Cisgenesis Is a Promising Approach for Fast, Acceptable and Safe Breeding of Pip Fruit

H.J. Schouten[1], J.M. Soriano[1], S.G. Joshi[1], A.J. Kortstee[2], F.A. Krens[1], J.G. Schaart[1], K. van der Linden[2], A.C. Allan[3], R.P. Hellens[3], R.V. Espley[3] and E. Jacobsen[1]
[1]Wageningen University and Research Centre, Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[2]Inova Fruit, P.O. Box 222, 4190 CE Geldermalsen, The Netherlands
[3]The Horticulture and Food Research Institute of New Zealand (HortResearch), Mount Albert, PB 92169, Auckland 1142, New Zealand

Keywords: *Malus* x *domestica*, apple scab, genetic modification, GMO regulation, crop improvement, biotechnology

Abstract

Introgression of traits from wild germplasm into pip fruit cultivars by means of classical breeding is painstakingly slow. Introgression of e.g., the apple scab resistance gene *Vf* from *Malus floribunda* 821 into marketable high quality apple cultivars took approximately 50 years. In the mean time the *Vf* resistance is being broken down in Europe. For durable resistance, different resistance genes should be accumulated. However, this may take another series of decades. This slow tempo is caused mainly by the long juvenile period of apple and the phenomenon that not only the allele of interest is inherited by the progeny, but also hundreds of unwanted alleles. The process would be much faster if only the allele of interest was inserted, without unwanted alleles. This can be achieved by cisgenesis. We defined cisgenesis as genetic modification of plants, inserting alleles of the plant itself or from crossable relatives. The allele should contain its native introns and should be flanked by its native promoter and terminator in sense-orientation. If the plant is equipped with foreign genes from outside the gene pool of the conventional breeder, the plant is named transgenic. Inquiries indicate that cisgenic plants are more acceptable to consumers than transgenic plants. As the phenotypic result of cisgenesis can, in principle, also be obtained by means of conventional breeding or translocation breeding, cisgenic plants are as safe as plants from conventional breeding or mutation breeding. Therefore we have proposed to treat cisgenic plants like conventionally bred plants, by exempting cisgenic plants from the GMO regulation. The number of isolated, functionally analysed genes and their alleles from fruit tree crops is increasing. Also technologies are available for introduction of these alleles without leaving selection genes behind. Cisgenesis is combining the knowledge of native alleles with marker free technologies. Cisgenesis is a promising path for utilizing the wealth of knowledge on plant genes to the benefit of the society in a fast, safe, and acceptable way.

INTROGRESSION OF A MAJOR RESISTANCE GENE IN APPLE TOOK HALF A CENTURY

In 1946, crosses were made for introduction of resistance to apple scab (*Venturia inaequalis*) into commercial apple varieties, using as source of resistance the crab apple *Malus floribunda* 821 (Hough et al., 1953). The progeny of the cross between *M. floribunda* 821 and susceptible cultivars segregated in a Mendelian fashion for resistance in a 1:1 ratio. The gene putatively underlying this resistance was named *Vf*-gene. 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. This was caused by linkage drag: not only the wanted resistance gene was inherited to part of the progeny, but also many unwanted alleles, leading to poor fruit quality and other unwanted 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 enough unwanted alleles from *M. floribunda*, yet keeping the desired *Vf*-gene for scab resistance. Approximately 50 years after the first cross, *Vf*-cultivars with a reasonable fruit quality were introduced onto the market (Anonymous, 1999). Therefore, it has taken half a century to introduce the *Vf*-gene and remove the linkage drag to an acceptable degree.

MORE RESISTANCE GENES NEEDED

In the mean time, *Venturia inaequalis* strains have been detected that are able to infect *Vf*-cultivars (Parisi et al., 1993). Especially in northwestern Europe, these strains are present and have spread (Parisi et al., 2006). As a result, several orchards that consist of *Vf*-cultivars have to be sprayed like orchards with susceptible cultivars (Trapman, 2006). Fifty years of breeding is fading away in ten years. Obviously, more individual resistance genes need to be accumulated for obtaining more durable resistance.

Fortunately, many loci that confer resistance to apple scab have been discovered in *Malus*, both major genes and QTLs (Calenge et al., 2004; Gardiner et al., 2006; Gessler et al., 2006; Schmidt and Van de Weg, 2005). The increase in number of mapped resistance genes has even required a new system for nomenclature of all these genes (Bus et al., this symposium). Therefore, sufficient genes for resistance are present in the germplasm of apple. Introgression of one resistance gene took approximately 50 years. Introgression of four or more genes for durable resistance will require more time, when the breeding is performed in the classical way. Would this imply that we are left with another 50 years of intensive fungicide applications to apple scab in conventional apple production or sulphur in organic apple production, before the more durably resistant cultivars are introduced? We regard it as our challenge to shorten this period significantly, by introducing the resistance genes and preventing the linkage drag.

ROUTES FOR INTROGRESSION OF MULTIPLE RESISTANCE GENES

One way to speedup the breeding process is marker-assisted breeding. This method can be of tremendous use and we advocate this approach, but still this will be time-consuming, because linkage drag has to be removed through crosses and meiotic recombination. Accumulation of resistance genes from four sources of resistance and sufficient removal of unwanted alleles from these sources, will probably require at least another five generations of apple. As long as the juvenile period and additional evaluation time in apple is about eight years, this would require a minimum of 40 years of breeding.

An alternative route is introduction of the resistance genes into susceptible elite cultivars in an asexual way without simultaneous introgression of unwanted alleles, so prevention of linkage drag, rather then removal of linkage drag. Then, durable resistance provided by several resistance genes is added to high quality cultivars in one step, preserving the proven fruit quality and other desired traits of these cultivars. We have named this process ‘cisgenesis’ (Schouten et al., 2006a).

DEFINITION OF CISGENESIS

A cisgenic plant is a crop plant that has been genetically modified with one or more genes isolated from an inter-fertile donor plant. A cisgene contains its native introns and flanking regions such as native promoter and terminator region in a sense orientation. We distinguish cisgenic plants from transgenic plants. Transgenic plants contain genes from non-inter-fertile organisms, synthetic genes or sequences, or artificial combinations of a coding gene with regulatory sequences, such as a promoter, from another gene (Schouten et al., 2006a; Schouten and Jacobsen, 2008).

Cisgenic plants can, in principle, also be obtained by means of classical breeding, as far as the phenotype is concerned. This indicates clearly its limits regarding breeding possibilities, but also its limits regarding possible biosafety risks. Cisgenic plants are as safe as conventionally bred plants or safer (Jacobsen and Schouten, 2007; Schouten et al., 2006b).

THE TREASURE CHEST OF ISOLATED ALLELES IS BEING FILLED AT AN INCREASING RATE

A prerequisite of cisgenesis is the availability of isolated functional alleles. Currently, the amount of DNA sequence information is increasing exponentially. Large EST-databases are available to everybody. In addition, whole plant genomes have been sequenced. After the whole genome sequencing of *Arabidopsis thaliana* and rice, many other crops will follow. For example, apple is being sequenced, and other fruit crops too. This provides unprecedented opportunities for identification of genes. In addition, numerous loci have been mapped genetically in diverse germplasms, including fruit crops (Kole, 2006). The information on genetic positions on the linkage groups, together with the whole genome sequences, and knowledge of genes from model plant species, offer us great opportunities to isolate alleles for desired traits at an increasing efficiency. We expect that, in the coming ten years, a vast number of major alleles for desired traits will be isolated in many crops, including fruit. So, the treasure chest of isolated alleles for cisgenesis will be filled at an increasing rate.

The already mentioned *Vf*-gene was isolated by means of map-based cloning and subsequently functionally analyzed (Belfanti et al., 2004). A closer analysis revealed that a tandem repeat of two gene copies provides the *Vf*-resistance (Malnoy et al., 2007). Therefore, the spelling '*Vf*-gene' should be updated to the plural form '*Vf*-genes'. In the meantime, several other resistance genes to apple scab are being isolated. As soon as the apple genome sequence becomes available to the scientific community, many more genes and their alleles will also be isolated and characterized, and will enrich the wealth of available alleles for cisgenesis.

CISGENESIS IS A WAY OF UTILIZING THIS TREASURY

After discovery of the *Vf*-genes, several research groups in Europe and the USA proceeded in inserting these genes with strong, constitutive promoters in susceptible cultivars, resulting in resistance. However, at nearly the same time, the genes were inserted with their own promoters. Apparently, the concept of cisgenesis was a logical step following the isolation of the *Vf*-genes.

Additional isolated resistance genes to the same or other diseases will also be inserted into apple by means of cisgenesis within a few years, in combination with the *Vf*-genes. The combination of these functional resistance genes will provide more durable resistance in elite cultivars. We regard cisgenesis as a way to apply the increasing knowledge about alleles to plant breeding, to the benefit of growers, consumers and the environment.

An extra advantage of cisgenesis in comparison with cross breeding is that susceptible cultivars can be used that already have a proven high fruit quality. Apple is self-incompatible. Crossing with apple germplasm scrambles the genetic composition of good cultivars, and restoring such a cultivar through crossings is virtually impossible. However, cisgenesis preserves the genetic assembly of the high quality cultivar, and adds some well-defined apple alleles. Subsequently, the enriched cultivar can be propagated vegetatively by means of grafting, which is a common practice in apple propagation.

OTHER TRAITS AND OTHER CROPS

We initiated cisgenesis in apple by introducing resistance genes with the aim of reducing fungicide applications significantly in view of durable apple growing. However, consumers accept genetically modified plants more in case of a direct benefit to the consumers, such as a health benefit. In view of this, Plant Research International is developing together with HortResearch and Inova Fruit a cisgenic apple with red flesh. Many crab apples have not only a red fruit skin, but have red flesh as well. This is caused by the production of anthocyanins in both the skin and flesh of the fruit. The anthocyanins have an antioxidant effect, and may therefore be beneficial to the health of the consumers. The red flesh allele was recently discovered to be a transcription factor (Espley et al., 2007). This allele, with its native promoter and terminator, will be introduced into high

quality cultivars, presumably leading to red-fleshed apples. It will be investigated whether these apples will preserve their good taste.

Cisgenesis can be applied to all kinds of crops. It is particularly attractive to crops that are cross fertilizers, and are propagated vegetatively, such as banana, grape, strawberry, and potato.

ROLES OF THE DIFFERENT APPROACHES

In our view, conventional breeding remains of crucial importance. It provides us high quality cultivars with many genes working together in a concerted action, in ways that mankind may understand poorly. History has proven that, even if there is a poor understanding of the underlying genetic network, breeders are still able to develop superior cultivars, just on the basis of phenotyping with experienced eyes, noses, and mouths. This empirical way of improving cultivars will remain important, because of the complexity of the functioning of living plants. Conventionally bred plants can be improved further with isolated major genes from wild germplasm, such as resistance genes.

During the development of cisgenic plants, the empirical knowledge of conventional breeders remains of crucial importance for selection of cisgenic plants without unwanted side effects that may have been caused by the transformation or regeneration process.

Genetic mapping of traits in segregating populations and pedigrees will be of increasing importance. It allows marker assisted breeding, but also it will be critical in searching and isolation of desired alleles for important traits in the breeder's germplasm. These alleles can be used for marker assisted breeding, but also for cisgenesis. Efficient and fast technologies for high throughput functional analysis of many alleles will be increasingly important.

Different technologies of genetic modification have been developed to introduce genes without leaving selection genes behind, such as genes for resistance to antibiotics or herbicides (Schaart et al., 2004). A remaining challenge is directed insertion of the cisgene in the host genome, e.g., by means of homologous recombination and zinc finger nucleases, for a directed genome surgery (Kumar et al., 2006), without insertion of foreign helper genes.

All these approaches will help in developing cultivars that can be grown in an ecologically sound way, and to the benefit of consumers and society.

Literature Cited

Anonymous. 1999. Variety List of Fruit Crops (in Dutch), Dutch Fruit Growers Organization.

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., Vinatzer, B.A., Gianfranceschi, L., Gessler, C. and Sansavini, S., 2004. The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc. Natl. Acad. Sci. USA 101:886-890.

Calenge, F., Faure, A., Goerre, M., Gebhardt, C., van de Weg, W.E., Parisi, L. and Durel, C.E. 2004. Quantitative Trait Loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in an apple progeny challenged with eight isolates of *Venturia inaequalis*. Phytopathology 94:370-379.

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E. Kutty-Amma, S. and Allan, A.C. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. 49:414-427.

Gardiner, S.E., Bus, V.G.M., Rusholme, R.L., Chagné, D. and Rikkerink, E.H.A. 2006. Apple. p. 2-62. In: C. Kole (ed.), Genome Mapping and Molecular Breeding. Volume 4: Fruit and Nuts. Springer-Verlag: Berlin.

Gessler, C., Patocchi, A., Sansavini, S., Tartarini, S. and Gianfranceschi, L. 2006. *Venturia inaequalis* resistance in apple. Crit. Rev. Plant Sci. 25:473-503.

Hough, L.F., Shay, J.R. and Dayton, D.F. 1953. Apple scab resistance from *Malus*

floribunda Sieb. Proc. Amer. Soc. Hort. Sci. 62:341–347.
Jacobsen, E. and Schouten, H.J. 2007. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends Biotechnol. 25:219-223.
Kole, C. 2006. Genome Mapping and Molecular Breeding in Plants. Volume 4. Fruits and Nuts. Springer-Verlag, Berlin.
Kumar, S., Allen, G.C. and Thompson, W.F. 2006. Gene targeting in plants: fingers on the move. Trends Plant Sci. 11:159-161.
Malnoy, M., Xu, M., Borejsza-Wysocka, E.E., Korban, S.S. and Aldwinckle, H.S. 2007. The role of *Vfa1*, *Vfa2* and *Vfa4* genes in resistance to *Venturia inaequalis* in transgenic 'McIntosh' apple plants. Acta Hort. 738:323-326.
Parisi, L., Lespinasse, Y., Guillaumes, J. and Kruger, J. 1993. A new race of *Venturia inaequalis* virulent to apples with resistance due to the *Vf* gene. Phytopathology 83:533-537.
Parisi, L., Laurens, F., Didelot, F., Evans, K., Fischer, C., Fouillet, V., Gennari, F., Kemp, H., Lateur, M., Patocchi, A., Schouten, H.J. and Tsipouridis, C. 2006. Geographical distribution of *Venturia inaequalis* strains virulent to the Vf gene in Europe. Bull. OILB/SROP 29:49-52.
Schaart, J.G., Krens, F.A., Pelgrom, K.T.B., Mendes, O. and Rouwendal, G.J.A. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol. J. 2:233–240.
Schmidt, H. and van de Weg, W.E. 2005. Breeding. p. 136-155. In: J. Tromp, A.D. Webster and S.J. Wertheim (eds.), Fundamentals of Temperate Zone Tree Fruit Production, Backhuys, Leiden, the Netherlands.
Schouten, H.J., Krens, F.A. and Jacobsen, E. 2006a. Do cisgenic plants warrant less stringent oversight? Nature Biotechnol. 24:753.
Schouten, H.J., Krens, F.A. and Jacobsen, E. 2006b. Cisgenic plants are similar to traditionally bred plants. EMBO Rep. 7:750-753.
Schouten H.J. and Jacobsen, E. 2008. Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends Plant Sci. 13:260-261.
Trapman, M. 2006. Resistance management in Vf resistant organic apple orchards. Bull. OILB/SROP 29:253-257.