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Engineering fire blight resistance into the apple cultivar 'Gala' using the *FB_MR5* CC-NBS-LRR resistance gene of *Malus* × *robusta* 5

Giovanni A. L. Broggini[1], Thomas Wöhner[2,‡], Johannes Fahrentrapp[1,†,‡], Thomas D. Kost[1,‡], Henryk Flachowsky[2], Andreas Peil[2], Maria-Viola Hanke[2], Klaus Richter[3], Andrea Patocchi[4] and Cesare Gessler[1,*]

[1]*Plant Pathology, Institute of Integrative Biology (IBZ), ETH Zürich, Zürich, Switzerland*

[2]*Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Fruit Crops, Dresden, Germany*

[3]*Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for Resistance Research and Stress Tolerance, Quedlinburg, Germany*

[4]*Agroscope, Institute for Plant Production Sciences, Wädenswil, Switzerland*

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**Correspondence* (Tel +41 44 632 38 71;
fax +41 44 632 15 72;
email cesare.gessler@usys.ethz.ch)
[†]Department Life Sciences and Facility Management, ZHAW Zurich University of Applied Sciences, Grüental P.B., CH-8820, Wädenswil, Switzerland.
[‡]Authors who contributed equally to this work.

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Summary

The fire blight susceptible apple cultivar *Malus* × *domestica* Borkh. cv. 'Gala' was transformed with the candidate fire blight resistance gene *FB_MR5* originating from the crab apple accession *Malus* × *robusta* 5 (Mr5). A total of five different transgenic lines were obtained. All transgenic lines were shown to be stably transformed and originate from different transgenic events. The transgenic lines express the *FB_MR5* either driven by the constitutive CaMV 35S promoter and the ocs terminator or by its native promoter and terminator sequences. Phenotyping experiments were performed with Mr5-virulent and Mr5-avirulent strains of *Erwinia amylovora*, the causal agent of fire blight. Significantly less disease symptoms were detected on transgenic lines after inoculation with two different Mr5-avirulent *E. amylovora* strains, while significantly more shoot necrosis was observed after inoculation with the Mr5-virulent mutant strain ZYRKD3_1. The results of these experiments demonstrated the ability of a single gene isolated from the native gene pool of apple to protect a susceptible cultivar from fire blight. Furthermore, this gene is confirmed to be the resistance determinant of Mr5 as the transformed lines undergo the same gene-for-gene interaction in the host–pathogen relationship Mr5–*E. amylovora*.

Introduction

Fire blight of pear and apple is a devastating disease caused by the enterobacterial phytopathogen *Erwinia amylovora* (Burill) Winslow *et al.* (1920). The fire blight disease is a worldwide problem for pome fruit growers because all popular apple cultivars are susceptible to the disease (Peil *et al.*, 2009). Recent epidemics caused relevant damages in different Western European countries, demonstrating the increasing economic importance of fire blight in this part of the world. In Switzerland, US $9 million of losses due to fire blight were calculated between 1997 and 2000, whereas in 2007, a CHF 50 million (i.e. US$ 54.1 million) loss arised from this disease (Hasler, 2002). For the Lake Constance region (Southern Germany), costs of US$1.6 million were reported in 2007 (Scheer, 2009). In 2013, approximately 600 ha of apple orchards and surrounding areas were infected in a small region in Eastern Germany.

Control of the fire blight disease may be achieved in years and at sites of high incidence and severity through severe pruning until eradication or preventive sprays with antibiotics, and failure may lead to the loss of the orchard. This incertitude puts the producer under a heavy stress during the time of the highest infection risk, just prior to and during flowering. Planting resistant cultivars seems to be an important measure available to manage the disease. Therefore, classical breeding is currently developing new resistant cultivars by introgressing resistance from accessions of wild *Malus* species into new breeding material. This material has to be back-crossed several times by different high-quality apple cultivars to remove most of the linkage drag until a new cultivar can be released (Flachowsky *et al.*, 2009; Schouten *et al.*, 2006). However, the fruit quality of such new selections may differ substantially from most commercially grown cultivars and may not readily be accepted by consumers. The success of classical resistance breeding is therewith not predictable. Thus, a solution could be to improve a popular cultivar by adding to it the resistance trait (gene) isolated from a donor genotype belonging to the native gene pool of apple.

The crab apple genotype *M.* × *robusta* 5 (Mr5) (Wöhner *et al.*, 2014) represents an ideal donor for fire blight resistance because it was described as resistant to all currently known European strains of the pathogen (Vogt *et al.*, 2013). The resistance is mainly inherited by a major QTL located on linkage group 3 (LG 3) of Mr5 (Peil *et al.*, 2007). This QTL was confirmed in different mapping populations with Mr5 in the pedigree and after inoculation with different *E. amylovora* strains (Gardiner *et al.*, 2012; Peil *et al.*, 2007, 2008). Although the QTL explains most of the phenotypic variations (Vogt *et al.*, 2013), the detailed genetics of this trait is still under discussion. Peil *et al.* (2007)

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proposed the presence of a single major gene in Mr5 conferring resistance to fire blight. However, the existence of multiple genes underlying the QTL region on LG 3 of Mr5 was also assumed (Gardiner *et al.*, 2012).

Experimental evidence exists that the resistance of Mr5 follows a gene-for-gene relationship (Gardiner *et al.*, 2012; Norelli *et al.*, 1986; Peil *et al.*, 2011; Vogt *et al.*, 2013). It was shown that *E. amylovora* strains usually express an $avrRpt2_{EA}$ effector. Furthermore, a specific allele polymorphism of this gene correlates with the virulence in Mr5. Mr5-avirulent strains encode for an $AvrRpt2_{EA}$ protein sequence with a cysteine residue at position 156 (C-allele), while strains encoding serine at position 156 (S-allele) or the $avrRpt2_{EA}$ effector deletion mutant strain ZYRKD3-1 (Zhao *et al.*, 2006) are virulent on Mr5 (Vogt *et al.*, 2013).

Very recently, a map-based cloning approach resulted in the detection of the *Fb_MR5* candidate resistance gene within the QTL region on LG 3 of Mr5 (Fahrentrapp *et al.*, 2013). The *FB_MR5* gene belongs to the CC-NBS-LRR resistance gene family and is located between the two flanking SSR markers Ch03e03 and Fem18. The present study is aimed at phenotypic characterization of *FB_MR5*-carrying transgenic plants of the fire blight susceptible cultivar 'Gala'.

The *FB_MR5* candidate gene was transferred into the genome of the apple cultivar 'Gala' via *Agrobacterium*-mediated transformation either under control of 2 kbp 5′-UTR and 1.5 kbp 3′-UTR sequences or of the constitutive CaMV 35S promoter and the ocs terminator. Artificial shoot inoculations evidenced the phenotypic resistance towards Mr5-avirulent strains of *E. amylovora* of the transformed lines. However, the tested lines were susceptible to the Mr5-virulent strain ZYRKD3-1.

Results

A total of three lines (M1401, M1402 and M1403) were obtained in 16 transformation experiments using vector 392p9N35s-Mr5FB1orf and seven lines (M1400, M1404, M1405, M1406, M1407, M1408 and M1409) in 11 experiments using 390p95N-Mr5FB1. All transgenic lines were derived from 'Gala' (4892 leaf explants), none from 'Pinova' (5712 leaf explants). At this stage, all regenerated plants were regarded as independent transgenic events and propagated vegetatively.

Molecular evaluation of transgenic events

All ten putative transgenic lines were positively tested on the presence of the *nptII* marker gene and the *FB_MR5* candidate gene for fire blight resistance (Figure S1) and absence of any *A. tumefaciens* contamination.

Subsequently, all putative transgenic lines were tested for transgene integration by Southern hybridization (Figure S2). Signals for *nptII* could be detected for all putative transgenic plants, although the signals for M1400, M1404, M1405, M1406 and M1407 were very weak. The experiment was repeated three times with nearly identical results using this probe. 'Gala' and all putative transgenic plants showed one unspecific band of high molecular weight when hybridized with the *FB_MR5* probe. An additional band was detected in Mr5 and in the putative transgenic plants. Plants of lines M1404, M1405, M1406 and M1407 showed nearly identical hybridization pattern for both *nptII* and *FB_MR5*. In the following, they were handled as one transgenic event designated as M1404. M1408 and M1409 also showed similar hybridization pattern. They were also handled as one transgenic line and designated as M1408. The same situation was found for M1402 and M1403. These plants were further handled as transgenic line M1402. The number of inserted copies was estimated combining the Southern blot hybridization results with nptII- and FB_MR5-probes as follows: single insertion in lines M1400, M1401 and M1402; two, possibly three, insertions in lines M1404 and M1408.

Evaluation on transgene transcription by reverse transcriptase (RT)-PCR

Transcription of the *nptII* marker gene was confirmed as all lines amplified fragments of the expected size (Figure S3). Transcription of the *FB_MR5* candidate gene for fire blight resistance was confirmed using primers 35S_f and Cand1-3f for lines M1401 and M1402 and using primers ATG-forward and $TGA_{(tomato)}$-reverse for lines M1404 and M1408.

Evaluation of transgenic lines on their resistance to Mr5-avirulent strains

The control 'Gala' was susceptible showing median stem necrosis (= percentage of shoot length with lesion, PLL) values varying between 80.4% (experiment 1, 28 dpi, Figures 1 and 2) and 62% (experiment 2, 36 dpi, Figure 3). The average stem necrosis of 'Gala' varied between 68.1 ± 35.0% (experiment 1) and 58.7 ± 22.9% (experiment 2). Contrary transgenic lines M1400, M1402, M1404 and M1408 inoculated with Mr5-avirulent strains Ea1189 and EA222_JKI showed significantly lower median PLL values (0% to 12%, average between 0% and 4%). Line M1401 only tested in the second experiment displayed a very broad variance with five shoots showing PLL values of 0% and five ranging between 25% and 83% when inoculated with Ea1189

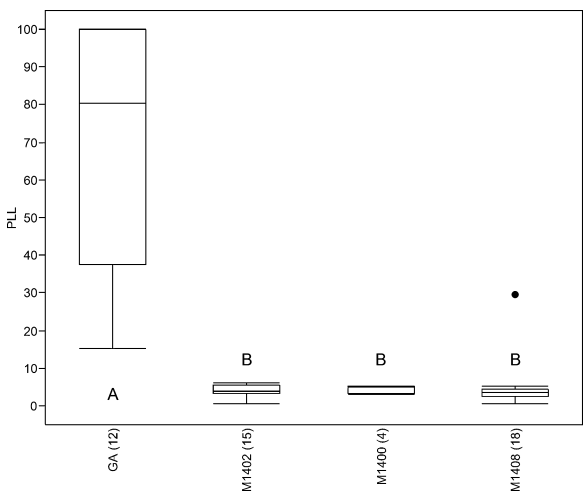

Figure 1 Boxplot representation of fire blight symptom severity expressed in percentage lesion length (PLL) of the shoot on 'Gala' (GA) and selected *FB_MR5* transgenic 'Gala' lines 28 days after scissors inoculation with a 10^9 CFU/mL suspension of the Mr5-avirulent *E. amylovora* strain EA222_JKI. Line M1402 contains the *FB_MR5* gene driven by the constitutive CaMV 35S promoter, whereas lines M1400 and M1408 contain the target gene driven by its native promoter region. The number of individual plants tested per genotype is given in brackets. Each box delimits values from the 25% to the 75% of the dataset. The horizontal line in the box represents the median of the data. Outliers are shown as black dots. Whiskers are drawn for obtained values that differ least from median ± 1.5 IQR. Letters indicate statistically significant difference by Steel–Dwass test ($\alpha = 0.05$).

Figure 2 Fire blight symptoms on representative shoots of nontransformed 'Gala' and 3 transgenic 'Gala' lines (M1400, M1402, M1408) containing the *FB_MR5* gene (experiment 1) 39 days after leaf inoculation with the Mr5-avirulent *E. amylovora* strain EA222_JKI.

(Figure 3). The lines M1404 and M1408 reacted differently to the avirulent and virulent strains showing susceptibility to the ZYRKD3_1 (PLL median 63.4% and average 49.9% for M1404, and 26.9% and 29.7%, respectively, for M1408) and immunity to Ea1189 (PLL median 0% and average 1.5% for M1404, and 0% for M1408, Figure 4).

Discussion

The *FB_MR5* gene was successfully transferred to the genome of the apple cultivar 'Gala'. In contrast, no transgenic line could be selected within all the experiments carried out with 'Pinova'. Currently, there is no scientific sound explanation for this problem. The p9N vector, which was used as backbone vector for the 392p9N35s-Mr5FB1orf gene construct, has been used successfully for transformation of 'Pinova' with other gene constructs, while using the identical transformation protocol (Tränkner *et al.*, 2010).

Using the two gene constructs, a total of five transgenic lines were obtained from 'Gala'. All lines expressed the *FB_MR5* gene. Experiments with two different Mr5-avirulent strains of *E. amylovora* gave evidence that the *FB_MR5* gene represents the fire blight resistance gene of Mr5. Further inoculation experiments performed with the two transgenic lines M1404 and M1408, which were inoculated with the *avrRpt2*$_{EA}$ gene deletion mutant strain ZYRKD3_1, indicated that the different transgenic lines undergo the same gene-for-gene relationship as Mr5 itself as shown by Vogt *et al.* (2013).

One single 'Gala' plant was free from symptoms; on the contrary, a single M1404 (of 40) showed severe symptoms corresponding to 62% PLL. A single swapping label error during the experiments may be the cause. A number of plants of the two transgenic lines M1401 (five of ten) and M1402 (six of 20) expressing the *FB_MR5* gene driven by the CaMV 35S promoter

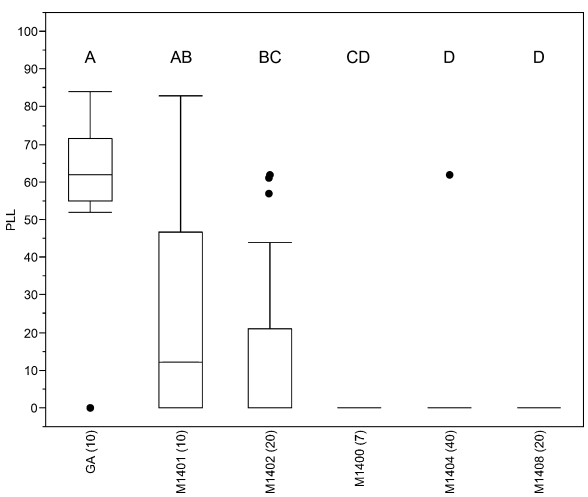

Figure 3 Boxplot representation of fire blight symptom severity expressed as percentage lesion length (PLL) on 'Gala' (GA) and on *FB_MR5* transgenic 'Gala' lines 36 days following inoculation using a 10^7 CFU/mL suspension of the Mr5-avirulent *E. amylovora* strain Ea1189. See legend Figure 1.

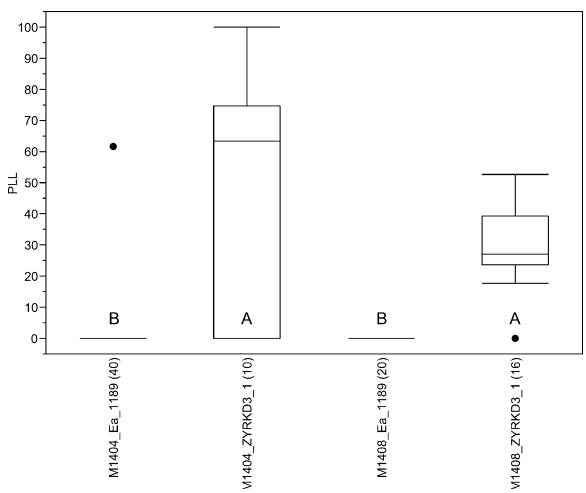

Figure 4 Boxplot representation of fire blight symptom severity on two *FB_MR5* transgenic 'Gala' lines induced by the two *E. amylovora* strains Ea1189 (Mr5-avirulent) and ZYRKD3_1 (Mr5-virulent) 36 days after inoculation using 10^7 CFU/mL suspensions. See legend Figure 1.

showed necrosis in the range of the 'Gala' plants after inoculation with the Mr5-avirulent strain Ea1189, whereas the other three lines with *FB_MR5* gene driven by the native promoter and terminator were more or less fully resistant. FB_MR5 resistance may be of a semi-qualitative nature, meaning that a reduced functionality of FB_MR5 combined with an unfavourable physiological state of the plants may result in single plant susceptibility. Such reduced functionality might be due to an insufficient transcription level or due to a truncated form of *FB_MR5* in the 392p9N35s-Mr5FB1orf gene construct caused by a wrong determination of the start or stop codon. Erroneous introns prediction may lead to such errors. Difficulties in correct intron prediction on *M. x domestica* sequences were reported already by Fahrentrapp *et al.* (2013).

The *FB_MR5* gene of *Malus* × *robusta* 5 was shown to confer fire blight resistance to the susceptible apple cultivar 'Gala', which is one of the leading cultivars on the global apple market. This gene has already been used intensively in classical apple and rootstock breeding (Peil *et al.*, 2009, 2011). The *FB_MR5* gene originated from a crossable donor and its regulatory sequences are identical to the chromosomal segments that are currently introgressed using classical breeding. On this account, this gene can be used to create cisgenic apple plants (Vanblaere *et al.*, 2011), which may encounter less opposition than transgenics (Gaskell *et al.*, 2011). A genetic engineered 'Gala' apple resistant to fire blight offers many advantages, including lowered costs of production due to effective disease management (e.g. fewer antibiotic applications, eradication costs) and lowering environmental footprint of apple production (lower antibiotic residues on fruits or in honey, less tractor rides). *E. amylovora* isolates overcoming this resistance were identified in Canada with a single amino acid substitution leading to virulence (Vogt *et al.*, 2013). In Europe, such strains were not yet detected. To avoid ephemerality of this resistance in Europe, further work will be needed to identify additional resistance genes enabling to build a resistance gene pyramid.

Experimental procedures

Plasmids, plant material and transformation

The coding region of the *FB_MR5* gene was amplified from start (ATG) to stop (TGA) with a length of 4167 bp using the primers ORFBam/ORFEco (Table 1) and cloned into the vector 392p9N35s using the restriction sites *Bam*HI and *Eco*RI, respectively (Figure S4). The *FB_MR5* open reading frame (ORF) is flanked from the CaMV 35S promoter and the terminator of the octopine synthase gene. A scheme of the resulting binary plant transformation vector 392p9N35s-Mr5FB1orf is shown in Figure S4. The *FB_MR5* ORF including 1995 bp of the genomic region upstream the ATG codon and 1547 bp of the region downstream the TGA codon was amplified using the primers FB1Bam/FB1Eco and cloned into the vector 390p95N using the restriction sites *Bam*HI and *Eco*RI, respectively (plasmid vector 390p95N-Mr5FB1, Figure S4). Both plasmids used for transformation were constructed by DNA-Cloning Service, Hamburg (Germany) and were cloned into *Agrobacterium tumefaciens* strain GV3101 using the freeze and thaw method as described by Holsters *et al.* (1978). For plant transformation, proliferating axillary shoot cultures of the apple (*Malus* × *domestica* Borkh.) cvs. 'Pinova' and 'Gala' were used. All plant material was cultivated with 16 h of light and 8 h darkness in an *in vitro* culture room at 20–24 °C. Plant transformation, regeneration and selection of transgenic 'Gala' plants at ETH Zurich were carried out as described by Szankowski *et al.* (2009) and Vanblaere *et al.* (2011). Plant transformation, regeneration and selection of transgenic plants at JKI Dresden (Germany) were carried out as described by Flachowsky *et al.* (2007). Once transgenic *in vitro* shoots were available, they were micrografted on actively growing shoots either of seedlings of the apple cv. 'Golden Delicious' or on M9 rootstocks as described by Vanblaere *et al.* (2011).

Table 1 Primers used in this study

Gene	Primer	5′→3′ sequence	*Ta* °C
FB_MR5	SBFBMR5F	CTACTAAAATGTTGAAACGTCAGATACAA	65
	SBFBMR5R	TGAAGGAAGATAAAAGTAGGTTAATCTCAG	
	ATG-forward	ATGGGGGGAGAGGCTTTTCTTGTGGCAT TCCTCCAAG	63
	$TGA_{(tomato)}$-reverse	TCAAATCATCTTCCAATCTATATCTATGTAAG	
	ORFBam	CCTTGGATCCTCCAAACAATGGGGGGAGAG	58
	ORFEco	GGGCCTTAAGTCGAAGTTTAGTAGAAGGTT	
	FB1Bam	CCCGGATCCAGTTTGTAAACTACACAGACTA	56
	FB1Eco	GGGCCTTAAGAAGTTCCTGCAGCA	
	35S_f	CCCACTATCCTTCGCAAGACCC	61
	Cand1-3f	CTTTCCGACTCCAGCCATAC	
nptII	nptII_F	ACAAGATGGATTGCACGCAGG	58
	nptII_R	AACTCGTCAAGAAGGCGATAG	
	NptIIF1	GGTTCTCCGGCCGCTTGGGTG	56
	NptIIR1	CGGCAGGAGCAAGGTGAGATGAC	
EF1α	EF1α_F	ATTGTGGTCATTGGYCAYGT	56
	EF1α_R	CCAATCTTGTAVACATCCTG	
VirG	VirG_F	GCCGGGGCGAGACCATAGG	60
	VirG_R	CGCACGCGCAAGGCAACC	

PCR and RT-PCR analysis

Genomic DNA was extracted from 0.1 g of leaf tissue using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). Transgene integration was evaluated using the primers nptII_F/R for *nptII* and primers ATG-forward and $TGA_{(tomato)}$reverse for *FB_MR5,* respectively (Table 1). Absence of bacterial contaminations was tested using the primers VirG_F and VirG_R.

Young leaves were randomly selected and frozen in liquid nitrogen. The total RNA was extracted from 100 mg leaf tissue using the Invisorb® Spin Plant-RNA Mini Kit (Invitek, Berlin, Germany). 1 μg of total RNA was treated with *DNase* I (Thermo Fisher Scientific Inc.©, Wilmington, DE) and subsequently tested for the presence of DNA residues by PCR using the primers EF1α_F and EF1α_R. The remaining RNA was reversely transcribed using oligo(dT) 18 primer and the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.©) according to the manufacturer's instruction. Successful reverse transcription was verified by PCR with 1 μL cDNA using primers EF1α_F and EF1α_R. Transgene expression was evaluated using the primers NptII-F1/R1 for *nptII*. The expression of *FB_MR5* was measured using the primers 35S_f and Cand1-3f_f or the primers ATG-forward and $TGA_{(tomato)}$-reverse, respectively. The PCR for detecting the genes *EF1α* using the primers EF1α_F/R, *VirG* using the primers VirG_F/R, *nptII* using the primers nptII_F/R and NptIIF1/R1, respectively, and *FB_MR5* using the primers SBFBMR5F and SBFBMR5R was performed as follows: The

polymerase chain reaction (PCR) mixture contained 25 ng genomic DNA or cDNA, respectively, 0.5 U DreamTaq DNA polymerase, 0.5 μM of each primer, 20 μM dNTPs (each) and 1× DreamTaq buffer. DreamTaq DNA polymerase, 10× Dream Taq buffer and dNTPs were used from Thermo Fisher Scientific Inc. ©. PCR was performed in a total volume of 25 μL. The PCR was performed by denaturation at 94 °C for 3 min, followed by 35 cycles of 30 s denaturation at 94 °C, 1 min annealing (depending on the primer, see Table 1) and 1 min extension at 72 °C. After a final extension at 72 °C for 5 min, the reaction was cooled down to 10 °C.

The PCR for detecting the *FB_MR5* gene using the primers ATG-forward/TGA(tomato)-reverse, ORFBam/ORFEco, FB1Bam/FB1Eco and 35S_f/Cand1-3f was performed as follows: the PCR mixture contained 25 ng genomic DNA or cDNA, respectively, 1U Phusion high-fidelity polymerase, 0.5 μM of each primer, 20 μM dNTPs (each) and 1× GC buffer. Phusion high-fidelity polymerase, 5× GC buffer and dNTPs were used from Thermo Fisher Scientific Inc.©. PCR was performed in a total volume of 25 μL. The PCR was performed by denaturation at 98 °C for 3 min, followed by 35 cycles of 30 s denaturation at 98 °C, 1 min annealing (depending on the primer, see Table 1) and 30 s/kbp extension at 72 °C. After a final extension at 72 °C for 10 min, the reaction was cooled down to 10 °C. All PCR products were separated and visualized on 0.8–1% agarose gels.

Southern blot analysis

The detection of integrated T-DNA copies was performed by Southern hybridization. 10 μg genomic DNA of each genotype was cut with 100 units of *Bam*HI (Thermo Fisher Scientific Inc.©) at 37 °C overnight. The cleaved DNA was separated on a 0.8% agarose gel and transferred onto a nylon membrane (Roche Diagnostics, Mannheim, Germany). PCR-amplified, digoxygenin-labelled probes from the coding region of the *nptII* marker gene and the *FB_MR5* gene were generated using the primers nptII_F and nptII_R for *nptII* and SBFBMR5F and SBFBMR5R for *FB_MR5*, respectively. Hybridization and detection were performed using the ECF-Random-Prime-Labeling and Detection Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's manual.

Artificial fire blight inoculation

The evaluation for resistance to fire blight was done as described by Flachowsky *et al.* (2008). In brief, vigorously growing shoots of transgenic plants and nontransformed control plants were inoculated by bisecting the two youngest unfolded leaves with scissors dipped in a bacterial suspension of *E. amylovora*. The severity of infection was then calculated as percentage of the total shoot length necrotized (per cent lesion length, PLL). Three experiments were performed: in the first one, active growing shoots of the lines M1400, M1402 and M1408 (two-bud scion grafted on M9T337 rootstock) were inoculated with a suspension of the Mr5-avirulent *E. amylovora* strain Ea222_JKI (Peil *et al.*, 2007) at a concentration of 10^9 CFU/mL and evaluated 28 days postinoculation; in the second one, lines M1400, M1401, M1402 and M1408 (micrografted on 'Golden Delicious' seedlings) were inoculated with the Mr5-avirulent strain Ea1189 (Zhao *et al.*, 2006) at a concentration of 10^7 CFU/mL and evaluated 36 days postinoculation; in the third experiment, lines M1404 and M1408 (micrografted on 'Golden Delicious' seedlings) were inoculated with the Mr5-avirulent strain Ea1189 and its deletion mutant Mr5-virulent strain ZYRKD3_1 (Zhao *et al.*, 2006) of *E. amylovora* at a concentration of 10^7 CFU/mL and evaluated 36 days postinoculation.

Statistical analysis

All statistical analysis was performed using the software JMP® 10.0 (SAS Institute Inc., Cary, NC) on Windows 7. As data were not normally distributed, the Steel–Dwass test (nonparametric version of Tukey HSD) was used for multiple comparisons of the data with $\alpha = 0.05$. Single values were defined as outliers if they differed more than 1.5 interquartile ranges (IQR) from the median.

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Author contribution

B. G. organized and supervised all the research, F. J. executed transformations and micrografting and contributed to inoculation experiments. K. T. and R. K. carried out the Fire Blight tests; W. T. performed the molecular evaluation of transgenic plants. F. H., P. A., H. V. furnished various materials, ideas and infrastructure; G. C. led the project and wrote the manuscript together with F. H.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 PCRs of putative *FB_MR5* transgenic shoots tested for the presence of the transferred genes.

Figure S2 Southern blot hybridization of *Bam*HI-digested DNA of *FB_MR5* transgenic plants with DIG-labelled probes of *nptII* (Figure a) and *FB_MR5* (Figure b), respectively.

Figure S3 RT_PCR results on mRNA transcription in selected transgenic lines (a) using primers NptIIF1 and NptIIR1 for marker gene *nptII;* (b) using primers 35S_f and Cand1-3f amplifying partially the *FB_MR5* candidate gene driven by the CaMV 35S promoter.

Figure S4 Scheme of the binary plasmid vectors used for apple transformation.