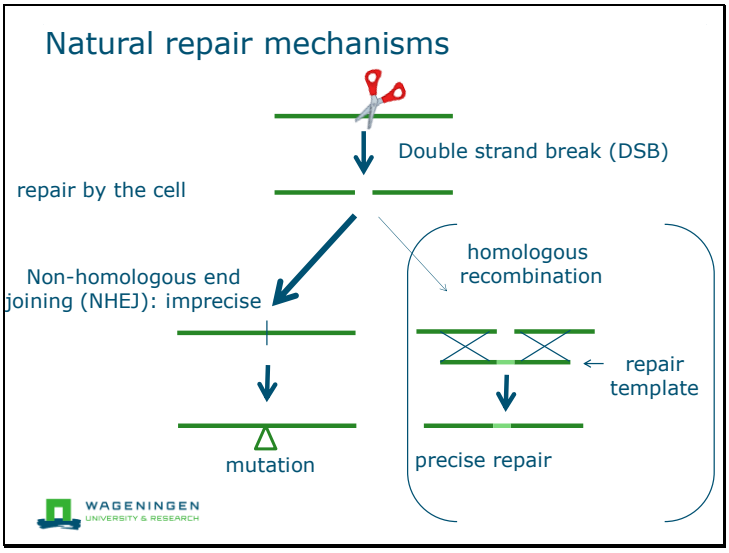
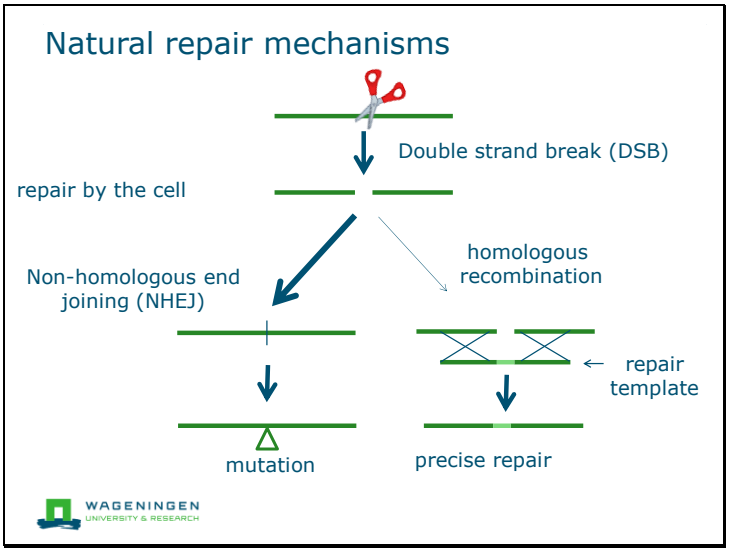
Slide 6



Homologous recombination or homology-directed repair (HDR) far less efficient in plants, therefore quite some research into developing systems improving its efficiency. Next slide shows an alternative animation of this slide, so as to separate NHEJ and homologous recombination in order to make the slide less full in the first instance and more comprehensible.

Slide 7



The site-directed nuclease makes a double-strand DNA break at a precise location in the genome. Two repair mechanisms are operable in the cell. The most frequently occurring is the NHEJ that is imprecise and may lead to various mutations. The other mechanism, homologous recombination or homology-directed repair (HDR), can use a repair template offered by the researcher. In order to have the template built in, it is provided with sequences at the ends that match those at either side of the DSB so that homologous recombination can proceed (indicated by the crosses in the picture). Homologous recombination or homology-directed repair (HDR) is far less efficient in plants (indicated by the slender arrow), therefore quite some research effort is put into developing systems improving its efficiency.

Slide 8

Molecular "scissors"

- Targeted DNA-cutting enzyme (nuclease)
  - Recognizing specific site in genome (DNA sequence) by a DNA-binding domain
  - Cutting domain
- Site-specific nucleases (SSNs), Site-directed nucleases (SDNs), Programmable nucleases, Designer nucleases....
- Zinc Finger Nucleases ~2005 (ZFN)
- TALENs ~2009
- CRISPR-Cas ~2012

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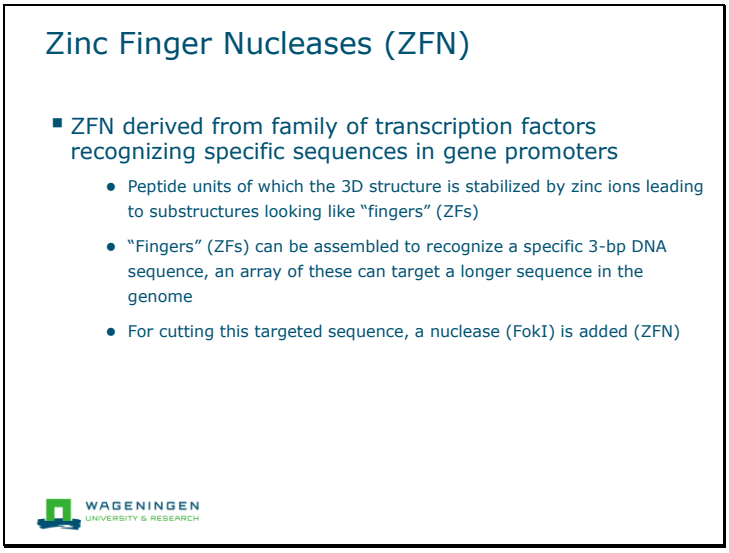
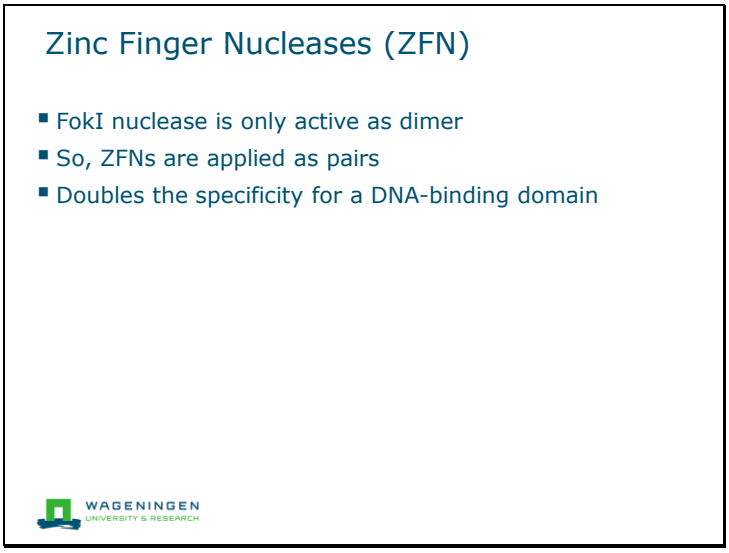
ZFN, TALEN, CRISPR-Cas are explained in following slides.

Slide 9



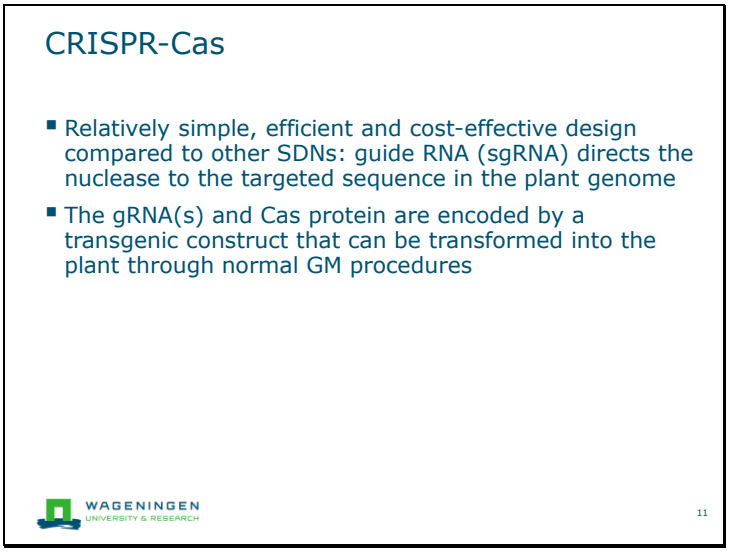
Transcription factors direct e.g. developmental programmes by regulating expression of a series of genes through targeting specific sequence elements in their promoters. The fingers are assembled together with the nuclease by transcription from a (transgenic) construct brought into the cell.

Slide 10



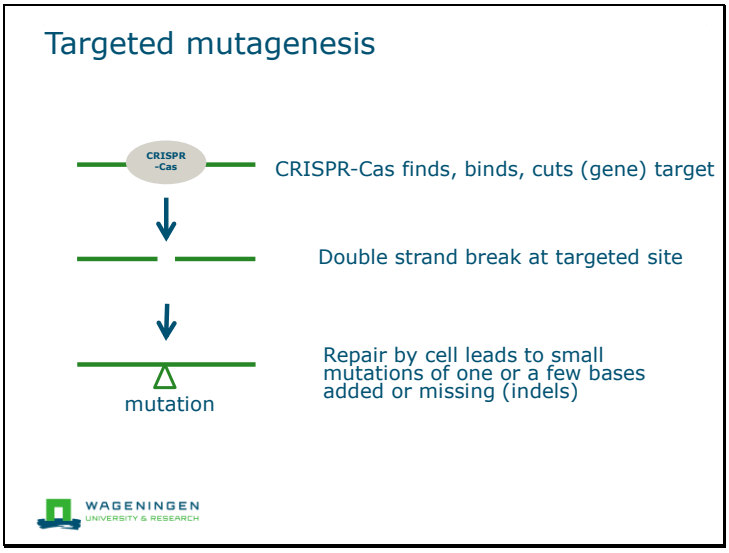
Likewise, TALENs (Transcription Activator-Like Effector Nucleases) are based on effector proteins (TALE) used by plant pathogens that also recognize specific sequence elements in promoters of plant genes, as do plant transcription factors such as the Zinc Finger family. In the case of TALE produced by pathogens, the gene's function is hijacked through its promoter for the purpose of accommodating the pathogen (e.g. a sugar transporter gene is manipulated to provide sugars to the pathogen instead of having its normal role in plant functioning, see the rice bacterial S gene in later slides). The TALEs can also be tailor-made for recognizing specific DNA sequences and combined with the cutting enzyme FokI (nuclease). TALEs have the advantage of being designable base by base, so for every conceivable sequence. ZFs are designed per triplet of 3 bases, with not all triplets designable; their advantage is being relatively small, which makes them easily fit in constructs for plant transformation.

Slide 11



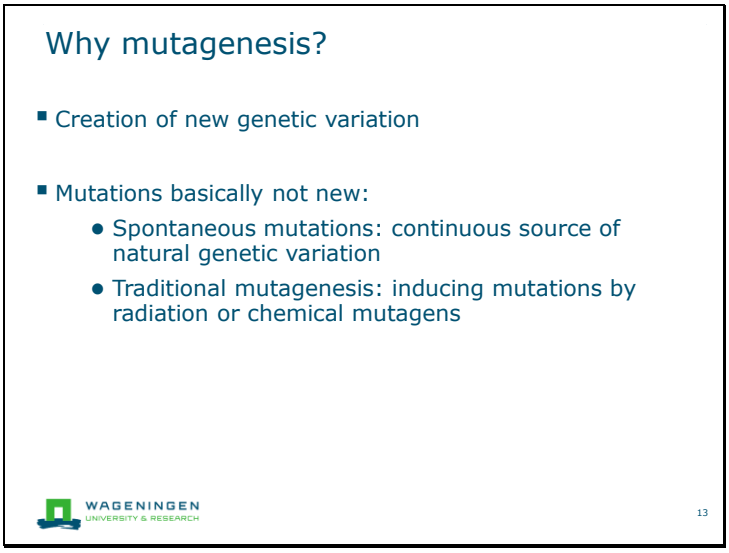
CRISPR-Cas: Clustered Regularly Spaced Palindromic Repeats-CRISPR associated protein 9 nuclease, based on a bacterial system providing resistance to bacteriophages. The system recognizes invading viral DNA from bacteriophages through RNAs from palindromic repeats in the bacterial genome. These repeats form a storage (“memory”) of specific sequences from bacteriophages. The RNAs recognizing the specific viral sequences through their homology to them are a guide for a Cas protein that will cut these specific viral sequences. For use in genome editing, so-called guide RNAs are designed by the researcher (instead of “provided” by bacteriophages) to direct Cas to the specific genomic site for cutting DNA at the desired sequence. One further limiting condition exists for the targeted genome sequence: in the neighbourhood of the targeted sequence, a so-called PAM site needs to be present, in the case of Cas9 with sequence NGG (N = any of the 4 nucleotides). The PAM site is the sequence used in the bacterial virus resistance system to distinguish the virus DNA from the palindromic sequences on its own genome (to avoid cutting its own genomic DNA). Other Cas proteins (e.g. the also becoming popular Cpf1) have different PAM sites. The great advantage of CRISPR is that no complex design of peptide sequences recognizing specific DNA sequences, as in ZFN or TALEN, is needed but that homologous gRNAs are designed simply based on the targeted plant genomic sequences. CRISPR-Cas construct can be introduced into plant cells by transformation as mentioned and later on, after mutagenesis has taken place, be removed by crossing. Alternatively, mutagenesis can also be performed by transient expression from a CRISPR-Cas vector (plasmid) brought into the plant cell without integration of the construct in the pant genomic DNA. It may even be possible to bring a CRISPR ribonucleoprotein complex (without DNA) into the plant cell for performing mutagenesis. In both latter cases, no transgene needs to be removed by crossing out, just in the case of using a plasmid vector for transient expression, the genome of the regenerated plant needs to be checked for vector sequences that may have been integrated by coincidence. For these two alternative methods, usually protoplasts are needed that are more difficult to generate and from which it can also be more difficult to regenerate mutated plants (this is not (yet) possible in all crops). Efficiency of HDR could also be raised by using modified geminivirus as vector for the CRISPR-Cas construct and repair template.

Slide 12



Indels = insertions and/or deletions of bases. With comparing sequences between plants/accessions/species, it is not clear whether such differences were caused by removal of a sequence (deletion) or addition of a sequence (insertion), thus the general term “indel” is often used for that. With targeted mutagenesis, whether bases were removed or added can be monitored by sequencing before and after the mutagenic treatment.

Slide 13



Mutations are not new, but the ones leading to a desirable trait cannot always be easily found in the existing germplasm or in natural populations of wild species. In natural populations, this might be related to natural selection against mutations disadvantageous under natural environmental conditions. That led to developing mutagenesis already early in 20th century, once the DNA damaging effects of radiation (e.g. X(Röntgen)-rays, later on, gamma-rays, and also UV) were discovered, later on followed by applying chemical mutagens.

Slide 14

Traditional mutagenesis

- Random, in order to have a chance of finding the desired one:
  - Many mutations needed
  - Mutation frequency: 1:50.000-500.000 bp (polyploid to diploid)
  - Large population of mutagenized plants needed (5.000-10.000)

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Traditional mutagenesis

- Random, after having found the desired one, additional crossings needed:
  - Mutation often only effective in homozygous state (e.g. 4 alleles in tetraploids, such as potato)
  - Removing undesirable mutations (840Mb/50Kb~17.000 mutations/haploid genome of potato for example)
  - Crossing (introgression) into elite varieties

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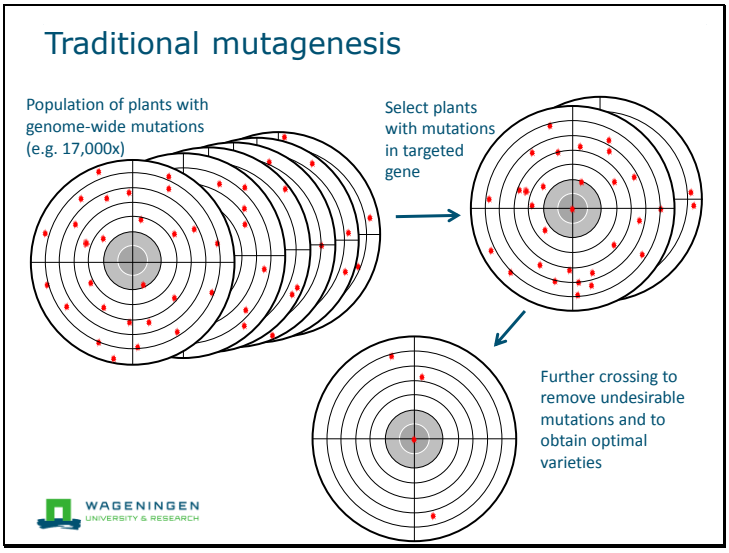
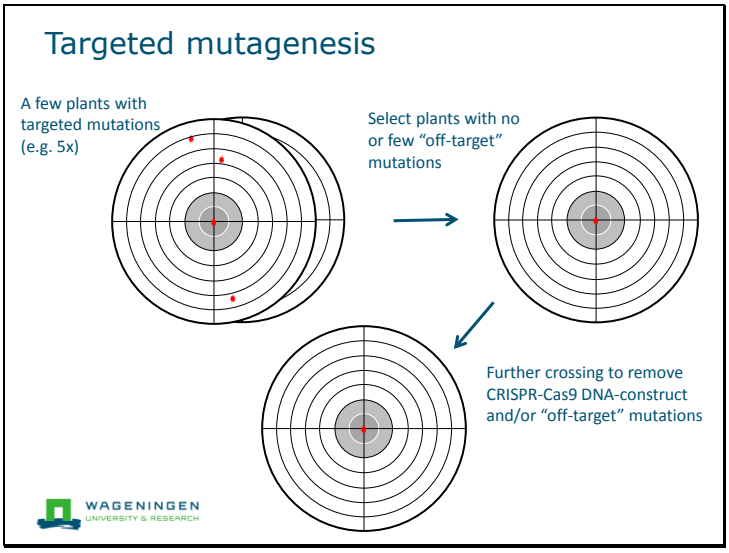
Mutations are most often gene knockouts that inherit recessively.

Slide 16



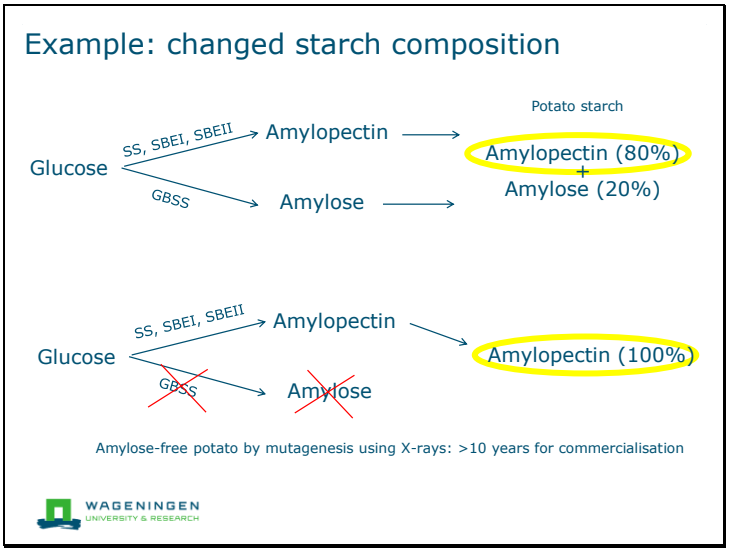
Comparison to shotgun: random mutation like a shower of shot (“schot hagel”), the target is hit at a series of random places, the desired mutation is like a hit (”toevalstreffer”) of the “bull’s-eye”, but still accompanied by other random hits, so additional “cleaning” is necessary, in reality achieved by crossing with elite plant lines.

Slide 17



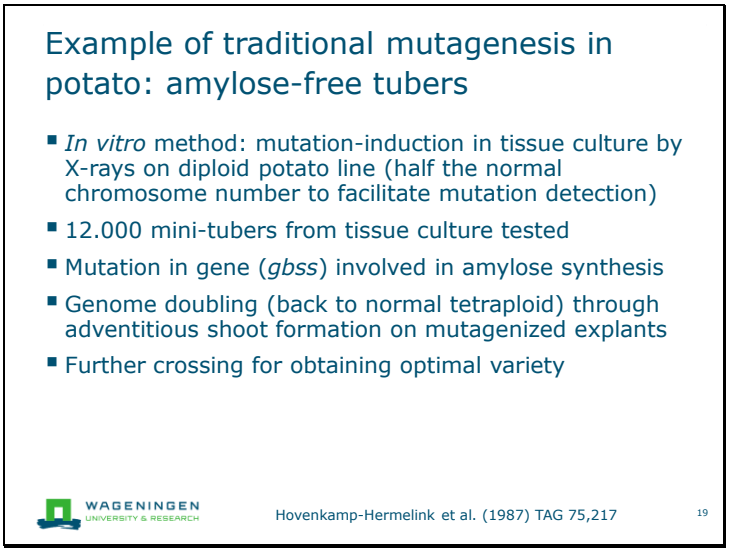
Comparison to shotgun: the targeted mutation is like a well-directed single bullet, the target is hit straight into the "bull's-eye" but may still be accompanied by other hits, so-called "off-target" effects that will mostly be due to similarities in sequences elsewhere in the genome to the sequence at the desired site. Additional "cleaning" for that may be necessary, in reality again by crossing with elite plant lines.

Slide 18



Amylopectin (branched starch) higher quality for use as thickening agent or stabilizer (a.o. lower tendency of gelling during storage). Having a potato with exclusively amylopectin saves costly and chemicals-demanding separation processes in the starch industry. GBSS: granule-bound starch synthase, enzyme producing amylose (unbranched). SS: starch synthase, SBE: starch-branching enzymes (I and II), involved in producing amylopectin. By knocking out *GBSS*, no more amylose is produced. It is by the way also possible to bring down amylopectin by targeting the SBEs, which could be desirable as amylose purportedly has dietary health advances (a.o. lower glycemic index).

Slide 19



For mutagenesis, monoploid leaf explants were used. Monoploids are also called dihaploids, they contain half of the normal chromosome number (in this case 2x from a tetraploid potato). They are obtained through haploidization techniques, i.e. anther culture or use of haploid-inducing pollinator *Solanum phureja*. This is necessary because knocking out a gene produces a recessive trait and the chance of finding such trait in a tetraploid such as potato is very low.

Targeted mutagenesis variant

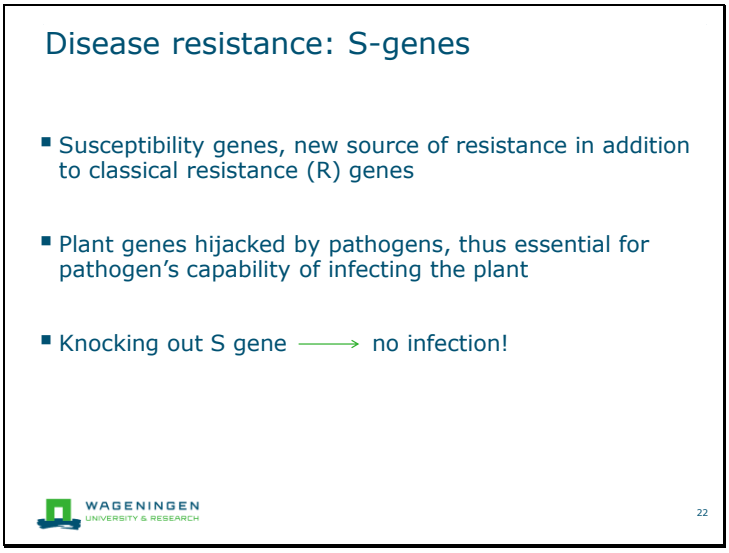
- Targeted mutagenesis
  - Mutation specifically in gene responsible for desired trait (e.g. *gbssI*)
  - Mutation in several alleles at the same time
  - Further crossing not needed (maintenance of elite variety make-up)

Amylose-free tetraploid potato by CRISPR-Cas

- All 4 *gbssI*-alleles mutated simultaneously
- Transient expression of CRISPR-Cas: expression from plasmid transferred into protoplasts, plants regenerated without CRISPR-Cas being transformed into plant genome
- No further crossing needed

Andersson et al. (2017) Plant Cell Rep 36,117

Slide 22



Classical R genes popular in breeding: simple dominant inheritance. R genes recognizing specific pathogen effectors, can be relatively easily circumvented by pathogens by changes in effectors etc. See further Cisgenesis below.

Slide 23

Disease resistance: S-genes

- Oldest example: barley *mlo* mutants resistant to powdery mildew
- *mlo* resistance effective in barley for more than 30 years!
- Other examples of *mlo* in: tomato, pea, grapevine...
- In hexaploid (6x) wheat, simultaneous knockout of six *MLO* alleles via gene editing

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MLO = mildew locus O

Slide 24

Disease resistance: S-genes

- Other S-genes identified in model plant *Arabidopsis thaliana*, crops such as maize, barley, pepper, tomato, potato
- Knockout of S-genes can have disadvantageous pleiotropic effects in the plant
- More precise mutagenesis by CRISPR-Cas of S genes may circumvent negative effects on plant performance?

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Slide 25

Disease resistance: S-genes

- S-gene for rice bacterial blight by *Xanthomonas oryzae* pathovar *oryzae* (Xoo)
- Silencing of *Os11N3*-expression: Xoo resistance! ...but *Os11N3* is essential gene in rice. Silencing results in stunted plants.

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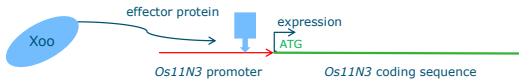
Li et al. 2012 Nat Biotechnol 30:390

Li et al. (2012) NatBiotech 30,390

Slide 26

Disease resistance: S-genes

- Xoo bacterium produces effector-proteins that activate the expression of *Os11N3*, which is responsible for sugar transport (also called SWEET14), to provide for its nourishment



- How could resistance be achieved without affecting the plant's performance, using targeted mutagenesis?

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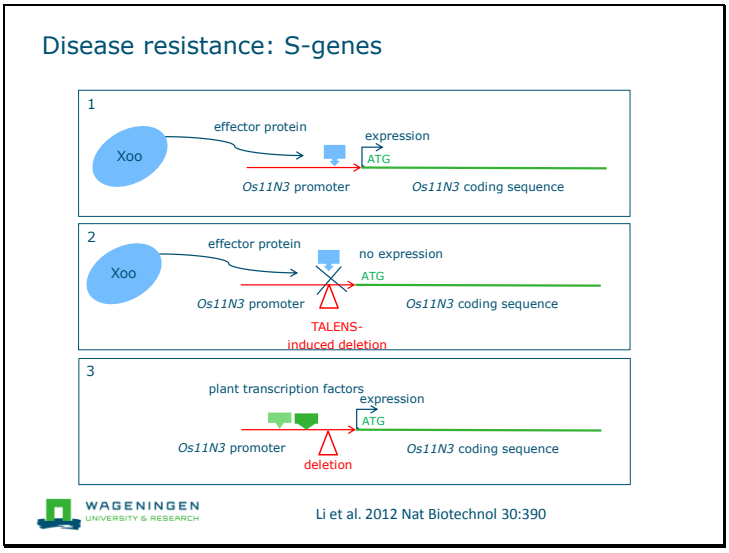
Li et al. 2012 Nat Biotechnol 30:390

Slide 27



The pathogen manipulates the plant to provide nourishment through its effector protein. The effector directs gene expression by targeting the plant's *Os11N3* (*SWEET14*) gene promoter. In order to knockout the pathogen's "abuse" of the plant promoter while keeping the plant's gene function intact, Li et al. (2012) tested which part of the promoter was essential for the pathogen's effector using TALENs targeted mutagenesis, i.e. by making small deletions at specific sites. Subsequently, the single deletion in the promoter found to be providing resistance to the pathogen was shown not to obviously affect the gene's function for the plant. Apparently, the plant transcription factors target different parts of the promoter, tentatively indicated by the two "TFs" in green in the third panel. Ironically, the targeted mutagenesis is performed with the TALENs system, which is based on combining a nuclease with an effector protein (Transcription Activator-Like Effector) similar to the one used by the pathogen to affect *Os11N3* gene expression (see the slides on ZFN & TALEN before). USDA regarded this plant as outside of its regulatory purview; field testing is presently ongoing for possible commercialisation (Hilscher et al. 2017).

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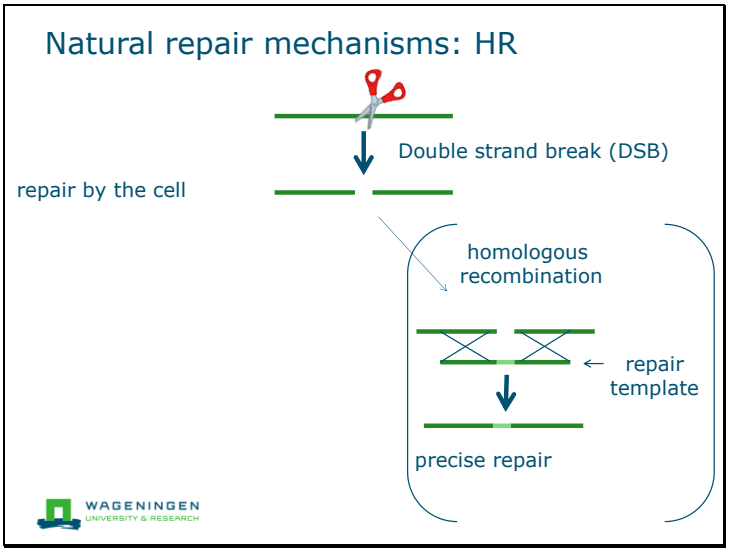
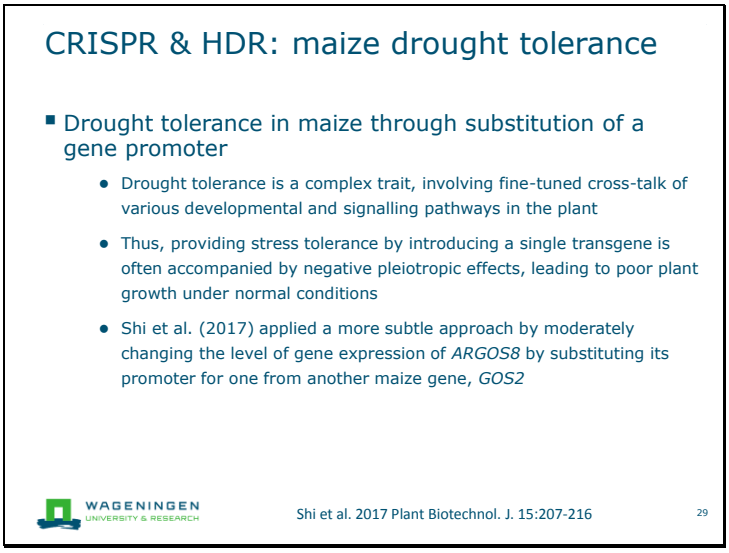


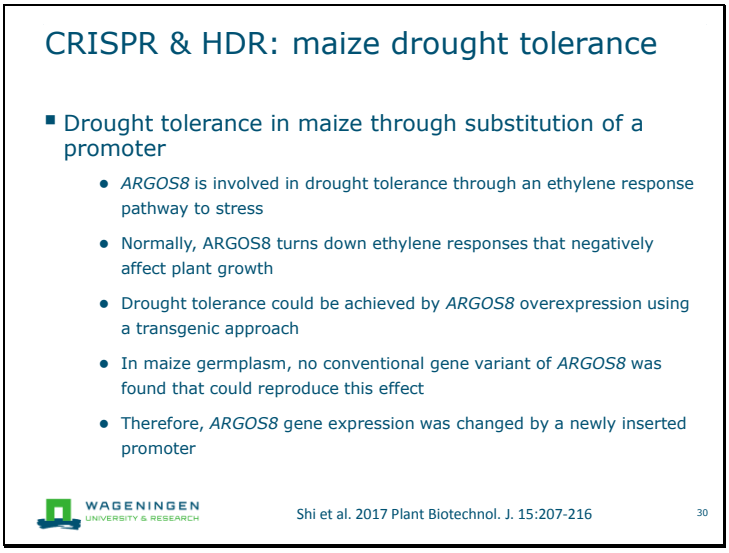
The alternative repair mechanism (apart from NHEJ), homologous recombination or homology-direct repair (HDR), can use a repair DNA template offered by the researcher. In order to have the template built in, it is provided with sequences at the ends that match those at either side of the DSB so that homologous recombination can proceed (indicated by the crosses in the picture). Homologous recombination or homology-directed repair (HDR) is far less efficient in plants (indicated by the slender arrow), therefore quite some research effort is put into developing systems improving its efficiency, e.g. by using geminivirus-based vectors.

Slide 29



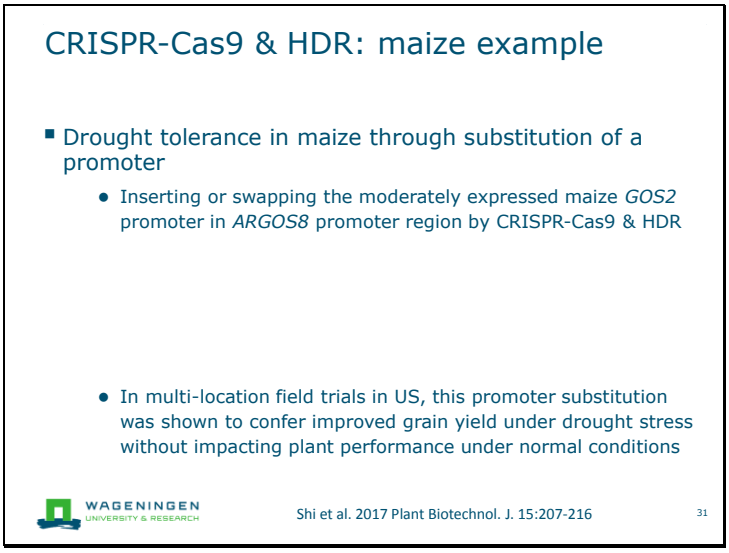
Up to now, the only commercially cultivated transgenic drought-tolerant crop is the Monsanto DroughtGard maize (based on a single bacterial gene, see GM examples ppt). Apart from introducing a transgene from another organism, simply overexpressing or silencing a single plant gene using GM can be applied to confer stress tolerance, but this will also often be accompanied by negative side-effects. Negative effects can be disturbance through cross-talk of other signalling pathways. A simple example could be that closing stomata can be a useful response to drought but when also artificially induced under normal conditions, normal growth will be affected. On the other hand, keeping stomata open under drought could be negatively selected for under natural conditions, but could help increase yields under agricultural conditions, provided that the plant is not lethally pushed across limitations of water use. Overexpressing a gene is usually performed by combining it with the constitutive 35S or ubiquitin promoters in a transgenic construct. Sometimes, negative effects can be reduced by using a stress-inducible plant promoter, such as rd29A, leading to the transgene only being expressed under stress conditions.

Slide 30



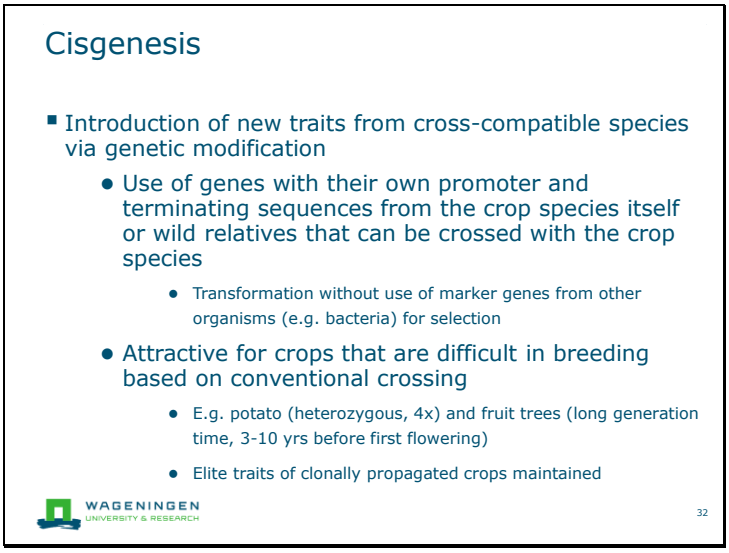
Ethylene is a plant hormone involved in several regulatory pathways, for instance fruit ripening, but also responses to abiotic stresses, e.g. reducing ethylene synthesis or the plant's sensitivity to ethylene can improve grain yield under stress situations, but this may come with a cost under non-stress conditions. Though standard transgenic overexpression of *ARGOS8* apparently did not show yield penalties under normal conditions tested, this study shows the possibilities of a more subtle engineering of plant responses through promoter adaptations. A drought-tolerant soybean recently (2015) authorised in Argentina, Verdeca HB4, is also based on a transcription factor supposedly targeting an ethylene pathway.

Slide 31



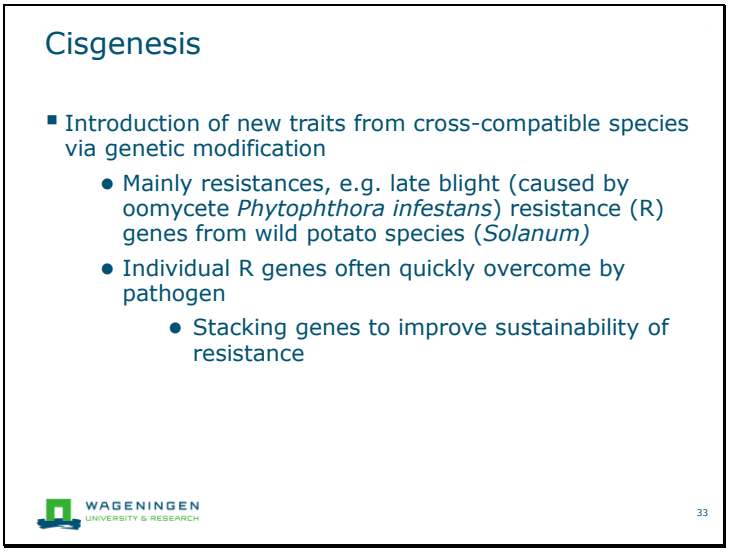
The engineered variants conferred higher grain yield under water stress during the flowering period (likely by an increase in kernel set), but not during grain filling (even in one case performing less than control in a situation of quickly developing drought stress).

Slide 32



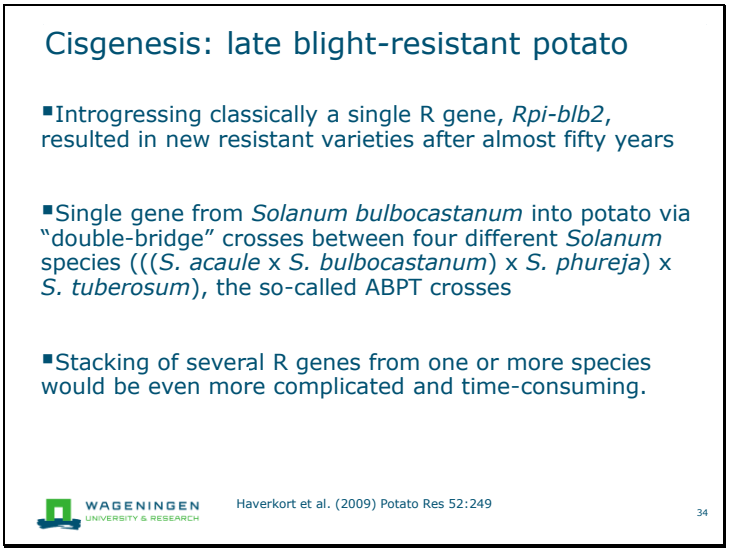
Elite varieties with long market life could be extended with resistances etc. (potato: e.g. Bintje still attractive because processing industry adapted to its characteristics, apple: e.g. Elstar, Golden Delicious are long standing varieties).

Slide 33



Several R genes are more difficult to overcome than a single R gene. For each R gene, an effector protein needs to become changed in the pathogen.

Slide 34



Summarized in Haverkort et al. (2009): After 46 years the first resistant varieties Bionica and Toluca were released for organic cultivation, based on a resistance gene from *Solanum bulbocastanum*. Before that was achieved, "double-bridge" crosses were performed between four different *Solanum* species (((*S. acaule* x *S. bulbocastanum*) x *S. phureja*) x *S. tuberosum*), the so-called ABPT crosses, at Wageningen University Plant Breeding. Tetraploid *S. acaule* was crossed with diploid *S. bulbocastanum* and the resulting triploid hybrid was doubled to a hexaploid using the mitotic inhibitor colchicine; the hexaploid was subsequently crossed with diploid *S. phureja*. A tetraploid hybrid was obtained that could be crossed to the crop species. This extensive programme led to the introgression of a single R gene, *Rpi-blb2*. Stacking of several R genes from one or more species would be even more complicated and time-consuming. Knowledge from the DuRPh programme (see below) working on the proof of principle of cisgenic late blight-resistant potato has been used to improve the effectiveness of a crossing programme for the organic market, the BioImpuls programme.

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Cisgenesis: late blight-resistant potato

- 26 *Rpi* genes in 9 different specificity groups identified
- Stacking R genes, e.g.:
  - *Rpi-sto1* (from *S. stoloniferum*)
  - *Rpi-blb3* (from *S. bulbocastanum*)
  - *Rpi-vnt1.1* (from *S. venturii*)

Field trial treated with *Phytophthora infestans*

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Rpi = Resistance against *Phtyophthora infestans*
Field treated with *Phytophthora infestans*: potato cultivar Bintje completely destroyed, some wild species accessions still thriving. For developing a proof of principle of a cisgenic late blight-resistant potato, Wageningen University & Research executed a ten-year programme, DuRPh (Durable Resistance against *Phytophthora*). In Dutch language, the acronym sounds like the word for “daring” or “guts”; an important aim of the project, next to a proof of principle, was to have an open discussion with the public about the pros and cons of this novel concept of using GM without introducing foreign DNA to significantly improve sustainability of potato cultivation by bringing down fungicide use.

Cisgenesis: late blight-resistant potato

- Producing various combinations of R genes ("cassettes") enable resistance management:
  - Choice of resistances as far as possible adapted to presence of specific *Phytophthora infestans* strains in the region
  - Monitoring of resistances in the field: when necessary, increase R genes' life span by fungicide treatment

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Demonstration field trial with Desiree containing varying numbers of R genes

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From front to back: Desiree, Desiree with 1 R gene, 2 R genes, 3 R genes. Left: support of resistance by fungicide spray; right: no spray.

Cisgenesis: apple scab

- Apple scab is major disease in apple, caused by fungus *Venturia inaequalis*
- Normal cultivation: 20-30 sprays per season

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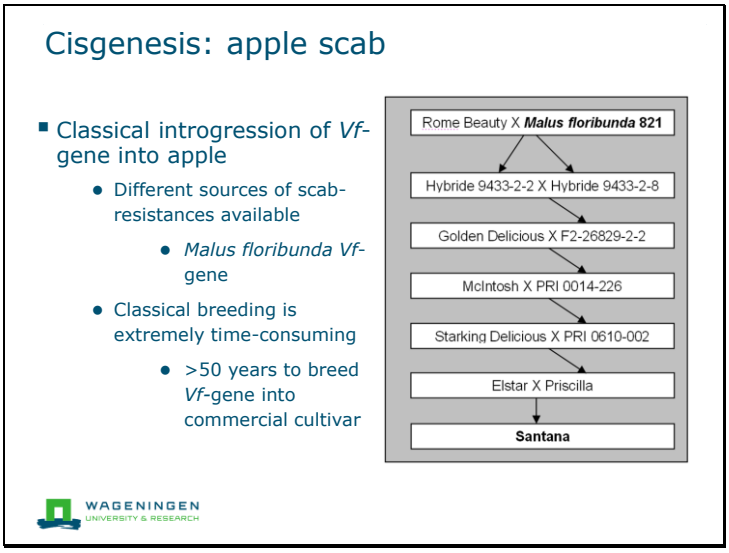

Malus floribunda produces small fruits unattractive for consumption, extensive backcrossing is needed to re-obtain a good quality apple, now provided with the resistance gene *Vf*. Backcrossing is extra time-consuming due to long generation time: apple only flowers 5-12 years after seed germination.

Cisgenesis: apple scab

- Scab resistance genes from wild apple species
  - *Rvi6* (*Vf*), from *Malus floribunda*,
  - *Rvi15* (*Vr2*), from *M. pumila*,
  - *Rvi18* (*V25*), from *M. pumila*

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Cisgenesis: apple scab

- Cisgenic trees in test orchard in Netherlands

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Gala (tree 21)

cisgenic Gala with *Vf* gene (tree 67)

Gala: scab spots on leaves; Gala with *Vf* gene leaves spotless

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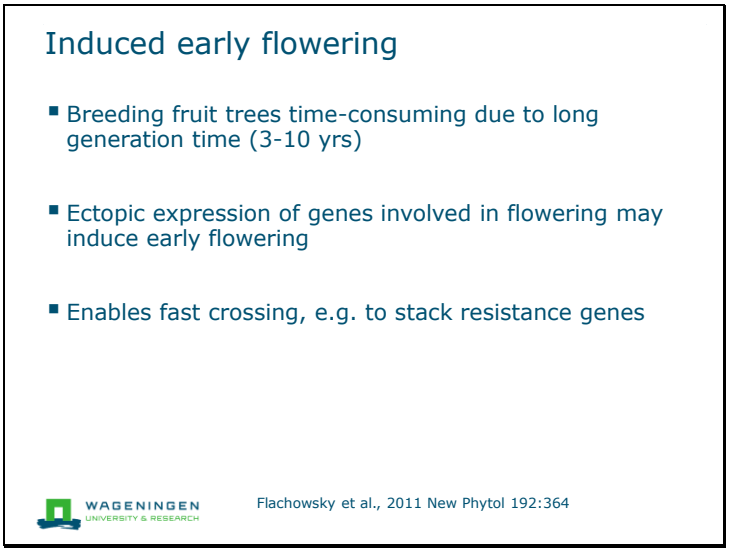

Ectopic expression is the expression in different tissues or at different time points than in a wild type plant, in this case usually overexpression compared to wild type.

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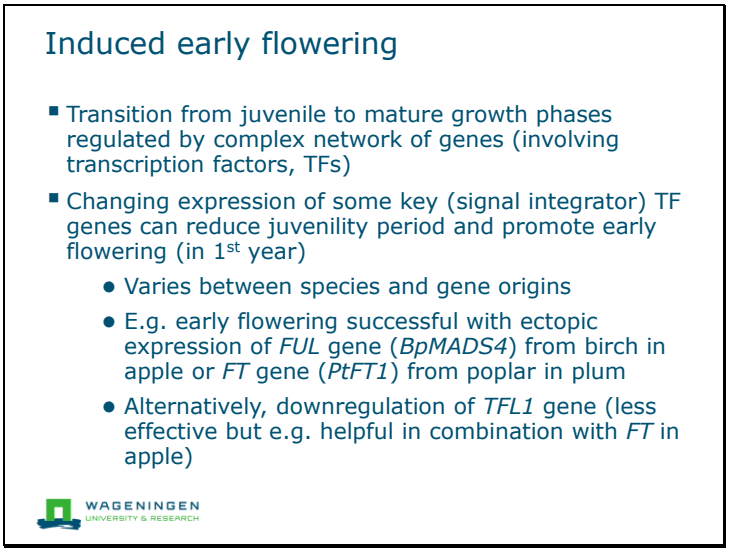

In apple, the *Betula pendula MADS4* gene (or *FUL* = *FRUITFULL*) is used under a constitutive promoter (35S), which could lead to flowering already starting in tissue culture. *MADS4* belongs to a gene family of TFs characterized by a specific sequence motif (MADS), in plants generally involved in developmental processes, among which flower formation and flower part specification. The chosen transgenic clone, T1190, flowered after 5.5. months with no reduction in vegetative growth or change in leaf shape but with a “corkscrew” growth pattern of the main shoot. In the F1 generation, seedlings flowered after 14-40 weeks and were able to produce fertile seeds. Flowering stopped under winter conditions, incl. short days.
In plum, transformation with the *Populus trichocarpa FT1* (*Flowering Locus T*) gene under a constitutive promoter (35S) also could lead to flowering already starting in tissue culture. Also here, there was a change in tree architecture, i.e. less upright with reduced apical dominance, and a change in flower phenotype but the plant was still producing viable seed, and flowered continuously for 3 years. F1 seedlings also flowered in the first year.
Silencing *TFL1* (*TFL1 = TERMINAL FLOWER 1* (by standard method using a hairpin construct of inverted repeats from the gene sequence inducing RNAi under a 35S promoter) in apple led to early flowering but often flower abortion, though there was some viable seed production, and to stopping of vegetative growth.

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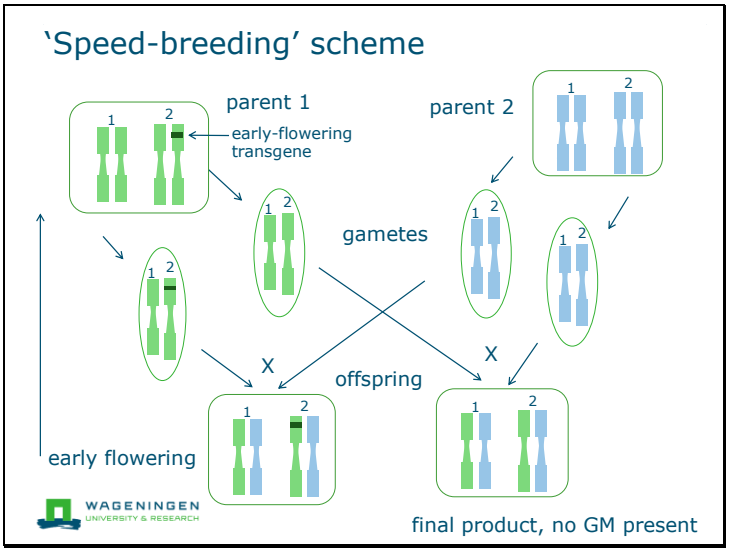

Basic scheme of induced early flowering: one parent is a hemizygous line transgenic for early flowering, which is dominantly inherited. The transgenic line itself can be propagated vegetatively. It is crossed with a conventional parent to quickly stack desired traits, such as resistances. As the early flowering line produces both transgenic (containing a green chromosome with a black band depicting the transgene) and non-transgenic gametes, progeny can be selected for early flowering to continue further fast crossing, but also for being free of the transgene, which is desirable for the final product. The final product being transgene-free is not only desirable to avoid drawbacks associated with GM, but also basic to product quality: the early flowering type will usually not be optimal for normal cultivation. For the final product, the early flowering line is thus only a helper in speeding up the crossing, the final plant's characteristics need to come mainly from elite cultivar material and useful genes from exotic materials, such as resistances (see detailed schemes below).

Induced early flowering: Apple "speed breeding"

- Stacking resistance genes:
  - 1st generation cross with *Malus fusca* (bacterial fire-blight resistance)
  - 2nd generation cross with cv. 'Regia' (resistances: 2 scab & 1 additional bacterial fire blight)
  - 3rd generation cross with Golden Delicious
- Screening for resistance genes using DNA markers
- Final selection: 'null-segregants' (plants without the transgene)

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Flachowsky et al., 2011 New Phytol 192:364

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Induced early flowering: other examples

- "FasTrack" in plum: *FT* gene from poplar
  - Improved sugar content combined with plum pox virus resistance in dried plum US
- Other woody perennials: pear, citrus, poplar, eucalyptus
- Also herbaceous perennials: *Gentiana triflora*, lisianthus

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FasTrack plum breeding: Yao (2011) Agr Res March, p. 16.

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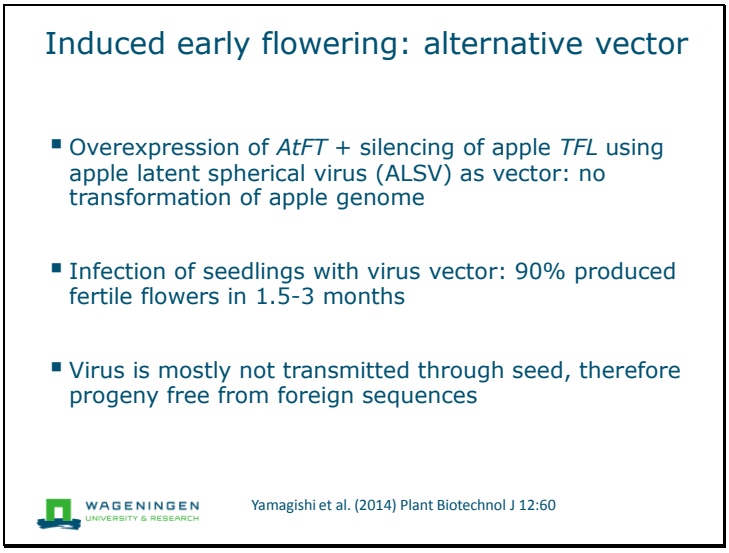

This could potentially be a highly versatile system: during the crossing programme, seedlings (progenies) can be flexibly chosen for induction of early flowering to be used in further crossing, provided that the seedlings can be genotyped. In the present system, flower induction by the vector was only tested by inoculation of cotyledons. It further depends on the reliability and efficiency of the method, in this study 90% early flowering was achieved by combining two gene constructs, but in 40% of seedlings flowering induction was lost within 6 months (Yamagishi et al. 2014).

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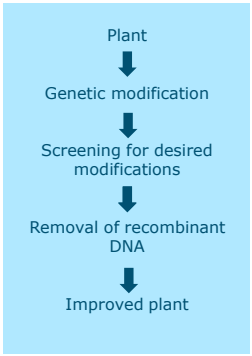
NPBTs can produce three types of improved plant

1. Plants that contain a new DNA fragment (copy of existing gene)
2. Plants that do not contain a new DNA fragment, but have a mutation or modification in their own DNA
3. Improved plants that do not contain a new DNA fragment or modification of their DNA



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Schaart et al (2016) Trends in Plant Science 21:438

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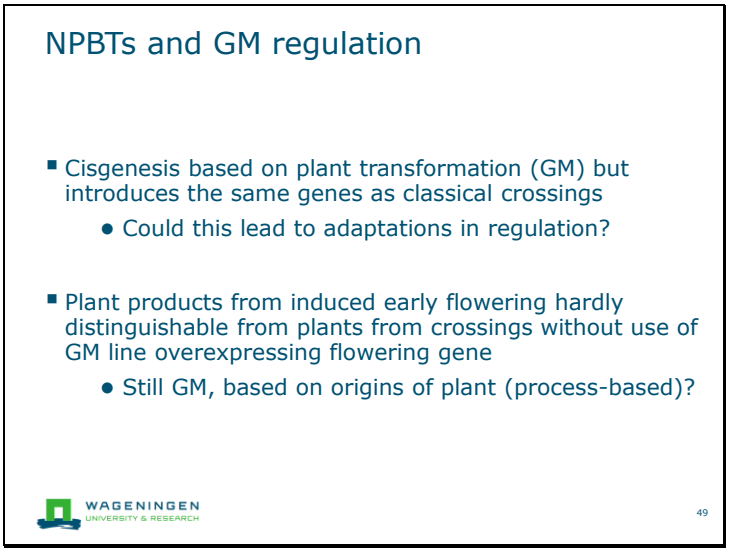

EFSA GMO Panel (2012): “The Panel concludes that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants. The Panel is of the opinion that all of these breeding methods can produce variable frequencies and severities of unintended effects. The frequency of unintended changes may differ between breeding techniques and their occurrence cannot be predicted and needs to be assessed case by case.” “It can be envisaged that on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment.”

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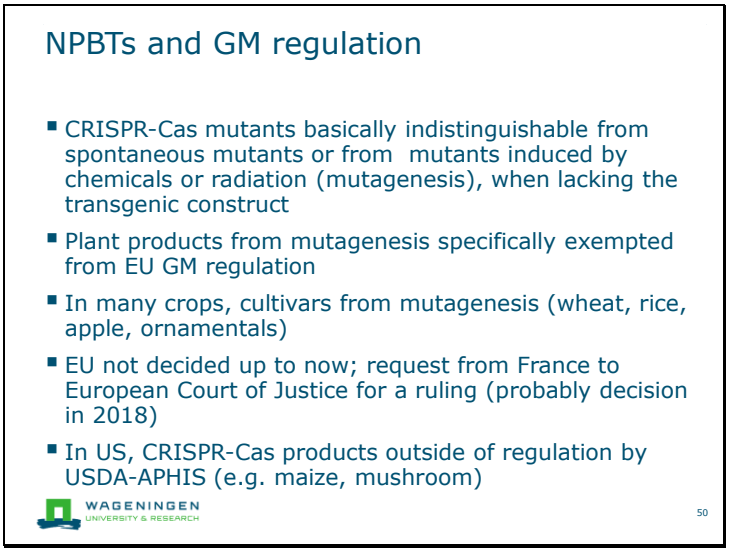

With prior knowledge, an individual plant from CRISPR-Cas editing can be identified based on a known mutant sequence; otherwise at most indirect clues, such as type of mutation (some more frequent with CRISPR-Cas9) and presence of PAM site, but no hard indications.

Conclusion

- New Breeding Techniques can significantly add to traditional breeding programmes:
  - More quickly introducing traits into existing varieties
  - Adding genetic variation
- Important issues:
  - Regulation in Europe
  - Consumer acceptance