Journal of Biotechnology 154 (2011) 304–311

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

The development of a cisgenic apple plant

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ARTICLE INFO

Article history:
Received 14 April 2011
Received in revised form 17 May 2011
Accepted 25 May 2011
Available online 1 June 2011

Keywords:
Cisgenesis
Marker-free plants
Apple scab
HcrVf2 resistance gene
Malus × domestica
Venturia inaequalis

ABSTRACT

Cisgenesis represents a step toward a new generation of GM crops. The lack of selectable genes (e.g. antibiotic or herbicide resistance) in the final product and the fact that the inserted gene(s) derive from organisms sexually compatible with the target crop should rise less environmental concerns and increase consumer's acceptance. Here we report the generation of a cisgenic apple plant by inserting the endogenous apple scab resistance gene *HcrVf2* under the control of its own regulatory sequences into the scab susceptible apple cultivar Gala. A previously developed method based on *Agrobacterium*-mediated transformation combined with a positive and negative selection system and a chemically inducible recombination machinery allowed the generation of apple cv. Gala carrying the scab resistance gene *HcrVf2* under its native regulatory sequences and no foreign genes. Three cisgenic lines were chosen for detailed investigation and were shown to carry a single T-DNA insertion and express the target gene *HcrVf2*. This is the first report of the generation of a true cisgenic plant.

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1. Introduction

Apple production worldwide is impaired by diseases such as apple scab, caused by the fungal pathogen *Venturia inaequalis* (Laurens, 1998). Disease control is achieved by a high number of chemical treatments during growing season (MacHardy, 1996). Such large chemical input is under critical scrutiny due to its potential environmental impact and ability to induce resistance in the pathogen. Natural resistance to diseases is known and classical breeding has developed scab resistant cultivars, mostly by introgression of *Vf* resistance from *Malus floribunda* 821 (Lespinasse, 1989; MacHardy, 1996). As apple cultivars are self-incompatible and highly heterozygous, the phenotype of a cultivar is unique and breeding produces genotypes with new and distinct characteristics (Gardiner et al., 2007). Contrary to most other crops, apples are recognized as a cultivar, e.g. 'Gala', 'Golden Delicious', and not as a crop, e.g. bananas. Therefore the popularity of the new cultivars carrying disease resistance genes is limited, as the traditional market is dominated with older established cultivars that have quality characteristics for producers, storage procedures and consumers that are difficult to equal. Most of the established cultivars are considered susceptible to apple scab, as they are being intensively grown in monoculture which leads to the selection of adapted pathogen populations (MacHardy et al., 2001).

In order to maintain the particular characteristics of a cultivar, single genes coding for enzymes or other proteins, which inhibit or reduce the development of scab and fire blight, were introduced by recombinant DNA technology. A large number of foreign genes coding for lytic enzymes from various sources (e.g. encoding lysozymes from bacteria, phages, and animals, chitinases and glucanases from fungi), have been successfully integrated into the apple genome and in several cases an increased significant resistance to the targeted pathogen was observed. Pathogen derived genes or pathogen induced promoters have also been used (reviewed in Gessler and Patocchi, 2007).

In all of these approaches, the incorporated genes and control sequences are exogenous and are coupled with marker genes needed for the positive selection of the transformed cells on selective media.

A large proportion of the consumers in Europe view genetically modified foods as a risk to both health and the environment (Gaskell et al., 2000). To overcome the notorious aversion against transgenics by European consumers, Schouten et al. (2006) proposed the use recombinant DNA technology to introduce genes (including introns and flanking regions such as promoter and terminator in a sense orientation) derived from a crossable donor plant. They defined

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0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.05.013

such plants as "cisgenics". A less stringent concept is the intragenic approach, as "intragenics" (Rommens et al., 2007) are considered transformations with "all-native" DNA where overexpression, down regulation and silencing by combining genetic elements of different origin, always from a crossable donor, are accepted. In any case, the presence of selectable marker genes, e.g. *nptII* or promoters such as the CaMV S35 promoter does not correspond to either definition. Currently no cisgenic plant corresponding to the definition is reported in the literature. The transformants described by Benjamin et al. (2009) and declared as cisgenic melon resistant to down mildew, contain the S35 promoter and the selection gene *nptII*, therefore they cannot be seen as cisgenic plants. In several cases the target genes and regulatory elements originate from a crossable donor (cisgenes) however the plants are declared correctly as "transgenics" as selectable marker genes and/or their regulatory sequences are from a not-crossable donor (Han et al., 2010; Joshi, 2010; Szankowski et al., 2009), however they are the relevant step toward creating cisgenic plants.

Several endogenous resistance genes are known in apple species/plants, but only one resistance encoding gene, *HcrVf2*, has been isolated and proven functional to date in cvs. Gala and Elstar (Belfanti et al., 2004; Szankowski et al., 2009; Joshi, 2010). The gene was functional under the CAMV 35S promoter and various lengths of native 5′UTR sequences, giving an identical type of resistance to that observed in classically bred *Vf*-cultivars. All, however, produced transgenic plants as the marker gene (*nptII*) and other regulatory sequences (Joshi, 2010) were also inserted to recognize successfully transformed cells. In order to develop marker-free plants, the chemically inducible recombinase system reported by Schaart et al. (2004) in strawberry was applied to apple. In this paper we represent the development of apple lines of the cv. Gala, transformed by *Agrobacterium tumefaciens* carrying the vector pMF1. The method is based on an *Agrobacterium*-mediated transformation followed by regeneration on a kanamycin selective medium. The recombinase, and therefore the excision of the cassette carrying the transgenes, is then chemically activated by addition of dexamethasone to the medium and recombinants selected on a negative selection medium containing 5-fluorocytosine. The T-DNA of this vector carried the cisgene *HcrVf2* along with its native up- and downstream regulatory sequences and a cassette flanked by recombination sites containing the R recombinase gene and a fusion of marker genes *nptII* and *codA*, allowing positive and negative selection on kanamycin- and 5-fluoro-cytosine selective medium respectively (Fig. 1). This is the first scientific report of the generation of a cisgenic crop.

2. Methods and materials

2.1. Gene isolation and vector construction

The entire ORF (2943 bp) with 5′ UTR (242 bp) and 3′ UTR (220 bp) of *HcrVf2* (Gene bank accession number AJ297740) was amplified from the BAC clone M18-5 (Vinatzer et al., 2001) using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Primers HcrVf2-F and HcrVf2-Term (Table 1), elongated to carry the restriction sites *Pac*I and *Asc*I for cloning, were used. 500 ng of the PCR product were digested overnight at 37 °C with 5 units each of restriction enzymes *Pac*I and *Asc*I in 20 μl 1X NEB buffer 4, purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and ligated overnight between the *Pac*I and *Asc*I sites of the binary vector pMF1 with an insert/vector ratio of 20:1 at 16 °C using 200U of T4 DNA Ligase (New England Biolabs Inc., Beverly, Massachusetts, USA) (Fig. 1). To verify the correct insertion of the PCR product in the vector, the pMF1 vector containing the *HcrVf2* gene was extracted from transformed *E. coli* clones (GenElute Plas-

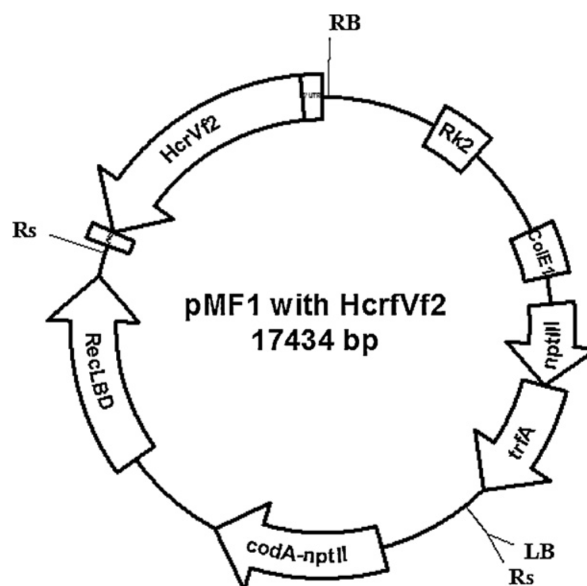

Fig. 1. Schematic representation of the pMF1 vector containing the *HcrVf2* gene controlled by its native regulatory sequences. The segment between the left (LB) and the right border (RB) is transferred into the plant cell, and the segment between the recombination sites (RS) is then removed on recombinase-mediated deletion, with the exception of one of the RS. *HcrVf2*, apple scab resistance gene from apple cv. Florina; fusion marker gene *codA*/*nptII*, hybrid gene for positive (*nptII*) and negative (*codA*) selection. RecLBD, translational fusion of recombinase R-LBD; Rk2 and ColE1, origins of replication. *trfA*; replication gene; *nptIII*, kanamycin resistance gene.

Table 1
Primers used for vector construction, PCR, RT-PCR and Southern blot Primer Sequence (5′ → 3′).

Primer	Sequence
HcrVf2-F[a]	ATACGTATTTAATTAACTAGCTAGTCCTAAATAGCCG
HcrVf2-Term[a]	ATCTAGATGGCGCGCCGGGGAGAACATAAACCTTACCC
codAfor	CGA TTC CGC ATT TTG AAT TT
codArev	TAC GCC CCG TTA TAG GAG TG
167nptIIfor	CCA CAG TCG ATG AAT CCA GA
367nptIIrev	AGC ACG TAC TCG GAT GGA AG
EF1for	TAC TGG AAC ATC ACA GGC TGA C
EF1rev	TGG ACC TCT CAT CAT GTT GT
RT1for	CAA TGC CTT ACG TGG TGA AA
RT2rev	CAG GGA TTC CAG CCA ATC TA
trfA FW2	GCG AGG AAC TAT GAC GAC CA
trfA REV1	CCA CAC CAG TTC GTC ATC GT
nptIIIfor	CCG GTA TAA AGG GAC CAC CT
nptIIIrev	GGA GTG AAA GAG CCT GAT GC
nptIIfor	AAT ATC ACG GGT AGC CAA CG
nptIIrev	GAA TGA ACT CCA GGA CGA GG
picA1	ATG CGC ATG AGG CTC GTC TTC GAG
picA2	GAC GCA ACG CAT CCT CGA TCA GCT
pmf_bb3	ATA AGT GCC CTG CGG TAT TG
pmf_bb4	GCA GCC CTG GTT AAA AAC AA
HcrVf2_SEQ1	TTG CTC ATA CAC ATC ACC TGC
HcrVf2_SEQ2	GTT TCT TTG GTT CTA TGA CAA G
HcrVf2_SEQ3	TCC GAT TCC CAA ATT GTT GT
HcrVf2_SEQ4	ACT AAG CTT GTC TGG TAC AGG AA
HcrVf2_SEQ5	GTA CCC GAT TGT TGG ATG AG
HcrVf2_SEQ6	CCG AGA TGC TTC CAC AAT TT
HcrVf2_SEQ7	CCA TGG AGC ATT CTT CTT TC
HcrVf2_SEQ8	GCT GCA ATT CTT GTT GAG A
HcrVf2_SEQ9	GTA AGT CCA GAC GCA ACC
HcrVf2_SEQ10	TTG GGA CAT TCC CAG TTA GG
HcrVf2_SEQ11	CGT TAG CAT TTT GAG TTG ACC A
HcrVf2_SEQ12	CCC CGA GAT TAA GAG TTG TAA GA
HcrVf2_SEQ13	TGC TTT AAA CTG AGC AAA GAA GG
HcrVf2_SEQ14	TGG TTG CAA TGG CTA GAA AC

[a] Restriction sites *Pac*I and *Asc*I are underlined.

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