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Approaches for Development of Cisgenic Apples

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ABSTRACT

Introgression of genetic traits from wild apple germplasm (*Malus* spp.) into commercial apple cultivars is a painstakingly slow process. For e.g. introgression of the *Vf* gene from *Malus floribunda* 821 for resistance to apple scab, caused by the fungus *Venturia inaequalis*, took more than 80 years due to genetic drag and the long juvenile period of apple. In order to speedup the classical breeding, molecular techniques can be applied to enrich existing commercial apple varieties with functional alleles from sexually compatible plants, preventing genetic drag and keeping the genetic makeup of the commercial cultivar. This concept is named "cisgenesis". This paper describes several approaches and considerations for development of cisgenic apples and stacking of genes. Also we provide an overview of isolated alleles from apple available for cisgenesis at the moment and in the near future.

Keywords: *Agrobacterium tumefaciens*, genetic modification *Malus x floribunda*, marker-free, *Venturia inaequalis*
Abbreviations: ***ALS***, *Acetolactate synthase;* **CaMV**, cauliflower mosaic virus; ***GBSSI***, *granule bound starch synthase*; **GFP**, green fluorescent protein; ***GUS***, *β-glucuronidase*; ***hpt***, *hygromycin phospho transferase*; ***HcrVf***, Homologues of *Cladosporium fulvum* resistance genes of the *Vf* region; ***nos***, *nopaline synthase gene*; ***npt*II**, *neomycin phospho transferase* II; **PGIP**, *polygalacturonase inhibiting protein*; ***PPO***, *polyphenol oxidase*; ***R***, *Recombinase*; **Rs**, Recombination site; **RNAi**, RNA interference; **RT-qPCR**, real time quantitative polymerase chain reaction; **T-DNA**, transfer DNA; **ZFN**, zinc finger nuclease; **GMO**, genetically modified organism

CONTENTS

CLASSICAL BREEDING

Genetic improvement of apples can be achieved in three ways, i.e. by making sexual crosses of selected apple genotypes, by mutation breeding, and by addition of genetic material through genetic modification.

Classical breeding of apple is achieved mostly by artificial pollination of selected genotypes with pollen from selected donors. The two gametes are then fused into a new genotype without involvement of other genetic sources. Many of the crosses are performed between genotypes producing high quality fruits with genotypes producing lower quality fruits but having interesting traits as disease resistance. However, there are some problems concerned with the classical breeding of apple. Firstly, development of a variety takes a lot of time as the juvenile period is very long, i.e. four to ten years (Ibanez and Dandekar 2007). Secondly, during crossing along with the trait(s) of interest other undesirable traits may be introgressed. In order to improve the quality of the genetic material it is necessary to perform sequential crosses with elite material for several generations. In 1914 crosses were made for introduction of resistance to

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apple scab into commercial apple varieties, using the crab apple *Malus floribunda* 821 as a source of resistance (Crandall 1926). The progeny of the cross between *M. floribunda* 821 and susceptible cultivars segregated for resistance in a Mendelian 1:1 ratio. The gene putatively underlying this resistance was named *Vf*-gene (*Venturia inaequalis*: *Malus floribunda*). However, the fruits of the resistant parent *M. floribunda* 821 were very small, approximately 1 cm. The apples of the progeny were also small, and did not have the fruit quality that was required for commercial cultivars (Crosby *et al*. 1990). This was caused by genetic drag: not only the wanted resistance gene was inherited by part of the progeny, but also many unwanted alleles leading to poor fruit quality and other undesirable traits. In order to get rid of the unwanted alleles, subsequent crosses had to be carried out between resistant progeny and susceptible high quality cultivars. About five generations were required to remove most unwanted alleles from *M. floribunda*, yet keeping the desired *Vf*-gene for scab resistance. Approximately 85 years after the first cross, *Vf*-cultivars with a good fruit quality were introduced onto the market, e.g. the varieties 'Santana', 'Topaz', 'Florina' (Backer *et al.* 1999).

The first bottleneck, i.e. the long juvenile period, can be solved to some extent by the induction of early flowering. Volz *et al*. (2009) put young seedlings in a climate chamber with high light intensity, high CO_2 concentration and optimal temperature for shoot growth, leading to many internodes. This led to flowering of one-year-old trees. Grasmanis and Edwards (1974) reported flower initiation in apple trees on MM104 rootstock in the first year of growth by short exposure to ammonium ions. After selection of the desired genotype, the trees can be vegetatively propagated under normal conditions in the orchard.

CROP IMPROVEMENT USING GENETIC MODIFICATION

The second mentioned bottleneck for fast breeding is genetic drag. This bottleneck can be circumvented by introduction of the wanted genes or alleles only, by genetic modification, without co-insertion of unwanted genes (Jacobsen and Schouten 2007). An additional advantage is that the genetic makeup of proven cultivars is maintained, as apple is self-incompatible and heterozygous, the genetic makeup of proven cultivars can never be fully restored in the progeny by traditional breeding. However, in case of cisgenesis only one or a few wanted genes are added to an existing cultivar.

Three types of genetic modification can be distinguished depending on origin and organization of the genetic material used to amend the target apple variety, e.g. transgenesis, intragenics and cisgenesis.

Transgenesis

Transgenesis involves transfer of gene(s) or part of the gene(s) from a heterologous organism, e.g. from microorganisms such as bacteria or viruses or from non-related plant species. James *et al.* (1989) were pioneers in genetic modification of apple. They developed transgenic apple using a disarmed Ti binary vector and studied the rooting of *in vitro* apple shoots by introducing *nos* and *npt*II genes. A few examples of transgenesis in apple are the introduction of a *puroindoline* gene from wheat endosperm under the regulation of the cauliflower mosaic virus (CaMV) 35S promoter (Faize *et al.* 2004), the introduction of a *chitinase* gene from *Trichoderma harzianum* under CaMV35S promoter (Bolar *et al.* 2000; Faize *et al.* 2003), both aimed at achieving apple scab resistance. Szankowski *et al.* (2003) introduced the *stilbene synthase* gene from grape vine under control of its own promoter or the *polygalacturonase inhibiting protein* (*PGIP*) gene from pear under the CaMV35S promoter into commercial apple varieties in order to obtain resistance to fungal pathogens. Norelli *et al.* (1994) has focused on resistance to fire blight. They introduced the *attacin E* gene from *Hyalophora cecropia* pupae into 'Malling 26' apple. This induced a significantly higher level of resistance than non-transformed 'Malling 26' in the greenhouse. Markwick *et al.* (2003) aimed at resistance to the light brown apple moth (*Epiphyas postvittana*), and introduced genes encoding the biotin binding proteins avidin and streptavidin. They observed a mortality of 80-90% in larvae in the transformants as compared to 14% in control plants.

Several publications report on the use of apple genes in sense or antisense orientation for improvement of apple. This type of genetic modification is often also transgenesis in case for the regulation of transcription heterologous promoter and terminator sequences are used. For example an antisense sequence of the resident apple *polyphenol oxidase* (PPO) gene which is responsible for enzymatic browning of apples, was used successfully to transform apple to reduce the enzymatic browning apples (Murata *et al.* 2001). Self fertile apples were developed by silencing the *S-RNase* gene which led to the inhibition of expression of the *S-RNase* gene in the pistil resulting in un-arrested self-pollen tube growth, and fertilization (Broothaerts *et al.* 2004). Flachowsky *et al*. (2009) described induction of early flowering in apple through transgenesis by inserting the *BpMADS4* gene from silver birch (*Betula pendula* Roth.).

The promoter of the *CaMV35S* gene, which drives transgene expression in plants to a high level and in a constitutive way, is very widely used in transgenesis. Also terminators of the *CaMV35S* gene and of *Agrobacterium tumefaciens* T-DNA genes, such as the *nopaline synthase* gene (*nos*) are being widely applied in transgenesis (Belfanti *et al.* 2004). First apple transformants using the apple gene *HcrVf2* with CaMV35S promoter for resistance against apple scab (*Venturia inaequalis*) were developed by Belfanti *et al*. (2004). Malnoy *et al*. (2008) developed apple transformants with *HcrVf1*, *HcrVf2*, and *HcrVf4* separately under control of their native promoters of at least 2 kb lengths, and used the *npt*II *gene* for selection. Because of this foreign selection gene, the plants were transgenic. Malnoy *et al.* (2008) described that the *HcrVf1* and *HcrVf2* transformants exhibited partial resistance to apple scab while *HcrVf4* transformants were susceptible. Silfverberg-Dilworth *et al*. (2005) were the first who studied different lengths of promoters of these *HcrVf* genes through *gus* activity. They reported that 312 or 632 bp for the *HcrVf1* promoter, 288 bp for the *HcrVf2* promoter and 332 bp for *HcrVf4* promoter provide good expression. Apple transformants with different promoter lengths of *HcrVf2* have been developed and studied by Szankowski *et al.* (2009). They reported that a promoter fragment of 288 bp for the *HcrVf2* gene is sufficient to confer a high level of scab resistance. Even in case the gene to be introduced is originating from the species itself, this may still be transgenesis if selectable marker genes have been introduced together with the gene of interest. Most often the selectable marker genes are of bacterial origin and may code for antibiotic resistances, herbicide resistances, or are giving nutritional advantage to the transformed cells. Gessler and Patocchi (2007) have reviewed transgenesis in apple crop for insect resistance, disease resistance, herbicide resistance and quality aspects like fruit ripening and self incompatibility.

Although transgenesis may speed up the breeding process considerably, it has given rise to three other obstacles: The first obstacle is Intellectual Property (IP). Genes and methods can be patented, and applying genes and methods owned by others can lead to serious financial dependence on the owners of the IP (Graff and Zilberman 2001). The second obstacle is the biosafety regulation. Genetically modified organisms (GMOs) fall under biosafety regulations. This requires extensive studies for safety of the environment, and depending on its usage, also for food and feed. According to a study of Schenkelaars (2008), the costs for market approval of a genetically modified crop have been on average 5.5 M€ in the USA and 6.8 M€ in the EU. Kalaitzandonakes *et al*. (2007) estimated similar costs. The third obstacle is the acceptance of GM food by consumers.

This is especially important in case of consumption of fresh products that are eaten directly without cooking or processing, such as apples. According to inquiries in The Netherlands, only 4% of the consumers were willing to buy and eat transgenic apples, containing genes from micro-organisms or animals (Jan Gutteling, pers. comm.). In case genes were introduced from a plant not belonging to the species of apple (*Malus* x *domestica*), the willingness to buy and eat increased to 20% while 37% was not willing to buy apples with genes from other plant genera. The other consumers were neutral. The willingness to buy and eat increased further to 35% when only apple genes were used for the genetic modification of apples. When the consumers received information from an independent organization about the genetic modifications, then the willingness to buy apples with own genes only, increased to 47%. 17% were still not willing to buy these apples, and the remaining 36% was neutral (Jan Gutteling, pers. comm.). This shows that the source of the genes has a strong impact on willingness to buy. Lusk and Rozan (2006) showed this also in an inquiry in the USA and France. Therefore the attention for genetic modification using 'own genes only' has increased, as is elaborated in the following paragraphs.

Intragenics / Intragenesis

The term intragenic was first used by Nielsen (2003). As the name suggests, intragenics means transfer of functional sequences "within the genera" by genetic transformation. Rommens *et al.* (2007) defined an intragenic plant as a genetically modified plant that only contains genetic elements derived from within the sexual compatibility group of that plant. Intragenics allows the generation of new combinations of donor DNA sequences. When e.g. the coding sequence of a certain gene from *Malus* is combined with a promoter of a different apple gene and subsequently used in genetic modification of apple, this is termed intragenics. By making new combinations of regulatory sequences and coding sequences one can alter the expression of the gene of interest to a desired level and pattern. The techniques of gene silencing involving the use of RNA interference (RNAi) and the use of anti-sense strands of resident gene sequences can be considered as examples of intragenics where the orientation of the sequences is not a critical factor (Russel and Sparrow 2008).

As new combinations of regulatory sequences and coding sequences can be made in intragenic plants, this may lead to gene expression levels and expression patterns that are not present in nature or non-GM breeding germplasm of *Malus.* From this point of view, intragenic plants may show phenotypic traits that are not feasible through classical plant breeding methods. Generally, plants from classical breeding are regarded as the baseline for GMO Regulations. As intragenic plants may deviate from this baseline, it could be argued that novel risks may emerge compared to the baseline, requiring additional biosafety tests for market approval (Schouten and Jacobsen 2008).

Cisgenesis

In order to take into account both consumers' attitudes and staying as close as possible to classical breeding, possibly leading to exemption from the GMO regulation, a strict case of intragenics was developed, i.e. cisgenesis. Schouten started to develop the cisgenic approach in 1999 as a result of discussions between ethicists, social scientists and plant scientists (Iversen 2000; Jochemsen 2000). The term 'cisgenesis' was invented by Jochemsen and Schouten in 2000 (Jochemsen 2000), when Schouten and Schaart developed strawberry plants with resistance to greymould, caused by *Botrytis cinerea*, using a native *PGIP* gene from strawberry (Schaart *et al.* 2004) but combined with a promoter of strawberry *Expansin* gene. The onset of cisgenesis was several years before the development of intragenics by Nielsen (2003) and Rommens (2004).

After the development of strawberries with its native *PGIP* gene, Schouten and Jacobsen further developed the concept of cisgenesis and made the definition stricter compared to intragenics. Cisgenesis is defined as the genetic modification of a recipient plant with natural gene(s) from a sexually compatible plant. The gene includes its native promoter and terminator in the normal sense orientation as in the donor plant (Schouten *et al.* 2006a, 2006b). In cisgenesis, no foreign genes are allowed in the final product.

There are two main differences between cisgenesis and intragenics. Firstly regarding the regulatory elements: Cisgenesis uses the native regulatory elements belonging to the target gene like promoters, introns and terminators. The inserted gene is an exact DNA copy of the complete natural gene. However, in case of intragenics new combinations of coding sequences and promoters are used. Intragenics has no requirements regarding introns or terminators, the only requirement is that the genetic elements are taken from within the sexual compatibility group. Secondly, unlike intragenics, cisgenesis does not apply the RNAi or antisense approach based on novel combinations of genetic elements (Schouten and Jacobsen 2008).

In the case of cisgenesis, where no novel combinations

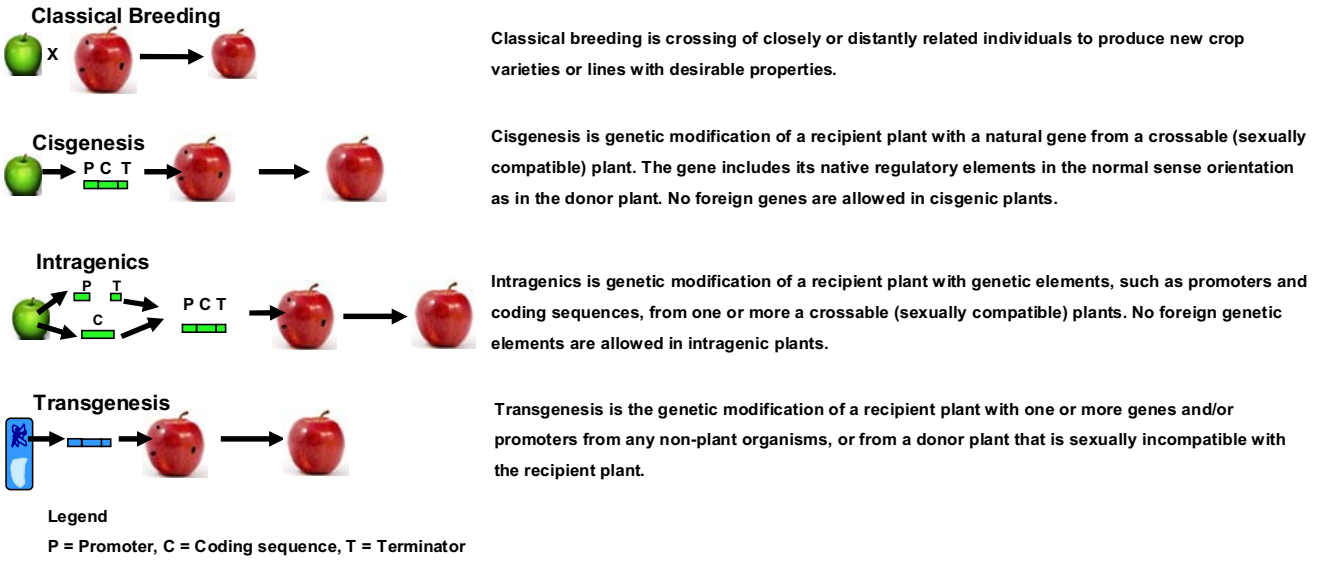

Fig. 1 Different breeding strategies for apple.

between coding sequences and regulatory elements such as promoters are made, but only complete genes are used from sexually compatible plants, the gene expression levels and patterns should be similar to expression of plants from classical breeding. If that is the case, it can be argued that the phenotypic traits of such plants can also be obtained through classical breeding. If no novel traits are introduced compared to classical breeding, it can be argued that such plants are as safe as plants from classical breeding, and should be exempted from the GMO regulation (Schouten *et al.* 2006a, 2006b; Jacobsen and Schouten 2007; Schouten *et al.* 2009). A cartoon for the different breeding strategies is given in **Fig. 1**.

METHODS FOR THE DEVELOPMENT OF CISGENIC APPLES

Transformation with *Agrobacterium*

Cisgenic apples are prepared through the process of genetic modification. In apple, like in many other crops, *Agrobacterium*-mediated transformation is the preferred method for gene transfer. After the introduction of genes into the plant cell genome, these particular cells with their newly acquired traits should be preferably multiplied and regenerated into entire plants. In order to ensure that all the regenerants contain the desired genes-of-interest, selectable marker genes are usually added as well. Within the concept of cisgenesis it is imperative that such genes, when derived from other, sexually non-compatible organisms, are not present in the final product.

Usage of marker genes

Use of selectable marker genes is a very important element in plant transformation (Komari *et al.* 2007). Some of the most common markers are genes conferring resistance to antibiotics such as kanamycin or hygromycin, or tolerance to herbicides, e.g. phosphinothricin or glyphosate. Kanamycin resistance has been most frequently used in transformation of many dicotyledonous plants, including apple. This selection gene has a bacterial origin and cannot be left behind in cisgenic apple plants.

Methods to generate marker-free transformed plants

Krens *et al*. (2004) discussed different methods to develop marker-free transformed plants. These methods include a) avoiding the use of any selectable marker; b) co- transformation; c) active marker-removal by recombination. Approaches a) and c) have been used in apple and will be dealt with in the following paragraphs. Co-transformation of plants with both a T-DNA vector carrying the selectable marker gene and a T-DNA vector containing the gene of interest might result in unlinked integration events, which can be segregated in the progeny. However, this requires sexual crossing which is not preferable in apple as it will destroy the genetic make-up of the cultivar. Avoiding the segregation step could be tried by co-transferring two T-DNAs, one carrying a marker gene and the other containing the gene-of-interest, followed by a selection step. The aim is that only the T-DNA with the gene-of-interest will integrate, while the other T-DNA with the selection gene will only be transiently expressed and active without integration into nuclear or plastid DNA (Rommens *et al.* 2004). This method appeared to be unsuccessful in apple (Giovanni Broggini, pers. comm.).

Transformation without a selection gene

Transformation without a selection gene will lead to the regeneration of many shoots originating from both untransformed and transformed cells. Vetten *et al.* (2003) demonstrated that marker free plant transformation is possible in the vegetatively propagated species potato, using PCR afterwards for selecion of the transformed plantlets. They transformed with an antisense construct for silencing the granule bound starch synthase (GBSSI) gene, using an additional upstream inverted copy of its 5′ region to obtain amylose free potatoes. Malnoy *et al.* (2007) employed this technique of transformation without selection in apple. Due to the fact that usually only a few cells within a tissue are successfully transformed most of the regenerated shoots will be non-transformed plantlets. In order to identify the genetically modified clones, all plantlets must be checked for the presence of the gene(s) of interest by PCR.

Because of the lack of selection pressure, there is a considerable risk of obtaining chimeric plants that only partly consist of genetically modified tissue.

Transformation with a foreign selection gene and elimination of the selection gene

This method makes use of site-specific recombination systems for removal of the selection gene after the transformation step (Dale and Ow 1991). The method comprises integration of T-DNA which carries a selectable marker gene. This marker gene is flanked by two recognition sequences specific for a recombinase whose activity can be controlled. Following successful T-DNA integration and selection of regenerants, the recombinase is activated and it excises the selectable marker gene.

There are several systems available consisting of a gene coding for a site-specific recombinase enzyme and specific recombination sites flanking the undesired sequences *viz*. the *cre-lox* system (Dale and Ow 1991), the R/Rs system (Schaart *et al.* 2004) and the FLP/FRT system (Lyznik *et al*. 1993). In the R/Rs system, the site-specific *Recombinase* (*R*) and the recombination sites (Rs) originate from the yeast *Zygosaccharomyces rouxii.* These Rs are 59-82 bp in length and consist each of three direct imperfect 12 bp repeats and one inverted repeat. The two Rs should be in the same orientation. After recombination a single Rs is left. The activity of *R* is inhibited at first by the combination with a ligand-binding domain. The Recombinase activity can be induced by the addition of dexamethasone (Schaart *et al*. 2004). The first report on active removal of a selectable marker gene was published in 1991 by Dale and Ow. They used the *cre/*lox site-specific recombination system and demonstrated in tobacco that the *hpt* selection gene flanked by two lox sites introduced in a primary transformation could be removed by a second transformation step with a binary vector carrying the *npt*II gene together with the recombinase *cre* gene. More details on the different systems including induction steps are given by Schaart *et al.* (2009). We use this technique for development of cisgenic apples.

A general consequence of the application of the mentioned recombination systems is that always a single recombination site, which is of foreign origin, is left behind in the recipient genome. Such recombination sites are usually 34 and 82 bps long in lox and Rs respectively and are expected not to be functional without a second recombination site and its corresponding specific recombinase.

CHIMERAS

Occurrence of chimeras

A chimera consists of sectors or tissues that differ in genetic constitution. Chimeras can arise from regeneration from multiple cells (transformed and untransformed ones) or after transformation of one cell in an already existing multicellular shoot meristem or embryogenic region. Gahan and George (2008) reported that in *P. sativum* transformed with the *gus* gene, the chimeric shoots are likely to be formed from at least one transformed and one untransformed cell. This will result in a plant that contains layers or sectors of transformed tissue. Chimerism can be a problem especially

in vegetatively propagated plants, where no fixation through a sexual phase occurs (Ahuja 1992). Identification of chimeras after genetic modification and subsequent regeneration is normally very difficult and generally not done. Chimerism in the protocol for the development of cisgenic apples can occur in a form where a plant is built up of non-transformed and transformed cells. Chimerism may also occur after the recombination, the chimeras will consist of both marker-free cells as well as of marker-containing, non-recombined cells and will be identified as non-recombined upon molecular characterization. Those individuals will not be used further and be discarded. Still, careful screening of putative marker-free plants is required.

A final possibility of chimerism is loss of function mutation of the transgene, intragene or cisgene itself. This type of chimerism will also be found in marker aided selection of GM plants. Russel *et al*. (1992) transformed *Arabidopsis* with the gene *acetolactate synthase* (*ALS*) encoding sulfonylurea (SU) herbicide-resistance. They placed the gene between loxp sites. After the recombination event they observed chimerism in the F_1 progeny of lox/ALS/lox/gus and cre/hpt plants. Some plants were completely sensitive to sulfonylurea in the stem callus assay, as the *ALS* gene was excised, but showed varying percentages of resistant or chimeric (intermediate resistant) progeny, indicating chimerism. In the cisgenic approach, all cells of a cisgenic plant need to be without the marker genes.

Chimerism can be minimized by optimization of the primary regeneration process or by subjecting the confirmed transformant to re-regeneration, grafting or budding and again testing for respectively the presence or absence of the gene-of-interest and the selectable marker gene. This can be a good method to identify chimeras in perennial crops like apple which in natural situation takes years together. In our experiments we have used the marker free system pMF1 (Schaart *et al.* 2004) and we have not experienced any kind of chimerism or somaclonal variation. If the binary vector system is not efficient, it may give rise to chimerism.

Identification of chimeras

Chimeras can be identified by the use of reporter genes, e.g. encoding *β-glucuronidase* (*gus*) (Jefferson *et al.* 1987), green fluorescent protein (GFP) (Chalfie *et al.* 1994), *luciferase* (Koncz *et al.* 2005), red fluorescent protein DS-RED E-5 (Mirabella *et al.* 2004) or *MdMYB10* (Espley *et al.* 2007). These reporter genes can then be placed between recombination sites. Transformants can be evaluated for chimerism by positive selection for uniform production of blue color (*gus*), green or red light emission (GFP), and red color (*MdMYB10*) respectively after transformation and uniform absence of these colors after recombination in cisgenic plants (Schaart *et al.* 2009). Faize *et al.* (2009) demonstrated that quantitative real time PCR (RT-qPCR) can also be a reliable molecular method to identify and quantify the chimeras in transformed apricots, using genomic DNA. This was based on quantitative real time PCR amplification of *npt*II as well as internal control (*actin*), used to normalize the quantity of *npt*II.

STACKING GENES/ALLELES

One of the advantages of marker-free and therefore also of cisgenic apple plants is the possibility to add more cisgenes to plants that already have obtained cisgenes earlier. In these secondary genetic modifications the absence of selectable marker genes allows the repeated use of the selection system that was optimized for apple.

Gene stacking can be done in three ways, 1) inserting all the genes of interest at once, so stacking the genes in a binary vector prior to the transformation event, 2) inserting the genes of interest consecutively, one after the other by retransformation or 3) by crossing two different transgenic lines and checking the progeny for the desired combination of genes. The latter option using sexual crossing is not well-suited for apple as it destroys the genetic make-up of elite genotype.

Retransformation was first described by Dale and Ow (1991). They transformed tobacco introducing the *luciferase* reporter gene and the selectable marker gene *hpt* for hygromycin resistance placed between two lox sites. Selection was on hygromycin. Established primary transgenic tobacco was retransformed introducing the *cre* gene together with the *npt*II gene for subsequent kanamycin selection. In this way they showed that selectable marker genes can be effectively eliminated by a system based on recombination and stacking of genes, i.e. *luc* and *cre* and *npt*II, is possible. However they removed the *cre-npt*II locus through self pollination and segregation and retained only *luc* gene. Stacking of the genes in plants without sexual crossing was also reported by Sugita *et al*. (2000) when they transformed *uidA* and *npt*II genes in primary transformation with *ipt* as selectable marker and then they excised the *ipt* gene which was between two recombination sites. They did secondary transformation of GFP by using same *ipt* gene as selectable marker. After secondary transformation and regeneration they were able to trace back *npt*II, *uidA* genes and GFP in the transformants. Retransformation with the same selection gene i.e. adding new genes to a transgenic line already equipped with other transgenes after removal of the selection gene, has not been attempted yet. Also transformation with binary vector containing both the old and new genes and transforming a crop with this new stacked construct, has not been done yet. Both require the full process of transformation and marker elimination and will take comparable amounts of time. Still, when introducing multiple genes at once, one will have to look for those cisgenic plants in which all genes will perform as necessary for the desired phenotype.

AVAILABLE GENES FOR THE GENERATION OF CISGENIC APPLES

Cisgenesis allows stacking of new desired genes coding for interesting traits, whenever they will become available, in elite cultivars. In this way, multiple traits can be brought together relatively fast by genetic modification. For e.g. at present, for apple scab resistance, the race specific resistance genes *HcrVf1* and *HcrVf2* have been isolated from apple (Vinatzer *et al.* 2001) and applied for cisgenesis. Once introduced in elite, originally susceptible apple cultivars, stacking can be done later with other apple scab resistance genes, providing resistance to other isolates of the pathogen *Venturia inaequalis*. An example of such a resistance gene is the *V25* gene (Soriano *et al.* 2009) that provides a broad spectrum for resistance against apple scab disease. By means of map based cloning, one Bacterial Artificial Chromosome (BAC) clone carrying three candidate genes for *V25* has been identified. These candidate genes will be functionally analyzed. Other examples of resistance genes to apple scab are the nearly isolated and functionally analyzed genes *Vr2* (Patocchi *et al.* 2004) and *Vm* (Patocchi *et al.* 2005). By stacking these resistance genes, a broad spectrum of resistance to apple scab can be realized leading to an increase in durability of resistance.

Other alleles may be added such as the apple transcription factor *MdMYB10* (Espley *et al.* 2007). This gene has a repeat in the promoter region onto which the *MdMYB10* protein binds, thus upregulating its own expression (Espley *et al.* 2009). This transcription factor regulates the anthocyanin biosynthesis pathway leading to production of anthocyanins and red coloration throughout the plant. This red coloration can be seen not only in the skin but also in the flesh of the fruit and even in the young plantlets after transformation and regeneration. This red color can also be helpful for selection of cisgenic plantlets even without antibiotic selection (**Fig. 2**). These red-fleshed apples have enhanced anti-oxidant capacity which may be health beneficial to consumers. The *MdMYB10* gene may be stacked along with scab resistance.

Fig. 2 Apple transformation with solely the apple *MdMYB10* gene. Cisgenic plantlets that are grown in the dark can be selected, based on their red color. For this transformation the *MdMYB10* gene (Espley *et al*. 2007) under control of its native promoter (Espley *et al.* 2009) was inserted into leaf material of 'Gala'. No additional gene was used for e.g. selective advantage. This picture shows regeneration of callus tissue that received the *MdMYB10* gene through *A. tumefaciens* mediated gene transfer. Picture: Aranka van der Burgh (H.J. Schouten et al, in prep.).

In view of the upcoming whole genome sequence of the apple cultivar 'Golden Delicious' (Ricardo Velasco, pers. comm.), numerous genetic mapping studies (Baldi *et al.* 2004; Calenge *et al.* 2005; Gardiner *et al.* 2009) and the present EST database for apple (Newcomb *et al.* 2006; Gasic *et al.* 2009) the number of isolated alleles that are functionally characterized and readily available for cisgenesis, will increase faster and faster. In the near future, a large number of genes will be isolated and made available for cisgenesis, such as alleles for fire blight resistance, columnar type architecture of the apple tree, and for health beneficial compounds.

FUTURE TARGETS

The draw back of the transformation is the random insertion of the cisgene into the genome. This may lead to change in the expression of the gene compare to native expression. Targeted insertion or allele replacement has been already applied to higher plant with success (Terada *et al.* 2002). Shaked *et al.* (2005) showed the homologous recombination-mediated integration of DNA segment into chromosomal target sequence by expressing the yeast *RAD54* gene in *Arabidopsis*. Zinc finger nucleases (ZFNs) proteins as molecular scissors can also be employed in targeted integration or allele replacement by homologous recombination (Durai *et al.* 2005). Mostly single genes were targeted by homologous recombination. However the applicability of such methodology to apple has not been yet tested.

CONCLUDING REMARKS

The main driver for genetic modification (GM) in apple is the painstakingly slow breeding process of classical breeding, due to the long juvenile period and genetic drag. GM is much faster, as it reduces genetic drag to a minimum. Moreover the genetic makeup of elite cultivars can be maintained. The second driver is the exponentially increasing number of functionally analyzed genes. The number of well characterized genes of apple will increase soon, especially because of the unraveling of the whole genome sequence of the apple cultivar 'Golden Delicious'.

There are also obstacles for GM: Not all forms of GM are accepted by consumers, especially in case of fresh fruits, such as apple. The willingness to buy apples with foreign genes is low. However, in case only apple genes are used for the genetic modification like in cisgenesis, the willingness to buy and eat increases strongly around the globe. Moreover, GM technologies are present that allow introduction and stacking of own genes without leaving any foreign genes behind.

The second obstacle for GM of apple for commercial purposes is the costly approval procedure of GM trees and apples for proving safety for environment and food. In case of cisgenesis, no novel traits are introduced compared to classical breeding. Solely well-known apple alleles are added to well-known apple cultivars, and consequently cisgenic apples are at least as safe as classically bred apples. Therefore cisgenic apples should be approved in a timely and cost effective manner, preferably by means of exemption from the GMO regulations (Schouten *et al.* 2006a, 2006b).

Another future challenge is the maintenance or repair of the epigenetic state of alleles, such as methylation pattern. A PCR reaction and cloning step strips of all methylation of the promoter and coding region. Also other non-native epigenetic changes may occur during isolation and insertion. This may have an effect on the expression pattern. In spite of these possible effects, the level of biosafety control of cisgenic apples is higher compared to classically bred apples.

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