Development of Cisgenic Apples with Durable Resistance to Apple Scab

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Keywords: *Malus* × *domestica*, *Venturia inaequalis*, marker-free plants, cisgenesis, gene stacking

Abstract

Most of the apple (*Malus* × *domestica*) growers are facing serious disease problems with apple scab which is caused by the fungus *Venturia inaequalis.* Developing a resistant variety in apple through classical breeding is very slow and inefficient. So, we aim at improving existing apple varieties through a new concept called "cisgenesis" which saves time and effort compared to classical breeding. *Malus floribunda* proved to be a good source of natural scab resistance genes. The genes *HcrVf1* and *HcrVf2*, consisting of promoter, coding and terminator sequences in their natural configuration, were isolated from *Malus floribunda* and cloned into the binary vector pMF1. Apple cv. 'Gala' was transformed with pMF1 containing *HcrVf1* and *HcrVf2*, individually or in combination. pMF1 can be used to obtain marker-free plants by recombinase-based excision of a fragment carrying undesired gene sequences, such as antibiotic-selection marker genes, leaving behind only the gene(s)-of-interest and one recombination site. Using this vector it is therefore possible to stack several genes by retransformation using the same selection procedure. In order to obtain durable resistance, we have the intention to combine different resistance genes from *Malus* either by stacking them one by one or by introducing them all together in one T-DNA. Performance of all different types of cisgenic plants will be evaluated by monitoring scab resistance levels phenotypically and by determining gene expression profiles through quantitative RT-PCR.

INTRODUCTION

Apple is one of the important fruit crops of the world. Most of the present day apple cultivars are susceptible to apple scab which is caused by the fungus *Venturia inaequalis* (Cooke) G. Wint. To overcome this disease, fruit growers may spray up to 20 times per season. There are some wild relatives within the genus of *Malus* which harbor several resistance genes for scab. One such species is *Malus floribunda*. Introgression of resistance genes from these wild species to the commercial elite susceptible cultivars takes a lot of time as the juvenile period of apple is very long. A second drawback of conventional breeding is that along with the gene(s)-of-interest other genes which code for undesirable traits also get introgressed due to linkage drag (Schouten and Jacobsen, 2008). So, in order to remove as many as possible of these unwanted alleles, the breeder has to do repeated backcrossing and select for resistant lines with the highest quality. A third problem is that apple is self-incompatible. As a consequence backcrosses are not possible, and the original set-up of the quality cultivar cannot be attained any more after making a cross. Use of biotechnological tools like isolating the gene-of-interest and transforming it into elite, susceptible apple cultivars through *Agrobacterium*-mediated transformation (Belfanti et al., 2004) is an interesting and promising new approach in fruit tree breeding, for solving these three problems. However, due to strict GMO regulations for the transgenic crops, e.g., with respect to the presence of bacterial selectable marker genes aimed at antibiotic resistance and other non-plant genes, and in view of the acceptance of consumers, a new concept has been developed, called "Cisgenesis" (Schouten et al., 2006a, b). Cisgenesis can be defined as the genetic modification of a recipient plant with only natural gene(s) from a crossable or sexually compatible species.

The gene includes its native promoter and terminator in the normal sense orientation as we see in the natural situation. In this case selection marker gene and other foreign genes present in transformants have to be removed, e.g., by a recombination-based system (Schaart et al., 2004) to retain only the gene(s)-of-interest and one recombination site.

MATERIALS AND METHODS

Gene Isolation and Vector Construction

Malus floribunda 821 is a source of scab resistance, and of the scab resistance genes. Both *HcrVf1* (Gene bank accession number AY397723) and *HcrVf2* (Gene bank accession number AJ297740) were isolated with their native promoter and terminator as one stretch per gene from a BAC library of a scab resistant apple selection from the Plant Research International breeding program. Promoters for the two *HcrVf* genes are being tested in two lengths (*HcrVf1* short promoter (*SP HcrVf1*) - 312 bp, *HcrVf1* long promoter (*LP HcrVf1*) - 2 kb; *HcrVf2* short promoter (*SP HcrVf2*) - 288 bp, *HcrVf2* long promoter (*LP HcrVf2*) - 2 kb). The genes were amplified using primers that flanked the gene sequences with unique restriction sites. A combination of *HcrVf1* and *HcrVf2* with the long promoters was also prepared. All these fragments were cloned to pGEMT-Easy and the complete inserts were checked by sequencing. Subcloning was done in a binary vector pMF1 which is based on pRCNG (Schaart et al., 2004) and which can be used to obtain marker-free plants. pMF1 containing the different genes were subsequently transferred to *Agrobacterium tumefaciens* strain AGL0.

Plant Transformation and Regeneration

Agrobacterium tumefaciens strain AGL0 was used for further transformations as delivery system for the gene(s) of interest to the recipient plant genome. The elite, scab susceptible variety ‘Gala’ was used as acceptor for plant transformation according to Puite and Schaart (1996). Initially, explants were co-cultivated for four days after which they were transferred to selection medium. Explants in regeneration went through different stages of subculturing. First they were cultured on shoot/callus induction medium (SIM) in the dark until they produce shoot like structures; then they were transferred to SIM under the light. After sufficient shoot production they were transferred to shoot elongation medium (SEM). When regenerants look like complete shoots, they were isolated and transferred individually to shoot propagation medium (SPM) for maintenance and multiplication (Fig. 1).

Characterization of Plant Transformants

Putative transformants were tested for the presence of the cisgene by PCR using *HcrVf*-specific primers. The copy number and gene expression of the cisgenes will be studied by Southern blot and RT-qPCR. The resistance level will be determined in bioassays using well-characterized isolates of *Venturia inaequalis*. Resistance levels and expression levels of the genes will be linked to the individual constructs for each gene and to combinations of the two genes obtained directly or after gene stacking.

RESULTS AND DISCUSSION

Several independent putative transformants were obtained through *Agrobacterium* mediated transformation with *SP HcrVf1* and *LP HcrVf1* (table. 1). Experiment with *SP HcrVf2* and *LP HcrVf2* and *LP HcrVf1 + LP HcrVf2* constructs is going on. All independent transformants were maintained separately and are being multiplied. DNA was isolated from the leaflets of these individual putative transformants and analysed by PCR by using *HcrVf1* specific primers (Fig. 2). Most of the putative transformants were PCR-positive and a few escapes were identified. For both *SP HcrVf1* and *LP HcrVf1* 13 independent PCR-positive lines were regenerated from 280 and 275 explants, respectively. Transformants will subsequently be used to give a dexamethasone treatment for induction of recombinase activity in order to remove the unwanted DNA sequences.

These sequences, including the selection marker, are located between recombination sites that are located on the transferred T-DNA of pMF1.

CONCLUSIONS

In this paper we presented the start of our research efforts on using cisgenesis as a new strategy of breeding of fruit trees. We aim to study the effect of different promoter lengths on expression and resistance level and the difference in performance, if any, of two genes combined directly or after stacking. The first, still transgenic, plants have been obtained, together with some cisgenic marker-free individuals that are generated thanks to spontaneous recombination events. All plants will be used in further characterization and in further research (stacking). Cisgenesis could be a better option compared to other methods of genetic modification such as transgenesis in dealing with GMO regulations and consumer acceptance.

ACKNOWLEDGEMENTS

I would like to thank TRANSFORUM, The Netherlands for the financial assistance of this Ph.D. project.

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Tables

Table 1. Total number of putative transformants.

Construct	Number of explants used	PCR positive shoots (*HcrVf*)	Escapes	Calli on selection medium
SP HcrVf1	280	13	2	
LP HcrVf1	275	13	1	
SP HcrVf2	300			80
LP HcrVf2	300			140
LP HcrVf1 + LP HcrVf2	300			

Figures

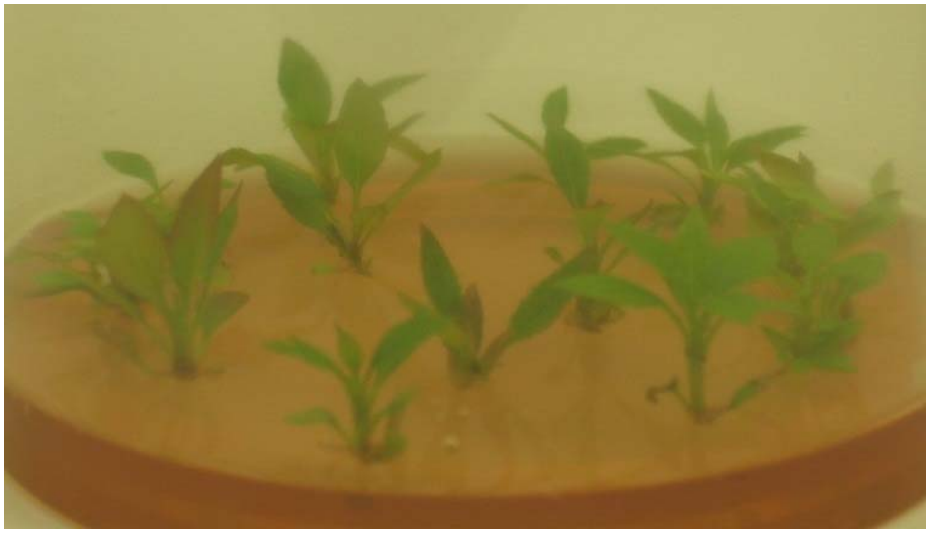

Fig. 1. Putative *HcrVf1* transformants on SPM.

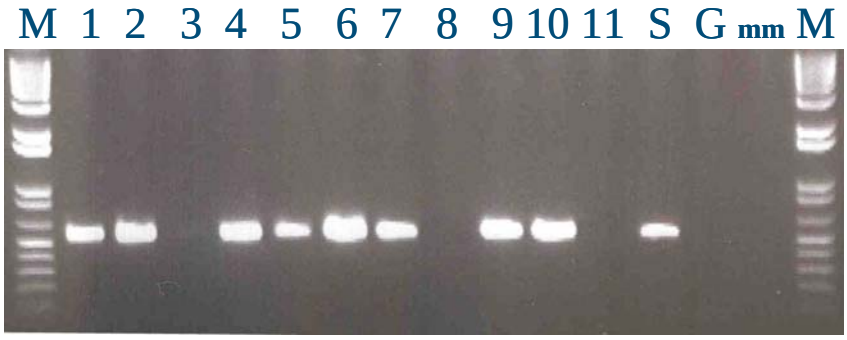

Fig. 2. PCR analyses of *HcrVf1* putative transformants. Amplification was obtained for lanes: 1,2,4,5,6,7,9. S-Santana (resistant cultivar), G- Untranformed Gala, mm-Master mix with out DNA, M-1 Kb+ DNA ladder