Generation of a cisgenic apple line of cultivar 'Gala' with increased fire blight resistance

T.D. Kost[1], M. Jänsch[2], C. Gessler[1], H. Flachowsky[3], A. Patocchi[2] and G.A.L. Broggini[1,2]

[1]Plant Pathology, Institute of Integrative Biology (IBZ), ETH Zurich, Zurich, Switzerland; [2]Agroscope, Institute for Plant Production Sciences, Wädenswil, Switzerland; [3]Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Fruit Crops, Julius Kühn-Institute, Dresden, Germany.

Abstract

To generate a fire blight (fb) resistant 'Gala' line, the fb-resistance gene *FB_MR5*, from wild apple *Malus* × *robusta* 5, was transferred to the highly fb susceptible cultivar 'Gala' by mean of *Agrobacterium tumefaciens* mediated transformation. The vector used to insert *FB_MR5* into 'Gala' in vitro plant's explants was designed to allow production of cisgenic lines by elimination of a cassette containing all transgenes supporting transformation and excision steps (e.g., recombinase, antibiotic resistance), after a heat induction step. 13 different transgenic 'Gala' lines were regenerated. A heating treatment, to activate the recombination and generate cisgenic genotypes, was then applied. So far one single cisgenic line was regenerated. Plants of this line were considered cisgenic as, after recombination, only the apple endogenous *FB_MR5* gene, in normal-sense orientation and under control of its native regulatory elements, was present. We focused on molecular characterization of this single line and determined a single insertion. Resistance of the cisgenic line against *Erwinia amylovora* was assessed performing pathogen inoculations by scissors in the greenhouse. The cisgenic line showed less disease symptoms on inoculated shoots compared to untransformed 'Gala' plants. To our knowledge this is the first prototype of a cisgenic fb-resistant 'Gala' apple line.

Keywords: *Malus*, FB_MR5, cisgenesis, transformation, genetic modification

INTRODUCTION

The whole apple production relies on a small number of commercial cultivars, which are all highly susceptible to several diseases like apple scab or mildew, but in particular, to fire blight (fb) (Norelli et al., 2003). An ecological disease management alternative to application of plant protective chemicals is breeding for resistances. This approach became increasingly important in different breeding programs (Peil et al., 2011). Introgression of resistance genes from wild apple genotypes by conventional breeding requires several backcrosses to remove undesirable properties inherited beside the resistance gene from the wild ancestor (e.g. fruit size and taste). Considering the long juvenile phase in apple, such introgression into a cultivar of commercial value can take 20 to 50 years (Flachowsky et al., 2009). The increasing number of apple resistance genes cloned and the availability of several methods to achieve marker free genetically modified plants fitting to the definition of cisgenesis offer an unprecedented opportunity to rapidly introgress resistance genes, possibly without changing any of the other cultivar characteristic that made it successful. Two vectors have been used to generate cisgenic apple lines: vector pM(arker)F(ree)1 (Schaart et al., 2004), used to generate cisgenic lines with the *Rvi6* scab resistance gene (Vanblaere et al., 2011) and the *Rvi15* scab resistance gene (Giovanni Broggini, Agroscope, pers. commun.); or vector p9-Dao-FLPi-HcrVf2, used to generate cisgenic apples of several cultivars also with *Rvi6* (Würdig et al., 2015). First field trials with cisgenic apples are ongoing (Krens et al., 2015). The first fb resistance gene *FB_MR5* has recently been mapped (Peil et al., 2007) and cloned (Fahrentrapp et al., 2012; Broggini et al., 2014). In this study, we present the generation of a fb resistant cisgenic 'Gala Galaxy' line carrying the *FB_MR5* gene using the vector p9-Dao-FLPi-FB_MR5. Molecular characterization of this line included the determination of the number of T-DNA integrations and transcription level of the

FB_MR5 gene in this line compared to conventional bred genotypes carrying *FB_MR5*. Finally plant shoot length of this line was compared to the untransformed genotype 'Gala Galaxy' as well as to other related genotypes.

MATERIAL AND METHODS

Transformation

Vector p9-Dao-FLPi-HcrVf2 (Würdig et al., 2015) with *FB_MR5* instead of *HcrVf2* was transferred in *Agrobacterium tumefaciens* strain GV3101pMP90RK. For this purpose we used *FB_MR5 (*4167 bps) flanked by its 1995 bps native 5'-UTR and 1547 bps native 3'-UTR and the same primers and restriction sites as described by Broggini et al. (2014) for generating 390p95N-Mr5FB1. Transgenic plants were produced and regenerated by transformation of in vitro explants of the cultivar 'Gala Galaxy' following the protocol of Szankowski et al. (2009) and Vanblaere et al. (2011). According to those protocols regenerants were selected on kanamycin containing media and put on fresh media every 4 to 6 weeks. In order to obtain cisgenic plants a heat shock (4 h at 42°C) was performed and explants were kept for two weeks in the dark on regeneration media without kanamycin to form calli. As soon as regenerants were available on the calli, they were put on elongation media without kanamycin and investigated by PCR. PCR was performed to test for the presence of *FB_MR5* using primers FB_MR5q1 F (TTTATGGAGAGTGCTCCTTGC) and FB_MR5q1 R (AGCGAATCAAGGTTCTCTGG) and for the absence of *nptII* using 167nptII-for (CCACAGTCGATGAATCCAGA) and 367nptII-rev (AGCACGTACTCGGATGGAAG).

Fire blight inoculation

Artificial fb inoculation was performed as described previously (Peil et al., 2007). Shoots were bud grafted on M9T337 rootstocks and the two youngest, completely unfolded leaves were transversally bisected using scissors dipped in buffer solution with around 10^9 cfu of *E. amylovora* strain EA222_JKI mL^{-1}. Only plants that reached 13.0 cm shoot length were considered.

Southern blot

Southern blot hybridization was performed as described by Vanblaere et al. (2014) using *Bsa*I for genomic DNA digestion and a *nptII* specific DIG-labelled probe to indirectly determine copy number of T-DNA insertion (therefore also of *FB_MR5*). As control genotype the non-cisgenic line T44.4.149 (a transgenic line in which recombination did not occur) was used.

Transcription level of *FB_MR5*

Transcription level of *FB_MR5* in different genotypes was determined by qPCR. A Taqman Probe (YYE-TGGCTTCCATTTCAAACGGATCACAGA-BHQ1) was designed to detect *FB_MR5* in combination with primers FB_MR5q1 F and FB_MR5q1 R (Fahrentrapp, 2012). As reference EF1 primers and the relative Taqman probe developed by Gusberti et al. (2012) were used. RNA was extracted in triplicates from young unfolded leaves of the following genotypes: C44.4.146, 'Gala Galaxy', that passed an in vitro phase, and the *FB_MR5*-carrying accession ACW 22161 and ACW 22176 from the Agroscope Swiss apple breeding program. Following RT-reaction, cDNA was then diluted 1:10 and 5 µL were used for qPCR on ViiA Ruo qPCR device (Thermo Fisher scientific Inc©, Waltham, USA) in a total reaction volume of 20 µL using the Taqman Fast Universal Master Mix (Thermo Fisher Scientific Inc©, Waltham, USA). Expression ratio was calculated according to the method of Pfaffl et al. (2001).

Comparison of shoot length

For comparison of shoot length, budwood of the following genotypes was grafted on M9 rootstock: C44.4.146, 'Gala Galaxy' that underwent in vitro culturing, 'Gala Galaxy' and both parents of 'Gala': 'Kidd's Orange' and 'Golden Delicious'. Following bud break, the length of the shoot was measured after eleven weeks. Only shoots that reached growth stage BBCH

31 and reached a length of at least 10.0 cm were considered.

Statistics

Nonparametric tests were used as not all data followed normal distribution. Boxplots and Wilcoxon rank sum test was performed (p-value < $7.0 \times e^{-7}$) using R version 2.15 (R Core Team, 2012), http://www.R-project.org. A Steel-Dwass test was performed in JMP®10.0 (SAS Institute Inc., Cary, NC) and barplots were created using Microsoft Excel. For all statistical analysis α=0.05 was used.

RESULTS

Transformations

Two transformation experiments (T44 and T45) were performed using agrobacteria carrying the vector p9-Dao-FLPi-FB_MR5 and 80 respectively 300 explants were generated. The first transformation led to eleven transgenic regenerants (efficiency of 13.5%) and the second transformation to two transgenic lines corresponding to an efficiency of 0.7%. Nine of those lines were propagated and used to generate explants, which were exposed to a 4 h heat shock. After regeneration, 447 putative cisgenic shoots were obtained. PCR showed that one of those regenerants carried *FB_MR5* but no more transgenes (data not shown). This regenerant named C44.4.146 was micrografted on a 'Golden Delicious' seedling and retested for the presence of *FB_MR5* and absence of the excisable transgenic cassette represented by *nptII* by PCR (Figure 1). As *FB_MR5* was present and *nptII* was absent we consider this line to be cisgenic.

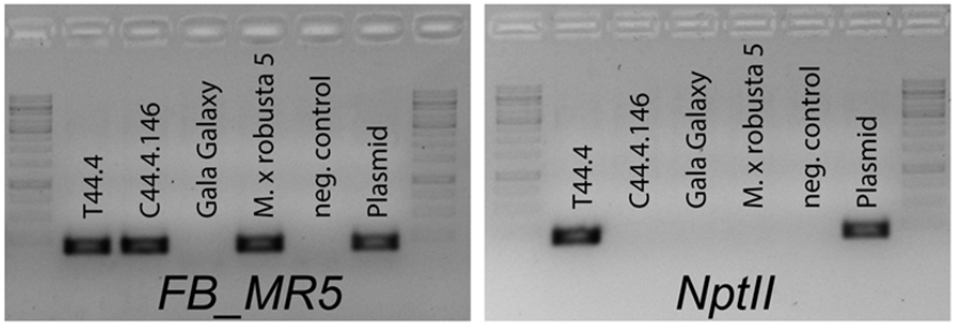

Figure 1. Amplicons aftePCR with primers to detect a) *FB_MR5*, b) *nptII* representing the antibiotic resistance gene. Samples were loaded in this order: transgenic motherline (T44.4), cisgenic line C44.4.146, 'Gala Galaxy', *M. robusta* 5, negative control and vector p9-Dao-FLPi-FB_MR5 (Plasmid). Marker is 1 kb ladder.

Fire blight resistance

This line was further used for bud-grafting on M9T337 rootstocks and for artificial fb inoculation tests in the greenhouse. 28 days post inoculation, all plants of line C44.4.146 showed a significantly lower percent lesion length (PLL) than the 'Gala Galaxy' plants that underwent a similar in vitro treatment (Figure 2). The average PLL was 12.4±17.1% in C44.4.146 and 77.0±13.5% in 'Gala Galaxy'.

Copy number

Using Southern hybridization we observed the single insertion of one copy of *nptII* in a sister line of C44.4.146, which stayed transgenic after heat shock treatment (Figure 3).

Transcription level of *FB_MR5*

We observed no significant difference in the transcription level of *FB_MR5* between line C44.4.146 and *FB_MR5*-carrying accession ACW 22161 and ACW 22176. Indicating that transcription level of *FB_MR5* is in the same range as in conventionally bred plants. No

transcription of *FB_MR5* was observed in the control plants of cultivar 'Gala Galaxy' (Figure 4).

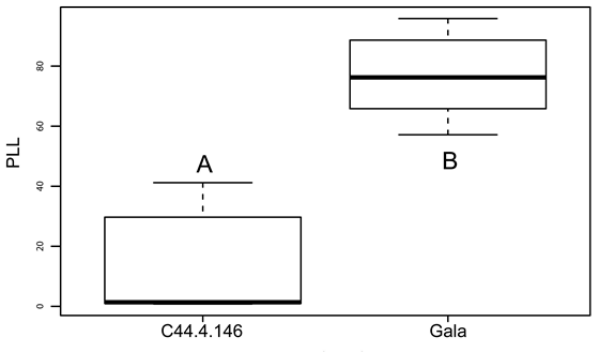

Figure 2. Percent lesion length (PLL) 28 days post inoculation with around 10^9 cfu mL^{-1} EA222_JKI. Data of twelve different shoots for each genotype. Letters indicate statistically significant difference by Wilcoxon test (α=0.05).

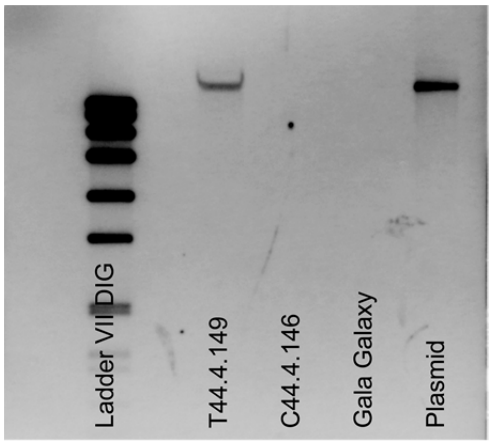

Figure 3. Southern blot hybridization with an *nptII* specific probe led to a single band in the linearized plasmid (Plasmid) and in the transgenic sisterline of C44.4.146 (T44.4.149) but to any bands in line C44.4.146 or 'Gala Galxy'.

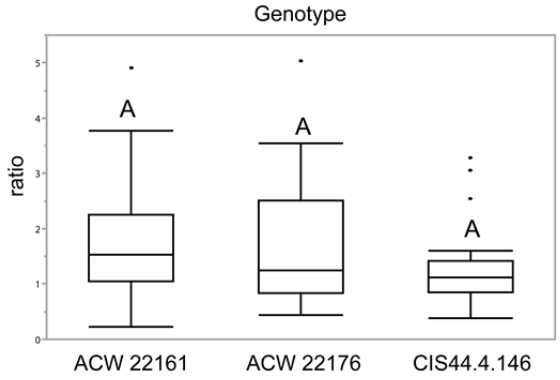

Figure 4. *FB_MR5* transcription ratio calculated using cDNA of two conventionally bred Mr5 accessions (ACW 22161 and ACW 22176) and cisgenic line C44.4.146. Similar letters indicate no statistically significant difference (Steel-Dwass test α=0.05).

Comparison of shoot length

Shoot length of plants of C44.4.146 after eleven weeks was in all comparisons similar to at least one of the parents of 'Gala' (Figure 5). Shoot length of plants of C44.4.146 was in the range between 'Golden Delicious' from nursery and 'Golden Delicious' that underwent an in vitro phase.

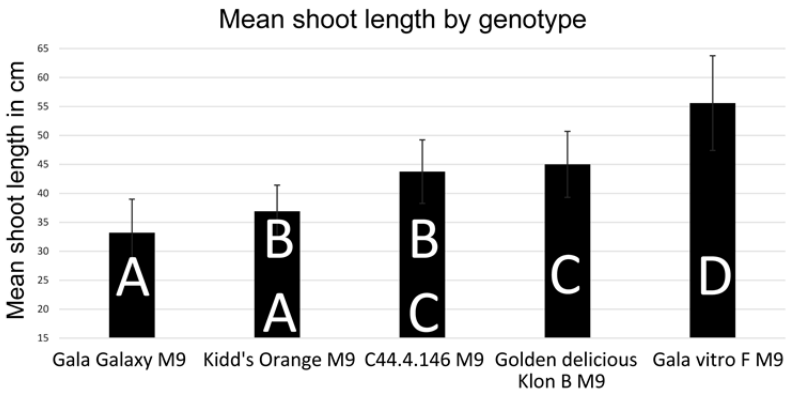

Figure 5. Mean shoot length of different genotypes measured on 23rd March 2015. Plant material was grafted on M9 rootstocks (18.12.2014) to produce ten to twelve plants per genotype. Plants were placed in a greenhouse cabin in the 5th January 2015. Only plants that reached growth stage BBCH 31 with a minimal shoot length of 10.0 cm were considered. Different letters indicate statistically significant differences between groups using Steel-Dwass test (α=0.05). Bars represent standard deviations.

DISCUSSION

Only a single cisgenic line, line C44.4.146, could be regenerated and investigated. PCR confirmed the presence of *FB_MR5* and absence of *nptII* in the final line C44.4.146 (Figure 1). Plants of this line showed significantly increased resistance to fire blight 28 days after inoculation with *Erwinia amylovora* strain EA222_JKI in the greenhouse (Figure 2). Southern blot hybridization allowed assessing a single T-DNA insertion in this genotype (Figure 3). *FB_MR5* transcription analysis revealed that the transcription in line C44.4.146 is not significantly different from the transcription in conventionally bred *FB_MR5* accessions ACW 22161 and ACW 22176 (Figure 4). Mean shoot length of this line after eleven weeks was always similar to at least one of 'Gala's parents, 'Kidd's Orange' or 'Golden Delicious' and in between 'Gala Galaxy' plants from nursery and 'Gala Galaxy' plants that underwent an in vitro phase (Figure 5).

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support by the Swiss Federal Office of Agriculture Project ZUEFOSII. We are grateful for the technical support of R. Blapp (Agroscope in Wädenswil), Ines Hiller (JKI Dresden) and J. Schneider (ETH Zurich) and we thank E. Chevreau from INRA Angers for providing the in vitro 'Gala Galaxy' plants.

Literature cited

Broggini, G.A.L., Wöhner, T., Fahrentrapp, J., Kost, T.D., Flachowsky, H., Peil, A., Hanke, M.V., Richter, K., Patocchi, A., and Gessler, C. (2014). Engineering fire blight resistance into the apple cultivar 'Gala' using the FB_MR5 CC-NBS-LRR resistance gene of Malus × robusta 5. Plant Biotechnol. J. *12* (*6*), 728–733. PubMed http://dx.doi.org/10.1111/pbi.12177.

Fahrentrapp, J. (2012). Fire blight resistance of *Malus* x *robusta* 5. PhD thesis (Switzerland: ETH Zurich), p.85–85.

Flachowsky, H., Hanke, M.V., Peil, A., Strauss, S.H., and Fladung, M. (2009). A review on transgenic approaches to accelerate breeding of woody plants. Plant Breed. *128* (*3*), 217–226 http://dx.doi.org/ 10.1111/j.1439-

0523.2008.01591.x.

Gusberti, M., Patocchi, A., Gessler, C., and Broggini, G.A.L. (2012). Quantification of *Venturia inaequalis* growth in *Malus* x *domestica* with Quantitative Real-Time Polymerase Chain Reaction. Plant Dis. *96* (*12*), 1791–1797 http://dx.doi.org/10.1094/PDIS-12-11-1058-RE.

Krens, F.A., Schaart, J.G., van der Burgh, A.M., Tinnenbroek-Capel, I.E.M., Groenwold, R., Kodde, L.P., Broggini, G.A.L., Gessler, C., and Schouten, H.J. (2015). Cisgenic apple trees; development, characterization, and performance. Front Plant Sci 6, 286. PubMed http://dx.doi.org/10.3389/fpls.2015.00286.

Norelli, J.L., Jones, A.L., and Aldwinckle, H.S. (2003). Fire blight management in the twenty-first century - Using new technologies that enhance host resistance in apple. Plant Dis. *87* (*7*), 756–765 http://dx.doi.org/10.1094/PDIS.2003.87.7.756.

Peil, A., Garcia-Libreros, T., Richter, K., Trognitz, F.C., Trognitz, B., Hanke, M.V., and Flachowsky, H. (2007). Strong evidence for a fire blight resistance gene of *Malus robusta* located on linkage group 3. Plant Breed. *126* (*5*), 470–475 http://dx.doi.org/10.1111/j.1439-0523.2007.01408.x.

Peil, A., Kellerhals, M., Höfer, M., and Flachowsky, H. (2011). Apple breeding—from the origin to genetic engineering. Fruit Veg Cereal Sci Biotechnol *5*, 118–138.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29* (*9*), 45e. PubMed http://dx.doi.org/10.1093/nar/29.9.e45.

Schaart, J.G., Krens, F.A., Pelgrom, K.T., Mendes, O., and Rouwendal, G.J. (2004). Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol. J. *2* (*3*), 233–240. PubMed http://dx.doi.org/10.1111/ j.14677652.2004.00067.x.

Szankowski, I., Waidmann, S., Degenhardt, J., Patocchi, A., Paris, R., Silfverberg-Dilworth, E., Broggini, G., and Gessler, C. (2009). Highly scab-resistant transgenic apple lines achieved by introgression of HcrVf2 controlled by different native promoter lengths. Tree Genet. Genomes *5* (*2*), 349–358 http://dx.doi.org/10.1007/ s112950080191-8.

Team, R.C. (2012). R: A Language and Environment for Statistical Computing (Vienna, Austria: R Foundation for Statistical Computing), ISBN 3-900051-07-0.

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G.A., and Gessler, C. (2011). The development of a cisgenic apple plant. J. Biotechnol. *154* (*4*), 304–311. PubMed http://dx.doi.org/ 10.1016/j.jbiotec.2011.05.013.

Vanblaere, T., Flachowsky, H., Gessler, C., and Broggini, G.A. (2014). Molecular characterization of cisgenic lines of apple 'Gala' carrying the Rvi6 scab resistance gene. Plant Biotechnol. J. *12* (*1*), 2–9. PubMed http://dx.doi.org/10.1111/pbi.12110.

Würdig, J., Flachowsky, H., Sass, A., Peil, A., and Hanke, M.V. (2015). Improving resistance of different apple cultivars using the Rvi6 scab resistance gene in a cisgenic approach based on the Flp/FRT recombinase system. Mol. Breed. *35* (*3*), 95–18 http://dx.doi.org/10.1007/s11032-015-0291-8.