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Field study of the fire-blight-resistant cisgenic apple line C44.4.146

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SUMMARY

Cisgenesis, the genetic modification of a plant with genes from a sexually compatible plant, was used to confer fire blight resistance to the cultivar 'Gala Galaxy' by amendment of the resistance gene *FB_MR5*, resulting in the line C44.4.146. To verify whether cisgenesis changed other tree-, flower- or fruit-related traits, a 5-year field trial was conducted with trees of C44.4.146 and multiple control genotypes, including members of the 'Gala' sports group. None of the 44 investigated tree-, flower- or fruit-related traits significantly differed between C44.4.146 and at least one of the control genotypes in all observation years. However, fruits of C44.4.146 and its wild-type 'Gala Galaxy' from tissue culture were paler in color than fruits of 'Gala Galaxy' that had not undergone tissue culture. There was no significant and consistently detected difference in the fruit flesh and peel metabolome of C44.4.146 compared with the control genotypes. Finally, the disease resistance of C44.4.146 was confirmed also when the fire blight pathogen was inoculated through the flowers. We conclude that the use of cisgenesis to confer fire blight resistance to 'Gala Galaxy' in C44.4.146 did not have unintended effects, and that the *in vitro* establishment of 'Gala Galaxy' had a greater effect on C44.4.146 properties than its generation applying cisgenesis.

Keywords: apple (*Malus* × *domestica* Borkh.), fire blight (*Erwinia amylovora* Burrill), *FB_MR5*, disease resistance, genetic modification, risk assessment, fruit metabolomics.

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) is an important fruit tree grown in the temperate zone. Because the apple global market is dominated by a few cultivars that are mostly susceptible to diseases, apple production involves a high input of plant protection products. The most devastating bacterial disease of apple is fire blight, caused by the bacterium *Erwinia amylovora* (Burrill; Winslow et al., 1920). The pathogen mainly enters trees via flowers (Vanneste, 1995), but also via wounds (Peil et al., 2009). Although pruning of infected branches helps to contain the spread of the disease, there is no effective treatment to combat fire blight once it has entered the tree, such that the use of fire-blight-resistant cultivars facilitates the management of the disease.

Breeding perennial crops like apple is challenging due to its long juvenile phase and gametophytic self-incompatibility, which promotes outcrossing and results in a high level of heterozygosity (Peace & Norelli, 2009). The development of a new cultivar requires 25–50 years (Luo et al., 2020). Another challenge with fire blight is that the majority of known major-effect resistance loci have been found in wild apple species (Norelli et al., 2003), which have poor fruit quality traits (Kellerhals et al., 2017) that must be removed through multiple (pseudo-) backcrosses (Schlathölter et al., 2018). The introduction of resistance genes from wild relatives through genetic modification could increase disease resistance in existing elite cultivars without introducing undesired traits. Thus, the development of genetically modified (GM) apple lines represents a rapid way to increase the disease resistance of a cultivar provided that the development of such resistance does not affect other cultivar characteristics.

Cisgenic plants, which are amended only with genes and their regulatory sequences from a sexually compatible plant (Schouten et al., 2006), have been developed for

several species [e.g. potato (Jo et al., 2014), barley (Holme et al., 2012) and apples (Kost et al., 2015; Vanblaere et al., 2011)]. Cisgenic crops have a higher public acceptance than conventional transgenic GM crops (Delwaide et al., 2015; Edenbrandt et al., 2018; Rousselière & Rousselière, 2017), genome-edited crops, or crops treated with natural or synthetic pesticides (Saleh et al., 2021). Although several highly effective fire-blight-resistance sources have been identified and mapped in wild apple accessions (Durel et al., 2009; Emeriewen et al., 2017, 2020, 2021; Emeriewen, Malnoy, et al., 2014; Emeriewen, Richter, et al., 2014; Peil et al., 2007), only the fire-blight-resistance gene *FB_MR5* from *M.* × *robusta* 5 (Mr5) has been cloned and functionally characterized (Broggini et al., 2014; Fahrentrapp et al., 2013). Unfortunately, *E. amylovora* strains able to overcome *FB_MR5*-based resistance have been detected in North America (Emeriewen et al., 2019; Vogt et al., 2013).

Using *FB_MR5*, Kost et al. (2015) generated the cisgenic line C44.4.146 through *Agrobacterium tumefaciens*-mediated transformation of the *in vitro*-grown fire-blight-susceptible cultivar 'Gala Galaxy'. They demonstrated that the cisgenic line had a single integration of *FB_MR5* on chromosome 16, and that *FB_MR5* was functional in C44.4.146, i.e. fire blight symptoms caused by artificial inoculation of shoots were less severe on C44.4.146 than on 'Gala Galaxy'. In Mr5, *FB_MR5* colocalizes with a strong QTL for floral fire blight resistance (Peil et al., 2019). Inoculation of C44.4.146 flowers could reveal whether *FB_MR5* also confers floral fire blight resistance, or whether this resistance is conferred by an additional gene tightly linked to *FB_MR5* in Mr5.

To assess the performance, composition and safety of a GM crop, researchers usually compare the GM crop with its non-GM counterpart or conventional cultivar(s) (Joint FAO/WHO, 2000; Kok & Kuiper, 2003). Studies on GM crops over the last 30 years suggest that no further risks are associated with the use of GM crops when compared with conventionally bred crops (EASAC, 2013; NAS, 2016). Nevertheless, there are examples showing that transgenesis may generate non-desirable phenotypic alterations (for review, see Kuiper et al., 2001; Cellini et al., 2004; Haslberger, 2003). The possibility of unintended effects, derived from the random integration of the introduced DNA or through the introduced DNA itself, remains a major public concern (Cellini et al., 2004; Kuiper et al., 2001), even though these effects are not necessarily related to safety. It follows that the assessment of both intended (introduced trait) and unintended effects is important for the evaluation of GM crops. Like non-GM cultivars, GM crops should be evaluated in field trials over several years to enable the study of environmentally dependent traits, such as tree growth, flower development and fruit characteristics, which are difficult to measure in the greenhouse.

In a greenhouse study, Jänsch et al. (2014) investigated the unintended phenotypical and molecular changes of a *Rvi6* cisgenic scab-resistant line (C11.1.53); three differentially expressed apple allergen genes were identified in this cisgenic line, but their expression did not translate into protein differences. In a field study, the line C11.1.53 and additional *Rvi6* apple scab-resistant lines were evaluated for resistance but not for tree growth or fruit-related traits (Krens et al., 2015). Borejsza-Wysocka et al. (2010), in contrast, assessed tree and fruit traits in a 12-year field trial. During that trial, seven transgenic GM apple lines producing the lytic, antimicrobial protein attacinE from *Hyalophora cecropia* pupae that is effective against fire blight exhibited no changes in fruit-, flower- or tree-morphology, or other fruit characteristics relative to the untransformed 'Gala Galaxy'.

The overall goal of the current study was to identify intended and unintended effects in the cisgenic line C44.4.146. To achieve this goal, C44.4.146 and several control genotypes (mainly sports of 'Gala'; Figure S1) were grown in the field. The following properties were assessed: (i) floral fire blight resistance; (ii) tree growth and fruit parameters; and (iii) flesh and peel metabolites from harvested fruits. For (ii) and (iii), the data collected from the control genotypes were used to put the deviations identified between C44.4.146 and its wild-type in the context of the natural variation observed within the 'Gala'-group grown in the same field trial. Only those deviations consistently found between C44.4.146 and the control group in all observation years were considered as unintended effects.

RESULTS

The resistance gene *FB_MR5* is functional and confers fire blight resistance when trees are inoculated through their flowers

Over two observation years (2018 and 2019, hereafter referred to as '18 and '19), a total of 228 flower clusters of C44.4.146 (CIS) and 219 of 'Gala Galaxy' (GG; obtained from a Swiss nursery) were inoculated with *E. amylovora* (Figure 1). The disease scores ranged from 0 (no symptoms) to 3 (floral and bourse infection) for CIS, and from 0 to 5 (infections expanding into the wood) for GG. A disease score of 0 was recorded in > 78% of the flower clusters of CIS and in a maximum of 25% of flower clusters of GG. The maximum disease score of 3 was recorded for CIS on 4.3% and 0.7% of the flower clusters in '18 and '19, respectively. A disease score of 5 was recorded on 37.9% ('18) and 44.8% ('19) of the flower clusters of GG. No significant differences in disease between years within the same genotype were found, but the two genotypes were statistically different from each other (Table S1). In both years, resistance to fire blight was significantly higher ($P < 0.001$) for

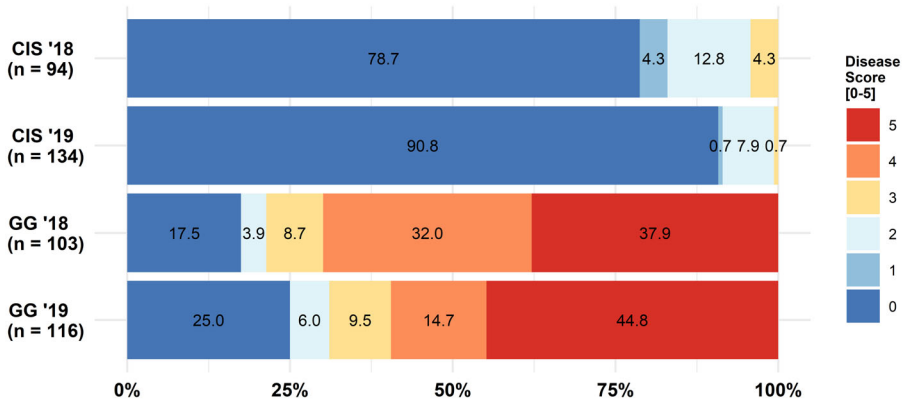

Figure 1. Distribution of floral fire blight disease scores of the cisgenic line C44.4.146 (CIS) and 'Gala Galaxy' (GG) 21 days post-inoculation in 2018 and 2019. The bar plots indicate the relative distribution of disease scores per genotype and year. Disease rating: 0 = no infection; 1 = floral infection; 2 = infection in flowers and peduncle; 3 = floral and bourse infection; 4 = floral, bourse and bourse shoot infection; and 5 = infection advanced up to 4 cm along the branch. Percentages may not total 100 due to rounding.

CIS than GG, with average disease scores for CIS versus GG of 0.43 versus 3.51 in '18, and 0.21 versus 3.23 in '19.

No trait assessed in the field trial differentiated CIS from the other 'Gala' genotypes over all observation years

Within-genotype comparison based on fruit peel coloration revealed eight GG and two 'Gala Galaxy' trees from an Italian nursery (GGB) that produced fruits with an abnormal over coloration for their genotype. These off-type trees were removed from the analyses. Of the 44 assessed traits (Table S2), 10 (no. 5, 12, 16, 17, 18, 21, 25, 35, 36, 41) were uniform within and between genotypes in the 2 years of observation, and three (no. 9, 43, 44) showed no genotypic effect in any investigated year (Table 1); these traits were not further analyzed. The qualitative traits 'ground color' (no. 29) and 'pattern of over color' (no. 32) were not statistically analyzed (Figure S2a, b). Ground color (no. 29) was "not visible" (score 1) in 'Gala Schniga® SchniCo red' (GSR) apples because they were completely red; fruits from all other genotypes had a predominantly yellow ground color (score 3). GSR apples mainly had a solid flush without stripes (score 1) as the 'pattern of over color' (no. 32); CIS, *in vitro*-cultured 'Gala Galaxy' (IVG, considered as the wild-type) and the original 'Gala' cultivar (GO) apples mainly had a "solid flush and strongly defined stripes" (45.0–68.1%, score 3), while 'Royal Gala' (RG), GG and GGB apples mainly had a "solid flush and weakly defined stripes" (52.5–95.5%, score 2).

Between CIS and IVG, differences in single years were found in six of 21 tree-related and 12 of 23 fruit-related traits (no. 6, 7, 13, 14, 19, 20, 22–28, 30, 31, 34, 37, 39; Table 1). CIS was outside the variability of the remaining 'Gala' genotypes for five traits (no. 13, 14, 28, 31, 34) in one observation year, and in the following three traits in two observation years: leaf drop (no. 19), percentage of yellow fruit over color (no. 27), and fruit sugar content at harvest (no. 39). No assessed trait was significantly different between CIS and all 'Gala' genotypes in all years of observation.

Analysis of fruit peel metabolomes identified metabolite features with a unique abundance in CIS compared with the 'Gala' genotypes in 2018, but not in 2019

The LC–MS/MS measurements of fruit peels detected 2166 metabolite features in '18 and 1482 in '19. Among them, one, two and 19 metabolite features were absent in RG, GSR or GG, respectively, but were present in all other genotypes (Table S3). No metabolite feature was present or absent only in CIS. Of the total number of metabolite features, 2145 ('18) and 1417 ('19) met the filtering criteria and were used for further analyses.

Comparisons within single years among all 'Gala' genotypes grown at the 'Protected Site' identified 302 (14.1%) and 31 (2.2%) differentially abundant features (DAFs) in '18 and '19, respectively. Principal cluster analysis (PCA) based on DAF abundance separated the genotypes into two or more clusters (Figure 2a,c) and were used for hierarchical clustering analysis (HCA), which separated the samples in two clusters in both years (Figure 2b, d). None of the clusters consisted solely of samples of a single genotype in '18, while in '19 one cluster consisted solely of most samples from GG (four of five samples). Within the clusters composed of several genotypes, genotype-specific sub-clusters separated from other genotypes were found for GSR in '18, and CIS and GSR in '19 (Figure 2b,d).

Comparisons between CIS and IVG identified 84 DAFs in '18 but none in '19 (Table 2). The average number of DAFs between the 'Gala' genotypes and CIS was 163 in

Table 1 Summary of the statistical analyses of all field phenotypic traits and number of genotype-specific traits

Trait no.	Trait name	Year	Data-set	Genotype effect		CIS	IVG	GG	GGB	**GSR**	RG	GO
Tree-related traits measured per tree (n = 20)												
1	Height increase	**'16**	R	**1.9 E-41**	***	e	be	ab	c	d	a	a
		'17	R	**1.2 E-15**	***	ac	c	abc	ab	d	b	ab
2	Stem to root-stock increase	**'16**	R	**0.05**	*	a	a	a	NA	NA	a	a
		'17	R	0.17								
		'18	R	**7.9 E-3**	**	b	ab	ab	NA	NA	a	a
		'19	R	0.49								
3	New branches (on 2-year-old stem)	**'16**	R	**8.4 E-12**	***	b	b	a	b	a	a	a
4	New branches (on 1-year-old stem)	**'16**	R	**5.5 E-13**	***	d	d	abc	ab	a	cd	bc
6	No. of internodes in 1-year-old shoots	**'16**	R	**0.01**	*	ab	a	ab	NA	NA	b	a
		'17	R	**0.06**								
		'18	R	**6.1 E-4**	***	a	b	ab	a	a	a	ab
7	Internode thickness	**'16**	R	**1.7 E-4**	***	b	ab	a	NA	NA	a	a
		'17	R	**4.1 E-7**	***	b	ab	a	a	ab	a	a
		'18	R	**9.1 E-3**	*	a	b	a	ab	ab	ab	ab
8	Internode length	**'16**	R	**0.02**	*	a	a	a	NA	NA	a	a
		'17	R	0.07								
		'18	R	0.35								
9	Leaf color L* (luminance)	**'16**	R	0.06								
		'17	R	0.27								
		'19	R	0.09								
10	Leaf color a* (green/red)	**'16**	R	**1.1 E-3**	**	b	b	ab	a	ab	b	ab
		'17	R	**2.1 E-3**	**	a	a	a	a	a	a	a
		'19	R	**0.02**	*	a	a	a	a	a	a	a
11	Leaf color b* (blue/yellow)	**'16**	R	**4.1 E-5**	***	c	abc	ab	a	abc	bc	ab
		'17	R	**6.8 E-5**	***	b	ab	ab	ac	ab	ab	c
		'19	R	**0.01**	*	ab	b	ab	a	ab	ab	ab
13	Leaf blade length	**'16**	R	**7.7 E-13**	***	d	b	ab	c	ac	b	ab
		'17	R	**8.9 E-9**	***	d	cd	abc	abc	bc	a	ab
		'18	R	0.08								
14	Leaf blade width	**'16**	R	**7.6 E-9**	***	d	ac	abc	ab	b	c	abc
		'17	R	**2.5 E-6**	***	b	ab	abc	a	a	a	a
		'18	R	0.40								
15	Leaf blade ratio length to width	**'16**	R	**0.04**	*	b	ab	ab	a	ab	ab	ab
		'17	R	**0.05**	*	ab	ab	a	ab	b	ab	ab
		'18	R	**0.03**	*	b	ab	ab	ab	ab	a	ab
19	Leaf drop	**'18**	R	**7.1 E-39**	***	c	b	a	a	a	a	a
		'19	R	**1.7 E-9**	***	d	bc	abc	a	ac	abc	bd
		'20	R	**2.7 E-17**	***	d	b	ab	a	c	ab	ab
20	Flower clusters	**'17**	R	**2.6 E-7**	***	a	b	a	a	a	a	a
		'18	R	**9.5 E-22**	***	ab	c	a	ab	b	a	a
		'19	R	**5.8 E-11**	***	a	b	a	a	b	a	a
		'20	R	**2.6 E-10**	***	a	b	a	a	b	a	a
Fruit-related traits measured per fruit (fruit measurements averaged per tree)												
22	Weight	**'18**	R	**1.2 E-17**	***	b	d	ab	ac	c	ab	ab
		'19	R	**4.3 E-21**	***	e	d	ab	a	c	be	a
		'20	R	**8.6 E-9**	***	ac	b	ab	a	c	ab	ac
23	Height	**'18**	R	**1.6 E-22**	***	b	d	ab	a	c	ab	ab
		'19	R	**8.0 E-17**	***	bd	d	ab	a	c	ab	a
		'20	R	**1.1 E-8**	***	a	b	ab	a	c	ab	ac
24	Caliber	**'18**	R	**3.9 E-8**	***	a	c	a	ab	b	a	a
		'19	R	**1.4 E-21**	***	b	d	ab	a	c	a	a
		'20	R	**8.9 E-9**	***	a	b	ab	a	c	a	ac
26	Percentage of green over color	**'18**	R	**6.1 E-31**	***	b	c	a	a	NA	d	b
		'19	R	**1.4 E-44**	***	b	c	a	a	NA	d	b
		'20	R	**2.3 E-28**	***	bc	c	a	a	NA	d	b

(continued)

Table 1. (continued)

Trait no.	Trait name	Year	Data-set	Genotype effect		CIS	IVG	GG	GGB	**GSR**	RG	GO
27	Percentage of yellow over color	**'18**	R	**2.0 E-52**	***	e	b	a	a	c	d	b
		'19	R	**2.2 E-62**	***	e	b	a	a	c	d	b
		'20	R	**1.3 E-50**	***	d	d	a	a	c	d	b
28	Percentage of red over color	**'18**	R	**1.4 E-48**	***	c	d	ab	a	b	e	c
		'19	R	**2.7 E-61**	***	f	d	ab	a	b	e	c
		'20	R	**6.2 E-58**	***	b	d	a	a	c	e	b
30	Relative area of over color	**'18**	R	**1.1 E-22**	***	b	c	a	a	a	a	b
		'19	R	**7.4 E-24**	***	c	c	a	a	a	a	b
		'20	R	**7.5 E-19**	***	c	c	a	a	a	a	b
31	Intensity of over color	**'18**	R	**1.4 E-44**	***	e	bd	a	a	c	d	b
		'19	R	**3.0 E-56**	***	c	be	a	a	d	e	bc
		'20	R	**5.5 E-51**	***	c	b	a	a	d	b	bc
33	Width of stripes	**'18**	R	**0.03**	*	ab	ab	a	ab	NA	b	ab
		'19	R	**4.1 E-6**	***	b	b	ab	ac	NA	c	b
		'20	R	NA	NA					NA		
34	Area of russet around stalk attachment	**'18**	R	**4.3 E-3**	**	ab	ab	a	ab	b	ab	ab
		'19	R	**2.6 E-6**	***	b	a	a	a	a	a	a
		'20	R	**8.6 E-6**	***	ab	c	ab	ab	ac	b	ab
37	Firmness	**'18**	R	**3.6 E-6**	***	a	b	ab	a	a	a	a
		'19	R	**0.02**	*	a	a	a	a	a	a	a
		'20	R	**1.6 E-4**	***	c	b	ab	abc	c	c	ac
38	Firmness (storage)	**'18**	R	**1.6 E-7**	***	bc	c	ab	ab	a	a	ab
		'19	R	**9.1 E-4**	***	ab	a	ab	ab	a	b	a
		'20	R	0.86		a	a	a	a	a	a	a
39	Sugar	**'18**	R	**1.9 E-12**	***	c	b	ab	a	ab	b	ab
		'19	R	**3.7 E-6**	***	b	a	a	a	a	a	a
		'20	R	**1.6 E-4**	***	b	ab	ab	a	a	ab	a
40	Sugar (storage)	**'18**	R	**7.4 E-10**	***	c	ac	abc	d	bd	abd	abd
		'19	R	**0.03**	*	b	ab	ab	ab	ab	a	ab
		'20	R	**0.02**	*	ab	ab	a	b	ab	ab	ab
Fruit traits analyzed per repetition resp. planting row (max $n = 4$)												
42	Acidity	**'18**	F	**0.02**	*	b	ab	a	ab	ab	ab	ab
		'19	F	0.45								
		'20	R	0.28								
43	Acidity (storage)	**'18**	F	0.22								
		'19	F	0.24								
		'20	R	0.33								
44	Seed germination	**'18**	F	0.92								
		'19	F	0.37								
No. of traits with genotype-specific differences in at least two years of observation						3	5	0	0	4	3	1
No. of traits with genotype-specific differences in all years of observation						0	1	0	0	2	2	0

Each trait was analyzed by year in the dataset without off-type trees (reduced dataset, R), except for traits no. 42–44 (full dataset, F). The *P*-values of the ANOVA output are presented as numbers and as significance codes as follows: 0 to ≤ 0.001 '***', 0.001 to ≤ 0.01 '**', 0.01 to ≤ 0.05 '*', 0.05 to ≤ 0.1 '.', 0.1–1 ' '. If a genotype effect was significant ($P < 0.05$), Tukey's tests were used to assign significance groups to the genotypes. Genotypes that are not significantly different to CIS are marked yellow, whereas genotypes that are different to all other 'Gala' genotypes are marked blue. Traits no. 29 and 32 are not indicated in the table as they were not statistically analyzed. The last two rows of the table sum up the number of traits in which a genotype showed statistically significant differences compared with all other assessed genotypes.

CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original.

Further information on the genotypes in Figure S1 and Table S6.

'18 and five in '19. The highest DAF count in a single comparison between CIS and a non-GM 'Gala' genotype was with GGB in '18 (238 DAFs) and GSR in '19 (15 DAFs). The number of DAFs identified among non-GM Gala genotypes in '18 and '19 ranged from one to 69 in '18, and from zero to 12 in '19. In '18 and '19, 41 and zero CIS-specific DAFs were found, respectively (Table S4). Among the 'Gala' genotypes, the number of genotype-specific DAFs ranged from zero to 10 in both years (GSR in '18 and GG in '19).

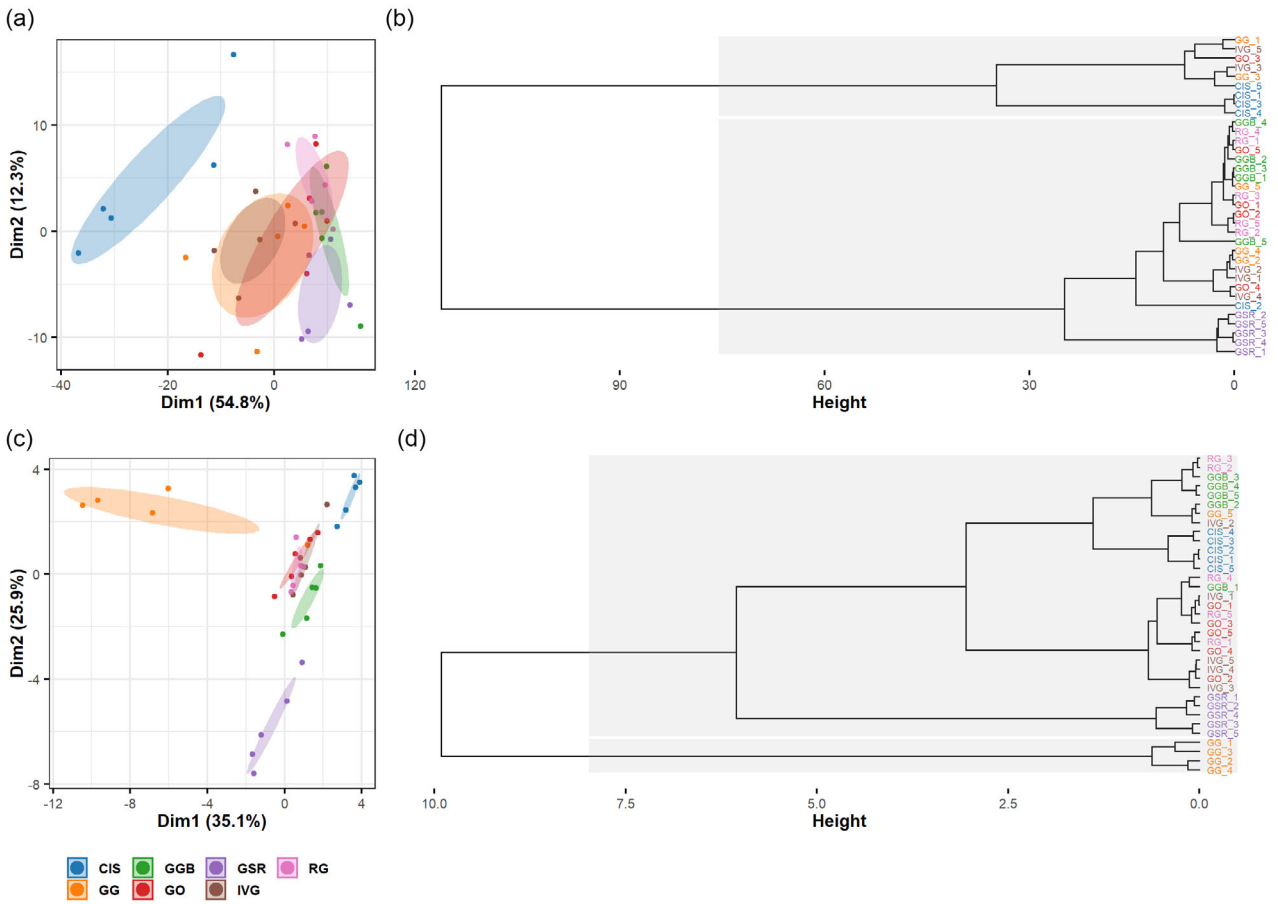

Figure 2. Principal component (PCA) and hierarchical clustering analysis (HCA) of the fruit peel metabolomes of seven 'Gala' genotypes calculated using the differentially abundant metabolite features (DAFs) identified in 2018 and 2019.
For the PCAs, 302 DAFs (a) and 31 DAFs (c) were used in 2018 and 2019, respectively. Confidence ellipses (confidence level 95%) were used to visualize the variability of the mean point per 'Gala' genotype. Hierarchical clustering was performed on the PCA of 2018 (b) and 2019 (d) using Euclidean distances and the Ward's criterion as the clustering method. The number of clusters chosen was based on k-means and the silhouette method. CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original.

Apples of 'Ladina' (LAD) and 'Gala Galaxy' (GGG) from two other growing locations were used for additional comparisons in '19; 122–189 DAFs were identified between LAD and the other genotypes, and from 67 to 82 DAFs between GGG and the other 'Gala' genotypes were found (Table 2). A total of 21 GGG-specific DAFs and 67 LAD-specific DAFs were identified (Table S4).

A search of metabolite databases (based on mass/charge values and fragmentation) provided putative identifications for five of the 41 CIS-specific DAFs (Table S5). Three metabolites features (15.88_502.3296 n, 16.91_517.3524m/z, 17.20_516.3452n) were more abundant in CIS than in the control genotypes, and the remaining two (6.90_516.1349m/z, 10.50_507.2801m/z) were less abundant in CIS than in the control genotypes. Up to nine different putative identifications for a single DAF (i.e. 15.88_502.3296n) were found.

None of the investigated metabolite features in fruit flesh extracts showed a unique abundance pattern in any of the investigated 'Gala' genotypes

The LC–MS/MS measurements of fruit flesh detected 335 and 1155 metabolite features for '18 and '19, respectively. Among them, no metabolite feature was found to be only present or absent in all samples of a single genotype while being absent or present in all other genotypes, respectively (Table S3). After filtering, the final datasets contained 333 ('18) and 1146 ('19) metabolite features.

Among the 'Gala' genotypes grown at the 'Protected Site', in each observation year 28 (8.4% in '18 and 2.4% in '19) of the flesh metabolite features were differentially abundant. The PCAs generated using DAF abundances separated the genotypes into two or more clusters (Figure 3a,c) and were used for HCA, which separated the

Table 2 Number of differentially abundant features among the investigated 'Gala' genotypes in the metabolomes of fruit peel extracts in 2018 (top number) and 2019 (middle number), and including GGG and LAD (bottom number)

	CIS	IVG	GG	GGB	GSR	RG	GO	GGG	LAD
CIS	–	84	78	238	212	223	143	–	–
		0	12	2	15	1	0	NI	NI
		4	12	1	11	10	1	74	135
	IVG	–	20	26	69	14	5	–	–
			11	0	5	2	0	NI	NI
			11	2	6	2	0	76	158
		GG	–	11	49	11	18	–	–
				11	12	10	12	NI	NI
				11	11	16	11	76	153
			GGB	–	15	1	11	–	–
					2	0	0	NI	NI
					1	4	2	74	151
				GSR	–	37	45	–	–
						6	6	NI	NI
						10	5	67	155
					RG	–	9	–	–
							0	NI	NI
							0	82	189
						GO	–	–	–
								NI	NI
								77	164
							GGG	–	–
									NI
									122
								LAD	–

NI, not included.

CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original; GGG, 'Gala Galaxy'; LAD, 'Ladina'.

samples into two and six clusters in '18 and '19, respectively (Figure 3b,d). None of the clusters consisted solely of samples of a single genotype in '18, while genotype-specific clusters for GSR and CIS were found in '19. Within the two clusters in '18, a genotype-specific sub-cluster was only found for CIS (Figure 3b,d).

Comparisons between CIS and IVG identified three DAFs in '18 and no DAFs in '19 (Table 3). The average number of DAFs between the 'Gala' genotypes and CIS was four in '18 and three in '19. The highest number of DAFs in a single comparison between CIS and a non-GM 'Gala' genotype was with GSR in '18 (eight DAFs) and GO in '19 (six DAFs). The number of DAFs identified among non-GM 'Gala' genotypes in '18 and '19 ranged from zero to 15 and from zero to nine, respectively. For CIS and all other 'Gala' genotypes, no unique DAF was found in either year (Table S4).

Analysis including GGG and LAD identified 43–80 DAFs between LAD and the other genotypes, and 22–45 DAFs between GGG and the other 'Gala' genotypes (Table 3). A single GGG-specific DAF and 20 LAD-specific DAFs were identified (Table S4).

DISCUSSION

FB_MR5 conferred floral fire blight resistance

This study shows that the cisgenic transfer of *FB_MR5* conferred fire blight resistance to the susceptible cultivar 'Gala Galaxy' also when trees were inoculated with *E. amylovora* through the flowers. Peil et al. (2019) reported mean resistance scores of field-grown, flower-inoculated Mr5 trees ranging from 0.08 to 0.28. Because the resistance scores in the current study were comparable to those of Peil et al. (2019; Table S1), we infer that the shoot and floral resistance QTL identified by Peil et al. (2019) using two segregating F1 populations of Mr5 can both be ascribed to the same gene, *FB_MR5*.

The high level of resistance shown by CIS should be sufficient to improve the management of fire blight in an orchard. However, because a single nucleotide change in the effector of *E. amylovora* led to the breakdown of *FB_MR5* resistance (Emeriewen et al., 2019; Vogt et al., 2013), the deployment of this particular resistance gene by classical breeding or GM approaches will require its pyramiding with additional resistance genes with different resistance mechanisms (McDonald & Linde, 2002) to preserve its durability.

Propagation methods explain the significant variation among 'Gala' genotypes

Despite being derived from the same cultivar, i.e. 'Gala Galaxy', trees of the genotypes GG and GGB (produced in two different nurseries) had significant differences in field phenotypic traits. Differences between GG and GGB were mainly found in the first year of observation ('16) in the tree-related traits height increase (no. 1), emergence of new branches in 1- and 2-year-old stems (no. 3 and 4), and leaf blade length (no. 13; Table 1). The effect of cultivation before field planting in the first observation year was stronger than the genotypic effect, as shown by the comparison between GSR and GGB, which originated from the same nursery. GSR showed similar trait expression levels as GGB in all traits mentioned above, except for the emergence of new branches on 2-year-old stems (no. 4). The effect of prior cultivation vanished in the next years, and GG and GGB showed a comparable height increase and leaf blade length in the next year ('17; Table 1).

GG and GGB also differed in the fruit-related trait sugar content after storage (no. 40) in two of three years. According to PCAs and HCA, these genotypes also differed in the abundance of metabolite features in the fruit peel, but less so in the flesh. These results show that the design, conductance and data depth in the current study were

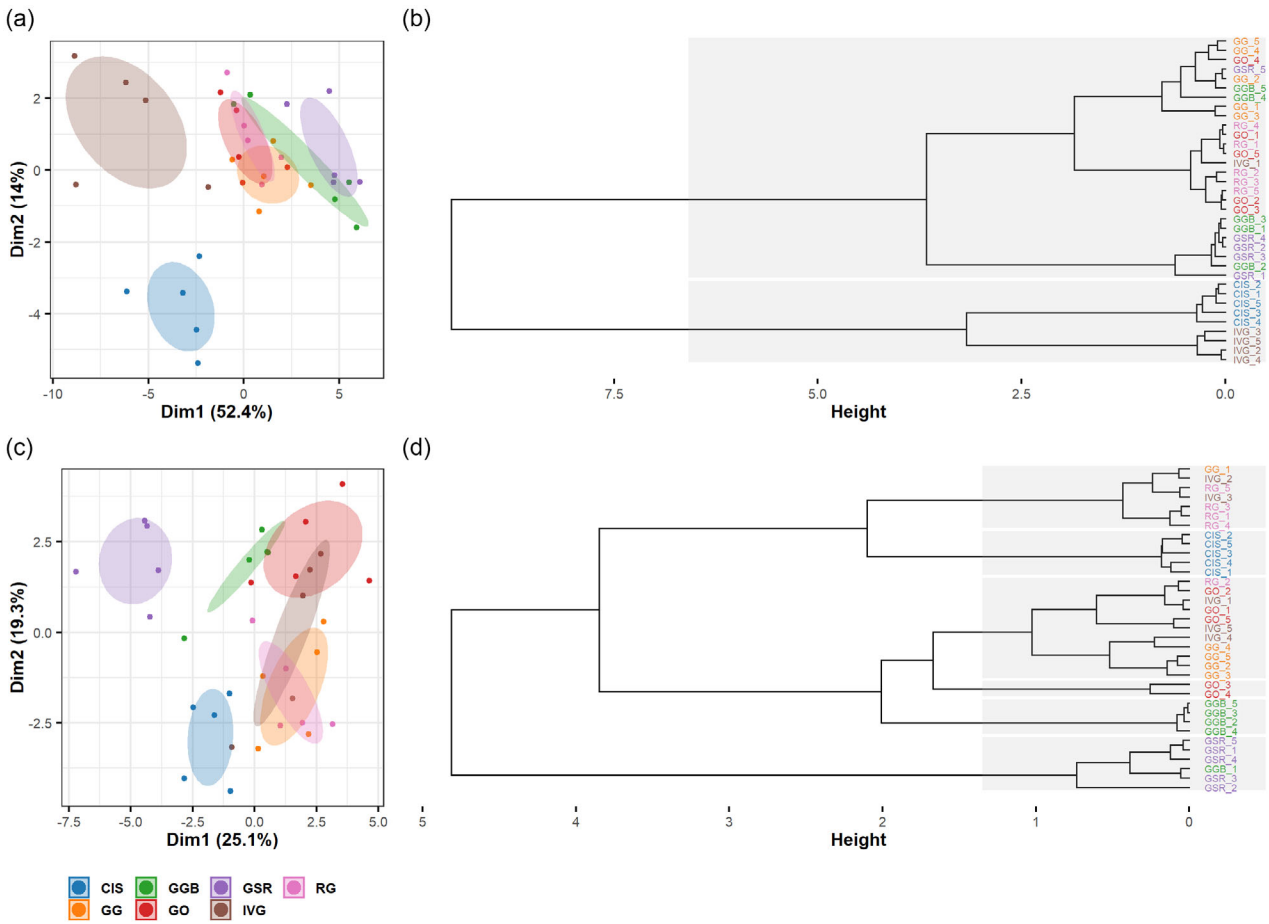

Figure 3. Principal component (PCA) and hierarchical clustering analysis (HCA) of the fruit flesh metabolomes of seven 'Gala' genotypes calculated using the differentially abundant metabolite features (DAFs) identified in 2018 and 2019. For the PCAs, 28 DAFs in both years were used (2018: a; 2019: c). Confidence ellipses (confidence level 95%) were used to visualize the variability of the mean point per 'Gala' genotype. Hierarchical clustering was performed on the PCA of 2018 (b) and 2019 (d) using Euclidean distances and the Ward's criterion as clustering method. The number of clusters chosen was based on k-means and the silhouette method. CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original.

sufficient to detect subtle differences like those caused by tree history before planting.

One major difference between IVG and GG/GGB was the reduction in the percentage of red over color (no. 28; Figure 4; Table 1). IVG was established *in vitro* following cell dedifferentiation and regeneration via organogenesis, and was maintained *in vitro* since its establishment in 1997 (Bidabadi & Jain, 2020; pers. communication Chevreau). A high frequency of somaclonal variation through *in vitro* culture has also been described for apple (Salvi et al., 2015). Somaclonal variation could have affected the expression of the flavonoid pathway (e.g. the genes *MdMyb1*/*MdMyb10*), which influences skin color in apple sports (Du et al., 2020; El-Sharkawy et al., 2015; Qian et al., 2014; Zhang et al., 2019). Most of the deeper red-skinned 'Gala' mutants were described to be L2-derived periclinal chimera (Dickinson & White, 1986), which could also be true for 'Gala Galaxy'. A possible explanation could be that the periclinal chimera was disrupted during the *in vitro* establishment. IVG could be a homohistont genotype that did not originate from L2-layer cells, and thus did not inherit the characteristic 'Gala Galaxy' skin coloration. Furthermore, 'Gala Galaxy' back mutations have been reported before (Sansavini et al., 1999), and were observed as off-type trees in our experiment. Thus, sport fruit properties may be disrupted by *in vitro* cultivation, although other groups did not report such effects when an *in vitro*-cultured 'Gala Galaxy' was used for transformation (Borejsza-Wysocka et al., 2010; Ko et al., 2000).

No consistent differences between CIS and 'Gala' genotypes across years

A comparison of CIS and IVG revealed differences that resulted from the generation of CIS or from the addition of

Table 3 Number of differentially abundant features among the investigated 'Gala' genotypes in the metabolomes of fruit flesh extracts in 2018 (top number) and 2019 (middle number), and including GGG and LAD (bottom number)

	CIS	IVG	GG	GGB	GSR	RG	GO	GGG	LAD
CIS	–	3	4	2	8	4	4	–	–
		0	2	3	4	0	6	NI	NI
		0	2	1	4	0	7	22	56
	IVG	–	10	6	15	1	2	–	–
			0	2	7	0	1	NI	NI
			0	1	5	1	2	22	67
		GG	–	0	0	0	0	–	
				3	7	0	1	NI	NI
				3	5	0	5	22	55
			GGB	–	0	0	0	–	–
					6	1	1	NI	NI
					4	2	2	22	61
				GSR	–	1	0	–	–
						9	8	NI	NI
						10	9	25	53
					RG	–	0	–	–
							1	NI	NI
							2	45	78
						GO	–	–	–
								NI	NI
								25	80
							GGG	–	–
									NI
									43
								LAD	–

NI, not included.

CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original; GGG, 'Gala Galaxy'; LAD, 'Ladina'.

FB_MR5 to this specific line. As CIS derived from IVG plant material should have inherited the same genotypic change (s) as IVG. The fact that the fruits of IVG and CIS were "paler red" (Figure 4; Table 1) than the fruits of 'Gala Galaxy' (GG and GGB) supports this hypothesis. In addition, two further steps of regeneration and a heat-shock treatment required for the generation of CIS could have caused other differences between IVG and CIS. Further comparisons of CIS and IVG with other 'Gala'-related genotypes are needed to determine whether such deviations are outside the observed natural variability in the same background of CIS. For this kind of evaluation, sports are ideal due to their common genetic origin, their limited number of trait modifications, and their close genetic relationship to each other and to the original cultivar. For example, yellow-colored 'Gala' mutants have been found and patented ['Delicia' (Aebischer & Aebischer, 2011), 'Aurora Golden Gala' (Hampson et al., 2005)]. Finally, we determined whether the CIS-specific differences were stable over the years of our observations. Only CIS-specific differences that were consistently found over all observation years were considered as potentially caused/derived from the genetic modification. Although no trait was found to be specific to CIS over all three observation years (Table 1), the following three paragraphs discuss three traits that were specific to CIS in two of the three years.

Leaf drop started about one week earlier for CIS than for the other 'Gala' genotypes in '18 and '20. Changing climate factors affect the signaling and/or synthesis of hormones that regulate the expression of senescence-related genes, which then affect the timing of leaf senescence (Santner et al., 2009; Zhang et al., 2020). Plant mutants with altered senescence phenotypes occur mostly as a result of altered hormone signaling (Lim et al., 2007). The basal, pathogen-independent expression of *FB_MR5* may activate a defense mechanism involving salicylic acid synthesis (Milčevičová et al., 2010) and the phenylpropanoid pathway (Jensen et al., 2012), which is involved in the synthesis of lignins and flavonoids.

The area of yellow over color was different in CIS relative to all other 'Gala' genotypes in '18 and '19, but was comparable in CIS to IVG and RG in 2020. RG and GO also showed a unique expression of this trait in at least one year of observation, underlining the high divergence in fruit coloration among 'Gala' genotypes. Yet, instead of CIS and IVG being similar to GG and GGB, the area of green, yellow and red over color indicated that both CIS and IVG are more similar to GO (Figure 4), supporting the hypothesis of a back mutation of IVG to GO. As described above, a plausible explanation for the additional change from IVG to CIS could be new somaclonal mutations that arose during the two regeneration steps that led to CIS (Kost et al., 2015).

The higher sugar content in CIS than in the other genotypes in the first year of harvest may have resulted from the harvesting of overripe fruits. Because change in coloration is an indicator of fruit ripening (Liu et al., 2013) and because CIS and IVG showed only a slight change in coloration, their harvest time may have been incorrectly determined. While we waited for the coloration to change in the first year of fruit observation, the fruits ripened and their sugar content increased. During the second and third years, we increased pre-harvest measurements of sugar content and starch degradation to ensure a standardized harvest time among the genotypes. Although higher levels of sugar were also observed in the fruits of the second harvest (2019), they were not significantly different in the third (2020). Sugar accumulation depends on environmental factors (Li et al., 2012) and the position of the fruit within the tree (Sestras et al., 2009), and could therefore vary between years and trees, and even within trees.

None of the three CIS traits discussed above has a known connection to biosafety concerns. Nevertheless, the

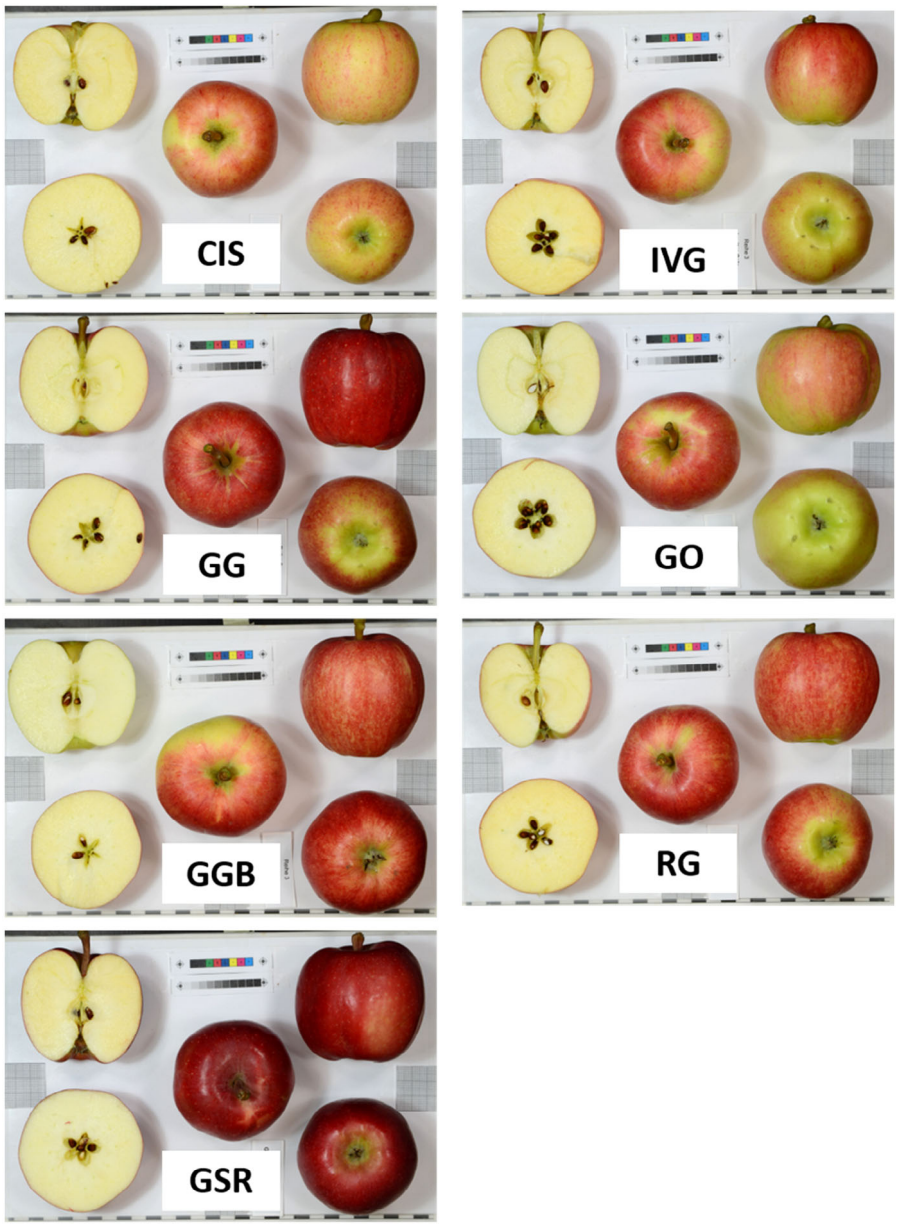

Figure 4. Representative photographs of fruits of the investigated 'Gala' genotypes harvested in August 2018.
Standardized picture panels per genotype were taken according to the guidelines described in Szalatnay (2006). CIS, C44.4.146; IVG, *in vitro* 'Gala Galaxy' (wild-type); GG, 'Gala Galaxy' produced in-house using scions from a Swiss nursery; GGB, 'Gala Galaxy' purchased in an Italian nursery; GSR, 'Gala Schniga® SchniCo red', purchased in the same Italian nursery as GGB; RG, 'Royal Gala'; GO, 'Gala' original.

reduced area of red over color could be negatively perceived by consumers, even though it was introduced by the tissue culture and not by the insertion of the cisgene *FB_MR5*.

No metabolites in CIS were consistently differentially abundant across the years

High variability of plant metabolomes between years as well as locations has been reported previously (Ma et al., 2019) as well as in the current study. In fact, differences between the years were detected, i.e. the metabolome of '18 was more variable than the one of '19 in fruit peel and flesh (Tables 2 and 3).

The number of both peel and flesh DAFs was about sevenfold higher between two 'Gala Galaxy' grown at different locations (GG versus GGG) than between two 'Gala Galaxy' from the 'Protected Site' (GG versus GGB; Tables 2 and 3). In all comparisons to the 'Gala' genotypes grown at the 'Protected Site', LAD, a 'Gala'-unrelated apple cultivar, showed an approximate twofold higher number of both

peel and flesh DAFs than GGG. In both cases, differences were larger than those observed among sports of a cultivar (including CIS) grown at the 'Protected Site'.

The analysis of genotype-specific differences identified 41 CIS-specific metabolite features in peel extracts in '18 (Table S4). A similar or higher (twofold) number of genotype-specific metabolites was found in '19 for GGG and LAD, respectively (Table S4). Of the 41 CIS-specific metabolite features, five had several putative identifications (Table S5). The three metabolite features with higher abundance in CIS could belong to the group of pentacyclic triterpenoids, plant metabolites that have been found to increase along with the bagging of apple fruits in the browning part of the peel (Wang et al., 2021). The two CIS-specific metabolite features with lower abundance could belong to the fatty acyl glycosides (10.50_507.2801m/z) and glucuronylglucosides (6.90_516.1349m/z).

No metabolite feature specific for CIS was found in peel extracts of CIS in '19 or in flesh extracts of CIS in '18 and '19. Consequently, no CIS-specific metabolite features were consistently found over the two observation years, indicating that the generation of CIS had no unintended effects on flesh and peel metabolites.

Conclusions

The amendment of 'Gala Galaxy' with *FB_MR5* produced a cisgenic line resistant to *E. amylovora* infections resulting from flower inoculation, which is the main entry mode for this pathogen into apple trees (Vanneste, 1995). This showed that the cisgenic approach allows rapid deployment of disease resistance in high-quality commercial cultivars.

Conducting the field trial for 5 years allowed the trees to adapt to field conditions (particularly important for CIS and IVG coming from *in vitro* culture), to mitigate the "nursery" effect observed for GGB and GSR and, when differences were found among the genotypes, to verify if these were consistently found over all over the observation years. The inclusion of sports as control genotypes enabled to assess the differences in relation to natural variation found in spontaneous sports. The generation process of CIS did not cause consistent significant differences over the years when studied in the context of its untransformed wild-type and the natural variation in 'Gala' and selected 'Gala' sports. The generation of cisgenic lines in apple, however, requires tissue culture, which could affect sport-specific properties as observed here. This must be considered because it can affect consumer-relevant traits, as was the case with the over color of CIS. This limitation could be overcome by the *in vitro* initiation of multiple lines of sports and their assessment in the field followed by the generation of multiple cisgenic lines in order to identify those that maintain the original characteristics. Here, we found that *in vitro* manipulation of the wild-type had more impact than the generation of the cisgenic line CIS. Even though only a single cisgenic line was investigated and *FB_MR5* resistance is of limited durability, our field assessment of CIS supports the use of cisgenesis as a complementary breeding tool for the development of new cultivars. The knowledge gained from this prototype will facilitate the development of new cisgenic lines using novel breeding techniques with pyramids of resistance genes for durable resistance.

EXPERIMENTAL PROCEDURES

Plant material for the field trial

The cisgenic line C44.4.146 (CIS; Kost et al., 2015), its background, i.e. the 'Gala' sport 'Gala Galaxy', two other 'Gala' sports, i.e. 'Royal Gala' (RG) and 'Gala Schniga® SchniCo red' (GSR), and the original 'Gala' (GO) cultivar were used for the field trial (Figure S1; Table S6). 'Gala Galaxy' was obtained from three sources: *in vitro*-cultured 'Gala Galaxy' (IVG), and 'Gala Galaxy' from a commercial nursery in Switzerland (GG) and Italy (GGB). Although derived from the same genotype 'Gala Galaxy', IVG, GG and GGB were considered independent genotypes for the experiment and for statistical analysis.

Scions of GG, RG and GO were derived from certified Swiss trees, while those of IVG and CIS were derived from *in vitro*-cultured, micro-grafted plantlets grown under containment in a greenhouse for 1 year. The budwood was grafted onto virus-free (vf) M9 T337 in 2015, and the trees were then grown in pots for one season in a greenhouse. One-year-old trees of GGB and GSR also grafted on M9 T337 vf were purchased in 2016.

Field trial setup

To investigate agronomic traits, 20 1-year-old trees per 'Gala' genotype were planted in spring 2016 in the 'Protected Site' at Agroscope Reckenholz (Zurich, Switzerland, www.protectedsite.ch; Brunner et al., 2021) as described in Schlathölter et al. (2022). The trees were distributed in four rows (3.5 m apart) in a randomized complete block design with five trees per genotype in each row (block) and 1.2 m between trees. Rows were oriented in a west–east direction. The whole experiment was bordered by three barrier rows. Two of these rows were in front (south) of and the third was behind (north) the four rows used for the experiment. The most southern and the most northern rows were used to grow potted (35 L) apple trees for the greenhouse assessment of floral fire blight resistance (see next section). The second-most southern row was planted with GGB trees, which were not further investigated. Due to vole damage in 2016, one CIS tree was replaced with a potted tree of the same age grown in the same plot. This tree was excluded from statistical analyses of the tree-related traits "height increase" and "flower clusters", because of a large height difference at planting compared with the other CIS trees.

According to governmental requirements (non-exhaustive list) for conducting the field trial, the whole orchard had to be surrounded with a screenhouse (hail net roof with 3-mm × 7-mm mesh and walls with insect-proof 0.9-mm × 1-mm mesh), and the flowers of the cisgenic lines had to be removed before or at the hollow ball stage (BBCH59; Meier et al., 1994). To produce and analyze fruits, about 15 flowers per tree of independent flower clusters of all genotypes were manually emasculated and pollinated with 'Golden Delicious' pollen.

Trees were pruned each winter and once in summer (2017). Anti-frost candles were lighted when needed to protect the emasculated flowers and/or young fruits from frost damage.

Assessment and statistical analysis of floral fire blight resistance

Three dormant, potted 3- (2018) or 4-year-old (2019) trees of GG and CIS were transferred from the field to a biosafety level 2 greenhouse (Agroscope, Wädenswil, Switzerland). Plants were grown under greenhouse conditions (17°C night, 24°C day, with additional light <30 klux between 07:00 hours and 18:00 hours) to induce flowering. After approximately 3 weeks, when most of the emerged flower clusters were in the hollow ball stage, they were tagged and manually pollinated with 'Golden Delicious' pollen. One day after pollination, each flower cluster was spray-inoculated with a suspension of *E. amylovora* EA222_JKI containing 1×10^7 cfu ml^{-1} in phosphate-buffered saline (PBS; pH 7.4). Three–five floral clusters per tree were mock-inoculated with PBS. Floral clusters were assessed for fire blight symptoms 21 days post-inoculation (dpi) using the harmonized scale of Peil et al. (2019). Only flower clusters that had at least one flower remaining attached at the end of the experiment were considered in the analysis. Flower clusters emerging from aphid-infested branches were excluded from analyses. Statistical analysis was done in R 4.0.5 (R Core Team, 2021) per genotype-year combination. Significant differences between genotypes were verified using the Kruskal–Wallis H-test ($P < 0.001$, 'stats' package R Core Team, 2021) followed by Games–Howell *post hoc* tests ($P < 0.005$, 'userfriendlyscience' package; Peters, 2017).

Assessment of tree- and fruit-related phenotypes

Branching was assessed in only 1 year, but 20 other phenotypic tree-related traits were assessed in at least 2 years between 2016 and 2020 (Table S2).

Mature apple fruits were harvested and phenotyped in 2018, 2019 and 2020. Twenty fruit-related traits were assessed on single fruits and averaged per tree; three other traits (acidity, acidity after storage, and seed germination) were measured at the block level (Tables S2 and S7). Four uniform and representative fruits per tree (80 fruits per genotype) were manually phenotyped (no. 25, 29–36, 41; Table S2). Sugar, acidity and firmness were measured on two uniform and representative fruits per tree (40 fruits per genotype) using a Pimprenelle automated apple quality control device (Setop, Cavaillon, France). Fruit acidity was determined by measuring 10 apples per block. Fruits were stored at 1°C and 95% relative humidity (RH) before assessment. Fruits from all trees were harvested on 20 August 2018, on 3 and 6 September 2019, and on 21 and 25 August 2020. A batch of the harvested fruits, if possible two fruits per tree, was stored under controlled atmosphere (CA) conditions at 1°C, 1% CO_2, 1% O_2 and 92% RH for 22 weeks in 2018, 24 weeks in 2019 and 23 weeks in 2020 to repeat sugar, acidity and firmness measurements after storage.

Statistical analysis of field data

All statistical analyses were conducted in R 4.0.5 (R Core Team, 2021). First, all genotypes were checked separately for the presence of so-called off-type trees (trees showing back mutations; Sansavini et al., 1999) using the percentages of red and green over color of fruits as criteria. Trees showing significantly lower red and higher green fruit coloration than the majority of trees of the same genotype [as indicated by Tukey's Honest Significant Difference (HSD) test, adjusted *P*-values ≤ 0.05] were considered off-type and were removed from the data set. Values of traits measured on several organs of the same tree (e.g. leaf length and all fruit-related traits) were averaged per tree for analysis. Trees with < 4 fruits were excluded from statistical analyses (Table S7).

The analysis to identify off-type trees as well as all single trait analyses of quantitative traits were done separately for each trait using a linear model (lm) or a generalized linear model (glm) depending on the trait (Table S2). Normality of residuals was visually assessed on quantile-quantile plots ('ggpubr' package; Kassambara, 2020) and tested via Shapiro Wilk's test (Shapiro & Wilk, 1965) using the 'stats' package (R Core Team, 2021). Homogeneity of variances was tested via Levene's test (Levene, 1960) using the 'car' package (Fox & Weisberg, 2019). If the residuals followed a normal distribution with an equal variance, linear regression was used. If the assumptions of linear regressions were not met, a generalized linear regression was used. The analysis to identify off-types was performed on data from all fruits of a single genotype using a model with year and "ID" (the unique tree identifier) as fixed effects. If the year effect was significant, the datasets were analyzed separately per year. Single traits (average values per tree) were subsequently analyzed using a model with genotype and row (if applicable) as fixed effects. The models were fitted using the 'lm' or 'glm' function of the 'stats' package. If over-dispersion was detected ('dispersiontest' function of the 'AER' package; Kleiber & Zeileis, 2008), the standard errors were corrected using a quasi-glm model (Cameron & Trivedi, 1990, 2013). The lm and glm approaches were both followed by an analysis of variance (ANOVA, 'stats' package). If the effect of genotype or tree was significant ($P < 0.05$), genotypes were compared using Tukey's HSD test ('glht', 'multcomp' package; Hothorn et al., 2008). The *P*-values of the Tukey's comparisons were converted to letters, where letters identified significance groups ('multcompView' package; Graves et al., 2019). Boxplots were generated using the 'ggplot2' package (Wickham, 2016).

Tree habit, ground color and pattern of over color traits (trait no. 5, 29, and 32) were visually assessed. According to UPOV guidelines (TGP/9/1, UPOV, 2008), these traits were directly compared using the relative distribution of the individual rating levels without statistical methods to interpret the results.

For all assessed traits, those with significant differences between CIS and IVG were first selected to identify traits that were potentially affected by the genetic modification. If differences were found, it was determined whether the values of CIS were also outside of the variability of the remaining 'Gala' genotypes and whether these differences were stable over the years of observation. Only traits matching all three criteria were considered to be specific to CIS and were considered to possibly result from the genetic modification leading to CIS.

Sampling and metabolite extraction of fruit tissue

Fruits for metabolomic analysis were stored at 1°C and 95% RH for 72 days (2018) or 90 days (2019), and were then transferred to room temperature 24 h before extraction. In 2019, apples of 'Gala Galaxy' (GGG) produced in Güttingen (Canton Thurgau, Switzerland) and of 'Ladina' (LAD) produced in Wädenswil (Canton Zurich, Switzerland) were investigated in addition to the apples produced at the 'Protected Site'. LAD, 'Topaz' × 'Fuji' (Leumann et al., 2013), is unrelated to 'Gala'. These apples were stored under similar conditions (1°C, 96–98% RH) for 87 (GGG) and 80 (LAD) days before extraction.

Before extraction, fruit surfaces were washed with distilled water (Petkovska et al., 2017). For sampling, a cylinder from the

equatorial, sun-exposed side of the fruit to the sun-averted side of the fruit was extracted by punching a cork drill (d: 8 mm) through the whole fruit. The peel discs (exocarp, approx. 20 mg) were separated from the flesh tissue (mesocarp) with a razor blade and placed in peel extraction solution [6.5 ml 1% (v/v) formic acid/methanol in 15-ml Falcon tubes]. Flesh tissue was collected from the outer 10 mm of tissue underneath the peel by cutting a 2-mm-thick disc from the sample (approx. 35 mg) and placing it in flesh extraction solution (11.0 ml 9:1 methanol–water in 50-ml Falcon tubes). Samples were incubated on an orbital shaker at 4°C in the dark for 24 h. Extracts were centrifuged for 20 min at 17 530 *g* and 4°C.

LC–MS/MS analysis

For investigation of fruit flesh metabolites, 20 μl of methanol extract was dried under an N_2 stream, reconstituted in 40 μl water, and diluted with 160 μl injection buffer (90% acetonitrile, 8.8% methanol, 50 mM NH_4-acetate). Flesh metabolites were separated with a BEH Amide HILIC capillary column (150 μm × 130 mm, 1.7-μm particle size; Waters, Milford, MA, USA) using the solvent system of buffer A (0.5 mM NH_4-acetate in water) and buffer B (0.5 mM NH_4-acetate in 95% acetonitrile). A nanoAcquity UPLC (Waters, Milford, MA, USA) was coupled to a Synapt G2Si mass spectrometer (Waters). Data were recorded with negative polarization in MS^E mode. For fruit peel metabolites like anthocyanins, flavonoids and lipids, methanol extracts were diluted with injection buffer (0.5% formic acid in water) to a final concentration of 5% water and were separated with an HSS T3 C18 capillary column (150 μm × 30 mm, 1.8-μm particle size; Waters) using the solvent system of buffer A (0.5% formic acid in water) and buffer B (0.5% formic acid in acetonitrile). A nanoAcquity UPLC (Waters) was coupled to a QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Data were recorded with positive polarization and DDA mode.

Data processing and statistical analysis of untargeted metabolomics data

Data were processed with Progenesis QI software (Waters). Polar metabolites were searched against the KEGG database (Kanehisa & Goto, 2000) with a precursor mass tolerance of 20 ppm and fragment mass tolerance of 50 ppm with possible adducts (M-H, M-H_2O-H). Apolar metabolites were searched against the LMSD database (Sud et al., 2007) with a precursor mass tolerance of 2 ppm and fragment mass tolerance of 20 ppm with possible adducts (M + H, M + Na, M + H-H_2O). Output files were processed further by in-house R scripts. Mass errors were corrected based on known reference compounds. Technical replicates of pooled samples were used to filter out metabolites with coefficients of variation (CV) > 25%. Only metabolite features with a peak intensity of 10 k (peel) or 100 k (flesh) in at least one analyzed sample were considered. The analyses were done separately per tissue (peel, flesh) and year. Mass errors were corrected based on known reference compounds, and the most probable identification was assigned to each observed mass. For identification of specific metabolite features, a search in Progenesis QI using the databases KEGG, ChEBI, LipidMAPS, NIST, HMDB, Pubmed and Wikipedia was performed. The parameters for the search were defined based on reference compounds analyzed as QC precursor tolerance: 2 ppm (< 1 ppm in reference compounds), fragment tolerance 20 ppm and isotope tolerance 70% (> 90% in reference compounds).

To test for DAFs among all genotypes, only metabolite features present in more than half of the samples per genotype were selected. Missing values (zeros) were imputed per 'Gala' genotype using the 'missForest' package (Stekhoven & Bühlmann, 2012). The intensities were log-transformed and were subjected to a one-way ANCOVA with genotype as fixed effect and injection order as covariate to control for the time the samples spent in the autosampler. *P*-values were corrected for multiple testing by Bonferroni–Holm. Tukey's HSD *post hoc* tests were performed to identify the differences between genotypes. Metabolite features were considered to show significantly different abundances in one-by-one comparisons if the corresponding Tukey's HSD *post hoc* tests resulted in an adjusted *P*-value < 0.05 and log2(fold change) < ¦1¦. Metabolite features showing significantly different abundances for a single genotype compared with all other genotypes were considered unique, i.e. genotype-specific DAFs. In addition, it was checked whether metabolites existed that were only present (in at least four of five samples) in a single genotype and absent (in all or four of five samples) in the remaining genotypes or vice versa (absent in only a single genotype).

The package 'FactoMineR' (Lê et al., 2008) was used to perform PCA and HCA on the DAFs, and 'factoextra' (Kassambara & Mundt, 2020) was used to visualize PCA and HCA results. The data were centered around zero using the z-score 'scale' function before performing the PCA. HCA was calculated by Euclidian distances and the Ward.D2 clustering method. The number of clusters was based on the silhouette method using the 'fviz_nbclust' function. Boxplots were generated using the 'ggplot2' package (Wickham, 2016).

ACCESSION NUMBERS

None.

AUTHOR CONTRIBUTIONS

IS: data curation, formal analysis, investigation, methodology, software, visualization, writing – original draft, writing – review & editing; GALB: conceptualization, funding acquisition, methodology, software, supervision, validation, writing – review & editing; SS: data curation, formal analysis, investigation, methodology, software, validation, writing – review & editing; BS: supervision, writing – review & editing; AP: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, writing – review & editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Lineage of the 'Gala'-derived apple genotypes and cultivars used in the field trial. The original 'Gala' cultivar GO (or 'Kidd's D-8') was obtained from a cross of 'Kidd's Orange' × 'Golden Delicious' and was patented in 1974 (McKenzie, 1974). The 'Gala' sport 'Royal Gala' (RG) (or 'Tenroy') was found and patented in 1977 (Ten Hove, 1977). 'Gala Galaxy' (GG) was patented in 1989 as an RG sport (Kiddle, 1989). The *in vitro* micro-propagated 'Gala Galaxy' line IVG was used to generate the cisgenic line C44.4.146 (CIS; Kost et al., 2015). 'Gala Schniga® SchniCo red' (GSR) is a sport derived from 'Gala Schnitzer'/Schniga®, a sport derived from RG; GSR was patented in 2017 (Gruber-Genetti et al., 2014).

Figure S2. Representation of the two qualitative fruit-related traits, i.e. (a) ground color (no. 29, Table S2) and (b) pattern of over color (no. 32, Table S2), visually graded on fruits of the seven investigated 'Gala' genotypes in the field for up to 3 years (2018–2020).

Table S1. Comparison of the disease scores of floral fire blight infections 21 dpi between the genotypes C44.4.146 (CIS) and 'Gala Galaxy' (GG) in two separate years (Games-Howell *post hoc* tests).

Table S2. List of traits examined in the specific years, including units and scale, as well as statistical tests used for analysis.

Table S3. Count of metabolite features absent or present in single genotypes only.

Table S4. Absolute number of genotype-specific DAFs per tissue (flesh and peel), year and progressive inclusion of GGG and LAD.

Table S5. Potential identifications of five out of 41 CIS-specific DAFs found in fruit peel extracts in 2018.

Table S6 Information on the 'Gala'-derived genotypes and the trees derived from these genotypes used in this study.

Table S7. Summary of the sample sizes for fruit trait assessments.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at https://www.ebi.ac.uk/metabolights/MTBLS5810/descriptors and https://doi.org/10.17632/bvrbsy79hj.1.

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