Plant Mol Biol Rep
DOI 10.1007/s11105-013-0682-0

ORIGINAL PAPER

A Phenotypic, Molecular and Biochemical Characterization of the First Cisgenic Scab-Resistant Apple Variety 'Gala'

Melanie Jänsch · Roberta Paris · Francis Amoako-Andoh · Wannes Keulemans · Mark W. Davey · Giulia Pagliarani · Stefano Tartarini · Andrea Patocchi

Abstract Scab resistance is one of the most important goals of apple breeding, typically achieved by time-consuming and expensive conventional breeding techniques. Cisgenesis, which is the genetic modification of a recipient organism with genes from a crossable—sexually compatible—organism, is a promising tool for plant breeding to develop disease resistance in a rapid way. A cisgenic, scab-resistant line of the apple variety 'Gala' expressing the native apple scab resistance gene *Rvi6* (formerly *HcrVf2*) under control of its own regulatory sequences has been recently developed. In this paper, we present the results from a phenotypic, molecular and biochemical evaluation of clonal replicates of this line (C11.1.53). The phenotype (shoot length, shoot diameter, internode length, number of leaves, leaf length and leaf width) of C11.1.53 was compared to that of the Gala parental background over a period of 108 days. Only a few statistically significant differences were detected, which are probably due to small differences in the quality of the budwood used for grafting rather than effects related to the presence of the cisgene. As the expression of a resistance gene can affect the downstream cascade of plant defence responses, a selection of apple defence-related genes was analyzed by quantitative real-time PCR analysis. These genes are also known as major allergen genes in apple. Even if three out of ten apple allergen genes tested in the leaves differed in the cisgenic line compared to both Gala (background) and 'Florina' (the variety from which the *Rvi6* gene was cloned), using 2D-PAGE, we were unable to find any significant difference in the expressed proteomes of the leaves of C11.1.53 compared to Gala. Results are discussed in the context of a possible use of cisgenic lines for fruit crop improvement.

Keywords 2D-PAGE · *Mal d* expression · qRT-PCR · *Venturia inaequalis*

Electronic supplementary material The online version of this article (doi:10.1007/s11105-013-0682-0) contains supplementary material, which is available to authorized users.

M. Jänsch · A. Patocchi (✉)
Plant Protection and Fruit and Vegetable Extension Division, Research Station Agroscope Changins-Wädenswil, Schloss 1, 8820 Wädenswil, Switzerland
e-mail: andrea.patocchi@acw.admin.ch

R. Paris · G. Pagliarani · S. Tartarini
Dipartimento di Scienze Agrarie, Università di Bologna, via Fanin 46, 40127 Bologna, Italy

F. Amoako-Andoh · W. Keulemans · M. W. Davey
Laboratory of Fruit Breeding and Biotechnology, Division of Crop Biotechnics, KU Leuven, Willem de Croylaan 42, Heverlee, B-3001 Leuven, Belgium

Present Address:
R. Paris
Consiglio per la Ricerca e la sperimentazione in Agricoltura-Centro di Ricerca per le Colture Industriali, via di Corticella 133, 40128 Bologna, Italy

Introduction

Apple scab, caused by the fungus *Venturia inaequalis*, is the most important disease of apple (*Malus* × *domestica* Borkh.) in temperate regions. Most of the cultivated varieties are susceptible to this disease and require up to 15 applications of fungicides per year for its control (Patocchi et al. 2004). The first, and so far, only cloned scab resistance gene (*Rvi6*, formerly *HcrVf2*) was isolated more than a decade ago from the classically bred cultivar 'Florina' (Vinatzer et al. 2001). Proof of functionality of *Rvi6* was provided by Belfanti et al. (2004) by expression of *Rvi6* under control of the strong constitutive CaMV35S promoter (CaMV35S:*Rvi6*) in the apple cultivar 'Gala'.

Published online: 13 November 2013

Recently, Schouten et al. (2006a) have proposed a cisgenic approach to generate a new class of genetically modified plants. According to these authors, a cisgenic plant is defined as 'a plant that has been genetically modified with one or more genes isolated from a crossable donor plant including introns, and flanking regions such as native promoter and terminator regions in a sense orientation'. Therefore, even once the relevant resistance gene had been cloned, two further milestones were still required before cisgenic, scab-resistant apples could be developed. These are (1) the isolation of the native *Rvi6* promoter sequence (work carried out by Silfverberg-Dilworth et al. (2005), Szankowski et al. (2009), Joshi et al. (2011)) and (2) the establishment of a transformation protocol that either does not require a selectable marker or which removes this marker from the final product. The inducible recombinase system developed by Schaart et al. (2004) for the transformation of strawberry fulfils this last requirement and was used to develop cisgenic apple lines expressing *Rvi6* (Vanblaere et al. 2011). In this approach, all DNA sequences (including selectable markers) that are flanked by the recombination sites are excised from transformed cells, leaving behind only the introduced gene of interest with promoter and terminator sequences, as well as the T-borders and part of the recombination sites.

In preparation of field trials, Vanblaere et al. (2013) have carried out a detailed molecular characterization of three cisgenic lines expressing *Rvi6* (C7.1.49, C11.1.53, C12.1.49) derived from the work of Vanblaere et al. (2011). The line C11.1.53 was shown to carry a minimal amount of foreign sequences. Specifically, line C11.1.53 has a single insertion site in chromosome 12, and the T-DNA is inserted in a putative gene (MDP0000310968) to which the gene ontology term 'microtubule cytoskeleton organization' has been assigned (Vanblaere et al. 2013). This insertion site in C11.1.53 has no right border and is missing the first five nucleotides of the native apple *Rvi6* promoter. The only foreign (non-coding) DNA present is a fragment of 72 bp (instead of the expected 140-bp one), derived from the vector cloning site and the recombination site. The expression of *Rvi6* in C11.1.53 was found to be about 500-fold lower than in Florina, a scab-resistant cultivar from which *Rvi6* was originally isolated. Artificial inoculation of C11.1.53 with *V. inaequalis* under controlled conditions resulted in a strong foliar deformation, chlorosis and in some cases also sporulating lesions. However, the quantity of sporulation in C11.1.53 was much lower than that in wild-type Gala under the same conditions, concluding that the gene was functional and resulted in increased resistance (Vanblaere et al. 2013).

In this paper, clones of line C11.1.53 have been analyzed and compared to its background (Gala) for (a) a variety of plant growth traits (phenotype), (b) total expressed leaf proteome via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and (c) the expression of members of the apple *Mal d* gene families in leaves (fruits are currently not yet available) by quantitative real-time PCR analysis.

For the phenotypic analysis, general aspects of plant growth were monitored during 108 days of growth. To verify if expression of the cisgene influenced protein abundance in the leaves of C11.1.53 relative to Gala, a 2D-PAGE proteomic analysis was carried out to identify differentially expressed proteins. Finally, we decided to focus on some apple *Mal d* genes which have been reported to be involved in the defence cascade activated by the *Rvi6* gene and to be affected by transgenesis in CaMV35S:*Rvi6* transgenic lines (Paris et al. 2012). *Mal d* 1, 2, 3 and 4 are also known as major apple allergen genes (Fernandez-Rivas et al. 2006), and expression levels of some of them were compared in line C11.1.53 relative to the cultivars Gala and Florina to determine whether they could impact on the allergenicity of the cisgenic line.

Materials and Methods

Plant Material and Sampling Procedure

For plant phenotyping, shoots from in vitro regenerated Gala and C11.1.53 plantlets, the later derived from the work of Vanblaere et al. (2011), were grafted onto M9 rootstocks and grown for 3–4 months. Budwoods obtained from plantlets of in vitro Gala (hereafter referred to as iGala) and from the cisgenic *Rvi6*-Gala line C11.1.53 were kindly provided by Prof. C. Gessler and Dr. G. Broggini (ETH Zürich, Switzerland) and were grafted onto M9 rootstocks to generate 11 plantlets of each genotype. Immediately after grafting, plants were transferred to the greenhouse where they were grown in 3-l pots filled with Dachgartensubstrat-Spezial (ProTer, Bern, Switzerland) supplemented with a fertilizer ('Osmocote'—Hauert, 15 % N, 9 % P_2O_5, 11 % K_2O; × g/l soil) under the following conditions: temperature (day/night) 21:18 °C, humidity (day/night) 70:90 %, light was added if the intensity was <250 W/m^2 (intensity 90,000 lumen).

For molecular and biochemical analyses, leaves were collected as follows. The first completely unfolded but still expanding young leaf (third to fourth leaf from the tip) from shoots of four individual clones (biological replicates) of iGala, C11.1.53 and the external control cv. Florina (also grafted on M9 and grown in the same conditions described above) were collected, snap frozen in liquid nitrogen and stored at −80 °C until shipment (in dry ice) to the University of Bologna for *Mal d* gene expression analysis and to the Katholieke Universiteit Leuven for 2D-PAGE proteomic analysis. The additional external control Florina, which was used for the *Mal d* gene expression analysis, was chosen as this cultivar naturally carries the *Rvi6* resistance gene under the control of a native promoter.

Plant Phenotyping

The following growth traits were measured: shoot length (cm), shoot diameter at 2 cm from the base (mm), number of leaves (excluding the leaf rosette and shoot apex), leaf dimensions (length and width in mm). The average internode length was calculated by dividing the shoot length by the number of leaves and the leaf area according to Bringe et al. (2006), i.e. leaf length × width × 0.71. Shoot diameter was measured using an electronic caliper ruler (Mitutoyo, Kawasaki, Japan). For each of the 11 plantlets per genotype, seven leaves, at the same relative position on each plantlet, were measured at every assessment date, as soon the leaves were fully unfolded. Leaf number 1 was the fifth leaf of the shoot (starting from the grafting point but excluding the leaves of the rosette) while leaves 2 to 7 were four leaves above the previous one. All parameters were measured at weekly intervals starting from the fourth week (28 days) post grafting and continuing up to 108 days.

Gene Expression Analysis of Selected *Mal d* Genes by qRT-PCR Analyses

Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, USA) from 100 mg fw of leaves from four different plants (biological replicates) of the cvs. iGala and Florina and from C11.1.53. RNA quantification and purity was measured at A260/280 and A260/230 using a Nanodrop™ ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was removed using on-column DNase Digestion (Sigma Aldrich, USA), according to the manufacturer's instructions, and cDNA synthesized according to Paris et al. (2009) starting from 1 µg total RNA. Quantitative real-time PCR (qRT-PCR) analyses were carried out on the StepOnePlus™ Real-Time PCR instrument (Applied Biosystems by Life Technologies, CA), with the Power® SYBR Green chemistry. Each biological replicate was analyzed in triplicate (technical replicates) using the *ubiquitin*-conjugating enzyme (MDP0000223660; primers UBC forward and reverse; Pagliarani et al. (2013)) as external reference gene and the standard curve method for absolute quantification (Larionov et al. 2005).

Ten *Mal d* genes were chosen for expression analysis as reported in Table 1, using the gene-specific primers reported by Pagliarani et al. (2009, 2013) and listed in Table 2. Each amplification reaction was performed in a total volume of 10 µl, containing 5 µl of Power SYBR® Green Master Mix 2×, 70–100 nM of each primer, PCR-grade water and 3 µl of a 1:9 dilution of the cDNA. Reactions were incubated at 50 °C for 2 min and at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 58/63 °C for 1 min with data collection at each annealing step. To ensure the specificity of amplification, each cycling programme was followed by a melting phase, according to the default settings of the StepOnePlus™ instrument (from 60 to 95 °C), and each melting curve was checked for the presence of a single peak of amplification to demonstrate specificity.

Protein Extraction for 2D-PAGE

Upon arrival, all leaf samples were lyophilized for 48 h and stored at −80 °C until use. For each leaf sample, two independent protein extractions were carried out essentially as follows. Between 17 and 40 mg of lyophilized leaf material were sequentially extracted three times, with 500 µl extraction buffer (500 mM Tris–Cl, pH 8.0, 100 mM KCl, 0.3 % Triton X-100, 50 mM EDTA, 2 % polyvinylpolypyrropolydone (PVPP), 1 % dithiothreitol (DTT), 0.7 M sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF)) by vortexing for 30 min at 2–4 °C. After centrifugation at 15,000*g* for 10 min at 4 °C, the supernatants were collected and pooled. An equal volume of pre-cooled Tris-buffered phenol, pH 7.9, was then added to the combined supernatants and vortexed as previously to re-extract proteins into the phenolic phase. Proteins in the phenolic phase were washed twice more with cold extraction buffer (without PVPP) and then precipitated with five volumes of ice-cold acetone containing 0.2 % DTT overnight at −20 °C. Precipitated proteins were collected at 15,000*g* for 60 min at 4 °C and washed twice with 100 mM ammonium acetate in methanol and once with 0.2 % DTT/acetone. Proteins were collected as pellets after each wash step by centrifugation at 15,000*g* for 30 min at 4 °C. Finally, remaining acetone was removed by drying under vacuum for 3 min at 84 kPa, using Millipore Vacuum pump XF 23050. Pellets were then resolubilized in 400–500 µl lysis buffer (5 M urea, 2 M thiourea, 2 % CHAPS, 2 % C7BzO, 20 mM DTT, 5 mM TCEP–HCl, 0.25 % ampholyte (pH 3–10), 0.50 % ampholyte (pH 4–7) and 1 mM PMSF) and yields quantified in triplicate using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

2D-PAGE

A total of 125 µg total protein in 340 µl of isoelectric focusing (IEF) buffer (5 M urea, 2 M thiourea, 2 % CHAPS, 2 % C7BzO, 40 mM DTT, 5 mM TCEP–HCl, 0.25 % ampholyte (pH 3–10), 0.25 % ampholyte (pH 4–7) and 0.002 % bromophenol blue) was loaded onto 18-cm Immobiline ReadyStrip, pH 3–10 (GE Healthcare) IEF strips for passive in-gel rehydration overnight at 22 °C. Rehydrated strips were subsequently subjected to first-dimension isoelectric focusing, using PROTEAN IEF cell (Bio-Rad) for a minimum of 54,000 Vh. For second-dimension SDS-PAGE, the following steps were carried out. IEF strips were first equilibrated for 20 min in 8 ml of equilibration buffer (6 M urea, 30 % glycerol, 2 % sodium dodecyl sulphate (SDS), 0.375 M Tris–

Table 1 List of apple allergen genes chosen for qRT-PCR analysis and the reasons for choice

Family	Gene	Reason for choice (from results reported in Paris et al. (2012))
Mal d 1	*Mal d 1.02* *Mal d 1.11*	The most highly expressed *Mal d 1* genes in leaves, induced in Florina after challenge with *V. inaequalis*
	Mal d 1.03A *Mal d 1.04* *Mal d 1.06A* *Mal d 1.07*	The most highly up-regulated genes in the CaMV35S:*Rvi6* transgenic lines relative to Gala and Florina. Moreover, *Mal d 1.06A* isoallergen gene has been chosen for a possible association between its allelic composition and the level of apple allergenicity (Gao et al. 2008)
	Mal d 1.10	The only *Mal d* 1 gene less expressed in CaMV35S:*Rvi6* transgenic lines with respect to Gala and Florina
Mal d 2	*Mal d 2.01*	The most highly expressed of the *Mal d 2* genes in leaves, down-regulated in Florina after challenge with *V. inaequalis*
Mal d 3	*Mal d 3.01*	The most highly expressed of the *Mal d 3* genes in leaves, down-regulated in Florina after challenge with *V. inaequalis*
Mal d 4	*Mal d 4.01*	The most highly expressed of the *Mal d 4* genes in leaves, down-regulated in Florina after challenge with *V. inaequalis*

Cl (pH 8.8), 0.002 % bromophenol blue, 130 mM DTT and 5 mM TCEP–HCl). Thiol groups were then alkylated using 135 mM iodoacetamide, and the strips were briefly rinsed with 0.025 M Tris, 0.192 M glycine and 0.1 % SDS and then loaded onto the top of 1-mm, 25×20 cm, 12 % cross-linked polyacrylamide gels. Strips were sealed in position using molten agarose containing bromophenol blue. Gels were subsequently subjected to discontinuous SDS-PAGE electrophoresis, using the Ettan DALTsix electrophoresis system (GE Healthcare) with running conditions according to the manufacturer's recommendations. Following SDS-PAGE, gels were removed from the glass plates, briefly rinsed in demineralized water and then stained according to Neuhoff et al. (1985). Essentially, the gels were fixed in a solution of 34 % methanol, 3 % phosphoric acid

Table 2 List of specific primer pairs for target and reference genes for qRT-PCR analysis

Gene	Primer name	Sequence 5′–3′	Length (bp)	Primer (nM)	Ta
Mal d 1.02	Mald102RTF[a] Mald102RTR[a]	CACACCAAGGGTGATGTTGAGA CTTGAACAAACCATGAGCCTTCT	75	100	58
Mal d 1.03A	qMd1.03AF qMd1.03AR	ATCTGAGTTCACCTCCGTCATT ACTGCTTGTGGTGGAATCTTT	96	70	63
Mal d 1.04	qMd1.04 F qMd1.04R	GGGTATGTTAAGCAAAGGGTCA TGATCTCAACATCACCCTTAGC	193	100	61
Mal d 1.06A	qMd1.06AF qMd1.06AR	CTATAGCTATAGCTTGATTGAAGGG TTCCAACCTTAACATGTTCTTCT	167	100	61
Mal d 1.07	qMd1.07 F qMd1.07R	CAACTTTGTGTACCAGTACAGTGTC TAGTGGCTGATGCTCTTGATAAC	126	100	61
Mal d 1.10	qMald1.10 F qMald1.10R	CAAGGCTTTCATCCACGAC GATTCTGTGCTTTACAAACCCT	157	100	61
Mal d 1.11	qMald1.11AF qMald1.11AR	GGAGGATGCATCTGTCATTTG CCATGAGATAGGCTTCCAAAACT	130	100	62
Mal d 2.01	Mald2RTF[a] Mald2RTR[a]	GCTTGCCTTGCGTTTGGT ACATGTCTCCGGCGTATCATT	66	100	58
Mal d 3.01	Mald301RTF[a] Mald301RTR[a]	CCGCTGACCGCCAGAC AGCCCTGCTGCATTGTTAGG	85	100	58
Mal d 4.01	Mald401RTF[a] Mald401RTR[a]	GGCCAAGCTTTGGTTTTC GCCTTGATCAATCAGGTAGTCT	99	100	58
Ubiquitin-conjugating enzyme	UBCFor UBCRev	CGAATTTGTCCGAAGGCGT CAATGATTGTCACAGCAGCCA	53	100	58/63

Ta annealing temperature

[a] Primers from Pagliarani et al. (2009). The other primer pairs were taken from Pagliarani et al. (2013)

and 17 % ammonium sulphate for 90 min. The gels were then washed overnight in 50 % methanol and 10 % acetic acid solution before staining with 0.1 % Coomassie Brilliant Blue G-250 in 18.0 % methanol, 2.0 % phosphoric acid and 8.0 % ammonium sulphate. Gels were then neutralized in 1.2 % Tris (pH 6.5) solution before finally being distained in the wash solution. Gels were individually scanned using an ImageScanner (Amersham Biosciences) at 150 dpi in transparent mode with blank filter and images saved in both TIF and MEL formats for analysis.

Data Analysis

Phenotypic Analysis

Plantlets displaying significantly different growth parameters (outliers) were identified by boxplot analysis performed for each measurement date separately with the software XLSTAT 2011 (Addinsoft, Paris, France). If the same plantlet was found to be an outlier at most measurement dates, its data were not considered for later statistical analysis. Leaf parameters were statistically analyzed when leaves reached the steady state (three measurement dates in a row without a change in length or width). Student's *t* test was used for pairwise comparison of iGala with C11.1.53 at each measurement date and for each parameter ($p \leq 0.05$, JMP software, SAS Institute).

Mal d Gene Expression

The expression levels of all *Mal d* genes were normalized with the transcript levels of *UBC*, and gene expression results were reported as absolute expression levels in arbitrary units (A.U.). The means, normalized expression levels and the standard error of the mean (SEM) were calculated among four different biological replicates. The statistical analysis was performed with ANOVA (SAS system) at a 0.05 significance level (p). When $p \leq 0.05$, means were compared with the *t* LSD tests to identify genotypes with significant differences at 95 % confidence level.

2D Gel Image Analysis and Differential Spot Identification

Six scanned gel images for both C11.1.53 and iGala were analyzed using the Progenesis SameSpots software v4.1 (Nonlinear Dynamics). Per sample, one gel image from the six available was selected as the 'reference', and the other five images automatically aligned to this reference. Gel alignments were then individually reviewed to manually improve selected regions of the gels. Gels were then normalized and an automated statistical analysis performed on all spots to identify statistically relevant spots using the 95 % confidence level of ANOVA ($p < 0.05$). Within the SameSpots software program, mean expression levels for each individual protein spot and for each of the two groups (plant types) are calculated from the six gels and subjected to statistical analysis to identify statistically significant differences. The statistical tests return results for p value, power, false discovery rate (FDR) adjusted p values (or q value) and fold change. Only spots with $p < 0.05$, FDR < 0.05 and power > 0.8 were considered, and only differences of ±2-fold between the two sample sets were considered to be differentially expressed.

Results

Phenotypic Evaluation

From a total of 11 clonal replicates of each genotype, 3 C11.1.53 and 3 iGala plants were excluded from the statistical analysis of the plant growth phenotype as they proved to be outliers according to the boxplot analysis.

For the remaining eight clonal replicates, no significant macroscopic differences or aberrations were observed within the C11.1.53 or within the iGala plantlets or between C11.1.53 and iGala over the 108 days of observation. However, budbreak of the iGala plants was on average 3 days later than that of C11.1.53 (10.1±1.4 days vs. 7.1±1.5 days, data not shown), and this difference was statistically significant ($p = 0.0017$). In addition, all seven iGala leaves were in general longer and wider compared to those of line C11.1.53 (Fig. 1a). These differences were only significant for lengths of leaves up until the fourth leaf and for widths up to the second leaf per shoot. iGala leaves generally had a larger surface than C11.1.53 (Fig. 1b), but these differences were only significant up to the third leaf of the shoot.

The average length of the shoots of C11.1.53 was greater at each time point than that of the shoots of iGala, and by the end of the observation period, the C11.1.53 plants were on average 119.0±6.7 cm long, while the iGala plants were on average 113.3±4.2 cm long. The difference in average length ranged from 2.8 cm at 35 days to 6.3 cm at 84 days. However, these differences were never statistically significant (Fig. 2a). Clones of line C11.1.53 also had more leaves on average than iGala (Fig. 2b). Here the differences in average leaf number ranged from 0.4 leaves at 70 days to 2.3 leaves at 108 days. These differences were only statistically significant at the last measurement date (108 days post grafting). In comparison, the shoot diameter of iGala was generally greater than that of C11.1.53 (Fig. 2c). The difference in average diameter between iGala and C11.1.53 ranged from 0 mm at 28 days to 0.7 mm at 98 days. These differences were significant at three evaluations days in the second half of the observation period (i.e. after 75, 84 and 98 days). Finally, no clear trend could be recognized for the mean internode length, which was identical (3 cm) for both iGala and C11.1.53 (Fig. 2d).

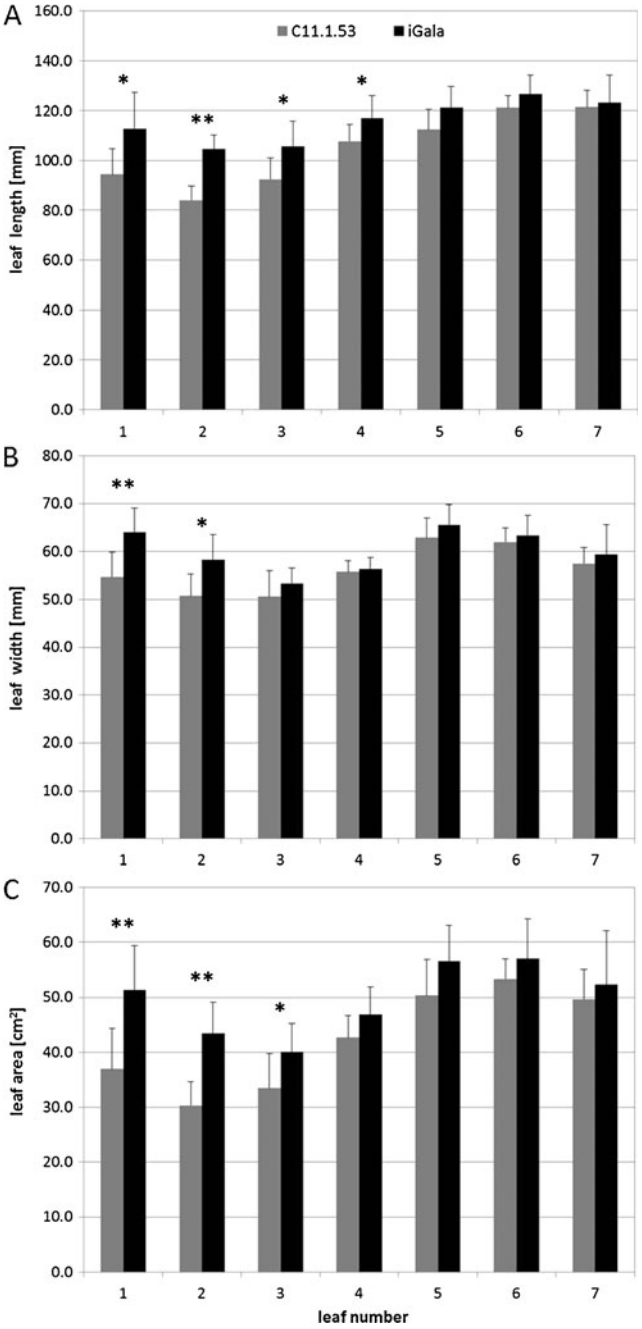

Fig. 1 Graphical representation of **a** average (and standard deviation) length, **b** average width and **c** average leaf area of the seven leaves per plant (eight in total) and genotype (iGala and C11.1.53). *Asterisk* indicates values that are statistically significantly different between iGala and C11.1.53 (*$p<0.05$, **$p<0.01$)

Mal d Gene Expression

The mean *Mal d* transcription levels in C11.1.53 plants were compared to those in both iGala and Florina (Fig. 3). Three genes (*Mal d 1.04*, *Mal d 1.07* and *Mal d 1.11)* displayed no significant differences in expression among the three genotypes. In four cases, namely *Mal d 1.02*, *Mal d 1.06A*, *Mal d 2.01* and *Mal d 3.01*, expression levels in iGala and the C11.1.53 line were not statistically different, but were significantly different from Florina. Finally, the expression levels of *Mal d 1.03A*, *Mal d 4.01* and *Mal d 1.10* were statistically different among the three cultivars. Specifically, levels of *Mal d 1.03A* and *Mal d 4.01* were higher in C11.1.53 relative to Florina and iGala, while the expression of *Mal d 1.10* in C11.1.53 was intermediate between those in Florina and Gala, the expression in C11.1.53 being lower than that in iGala.

2D-PAGE

2D-PAGE analysis was used to compare the total soluble proteomes of the leaves of C11.1.53 and iGala (Fig. 4). In total, 1,064 protein spots were identified, out of which 94 were significantly different ($p<0.05$), with varying molecular weights and pI values (Tab. S1). Thirty-seven of the significantly different spots have a statistical power >0.8, and of these, three (i.e. spots 482, 1,098 and 1,099) have FDR values <0.05 (Table 3). However, none of these three proteins showed a greater than 2-fold difference in expression, which is generally considered to be the minimum reliable cutoff value for differential expression in Coomassie-stained gels, and according to this assumption, they were not considered as differentially expressed proteins.

Discussion

Phenotype

The overall growth of C11.1.53 and iGala was monitored for a period of 108 days following grafting, and no aberrations or evident macroscopic differences were observed. However, budbreak of C11.1.53 plants was statistically different from that of iGala, occurring on average 3 days earlier than that in iGala. This delay in the start of growth of iGala could not be recovered by iGala even after more than 100 days of growth, and the average length of iGala plants remained lower than that of C11.1.53 plants. However, these growth differences are not statistically significantly different at any measurement time point. We consider that the reason for the delayed budbreak of iGala could be due to the use of an initial budwood that was at a slightly different 'maturity' stage. Therefore, in the future, biological replicates should be derived from plants obtained from the grafting of several different budwoods.

Regarding the leaf number, only one statistically significant difference was observed between iGala and C11.1.53, and this was at the last measuring point, 108 days post grafting. If a linear regression is calculated for both genotypes and the average number of leaves (absolute value) is calculated for the 11 measurement dates and then corrected for the

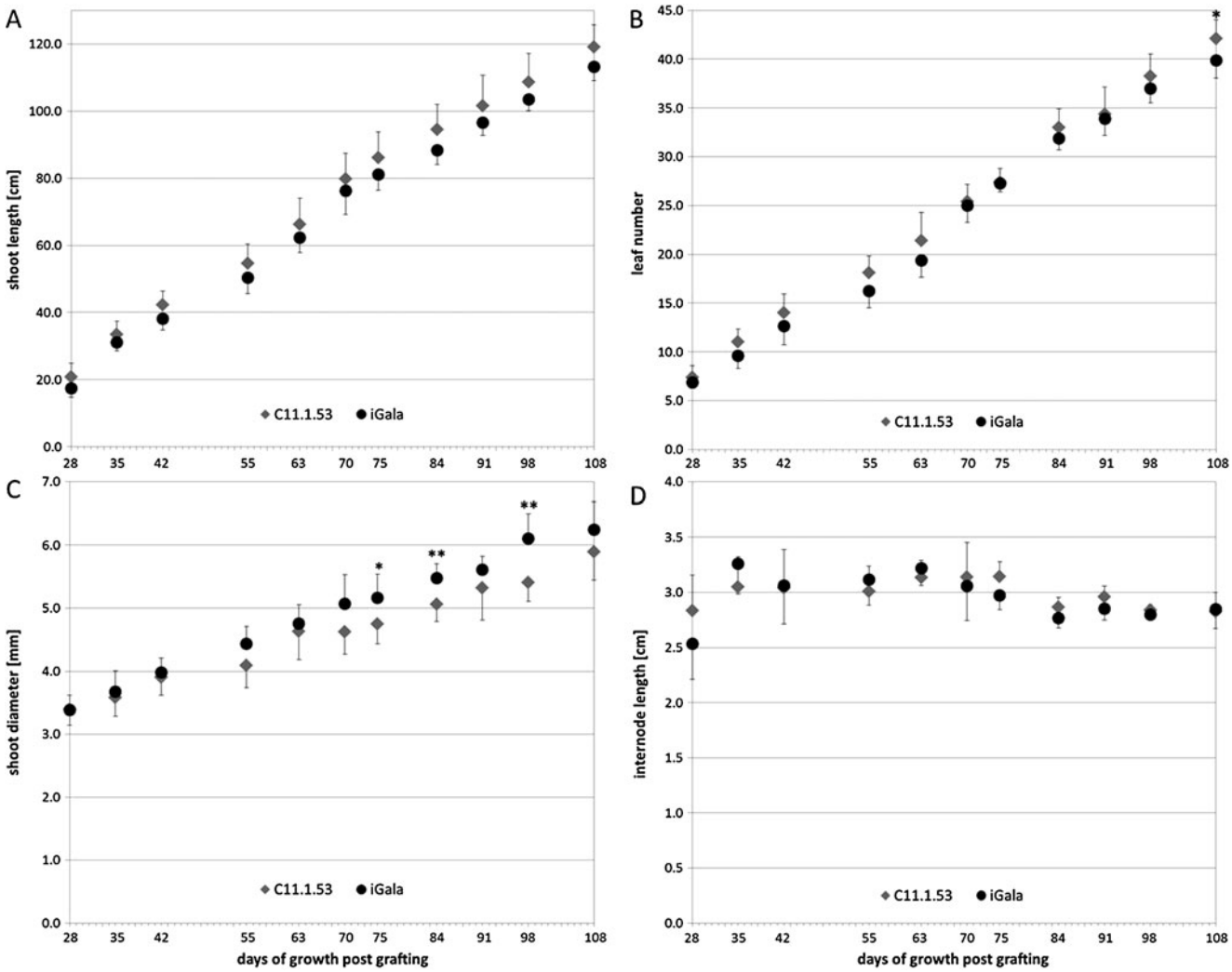

Fig. 2 Average values and standard deviation from 28 to 108 days after budbreak of iGala (*black dots*) compared to C11.1.53 (*grey diamonds*). **a** Shoot length, **b** number of leaves, **c** shoot diameter and **d** internode length. *Asterisk* indicates values that are statistically significantly different between iGala and C11.1.53 (*$p<0.05$, **$p<0.01$)

differences in budbreak, then exactly the same number of leaves is found for both genotypes at all time points. Therefore, the difference in leaf number observed after 108 days is entirely due to the shift in budbreak between the two genotypes.

The shoots of iGala plants were on average thicker than those of C11.1.53 plants, and at three dates, significant differences were observed. However, it should be noted that the magnitude of these differences is small (a maximum difference of 0.7 mm), and such differences can easily be obtained by exerting a little extra pressure on the caliper ruler during measurement. Therefore, we consider these differences to be due to technical variations during measurements rather than representing true biological differences.

The leaves of C11.1.53 closer to the grafting point were on average longer and wider than those of iGala, and these differences in length were significant up to the fourth leaf from the base. Unsurprisingly, the leaf surface area exhibited a similar trend. The variability of all three leaf parameters tended to be higher within both genotypes up to the fourth leaf and then lower for the remaining upper three leaves (Fig. 1a and b). Since values are more variable up to the fourth leaf within both genotypes, it is reasonable to only compare the two genotypes from the point where this intra-genotype 'instability' is lowest. Under these conditions, there are no significant differences for any of the leaf parameters measured.

Allergens

Apples are frequently involved in allergic reactions whose severity is related not only to the sensitivity of the individual but to genetic determinants (Bolhaar et al. 2005; Ricci et al. 2010; Vlieg-Boerstra et al. 2011) that are not fully understood yet. At the genome level, apple allergens are encoded by four

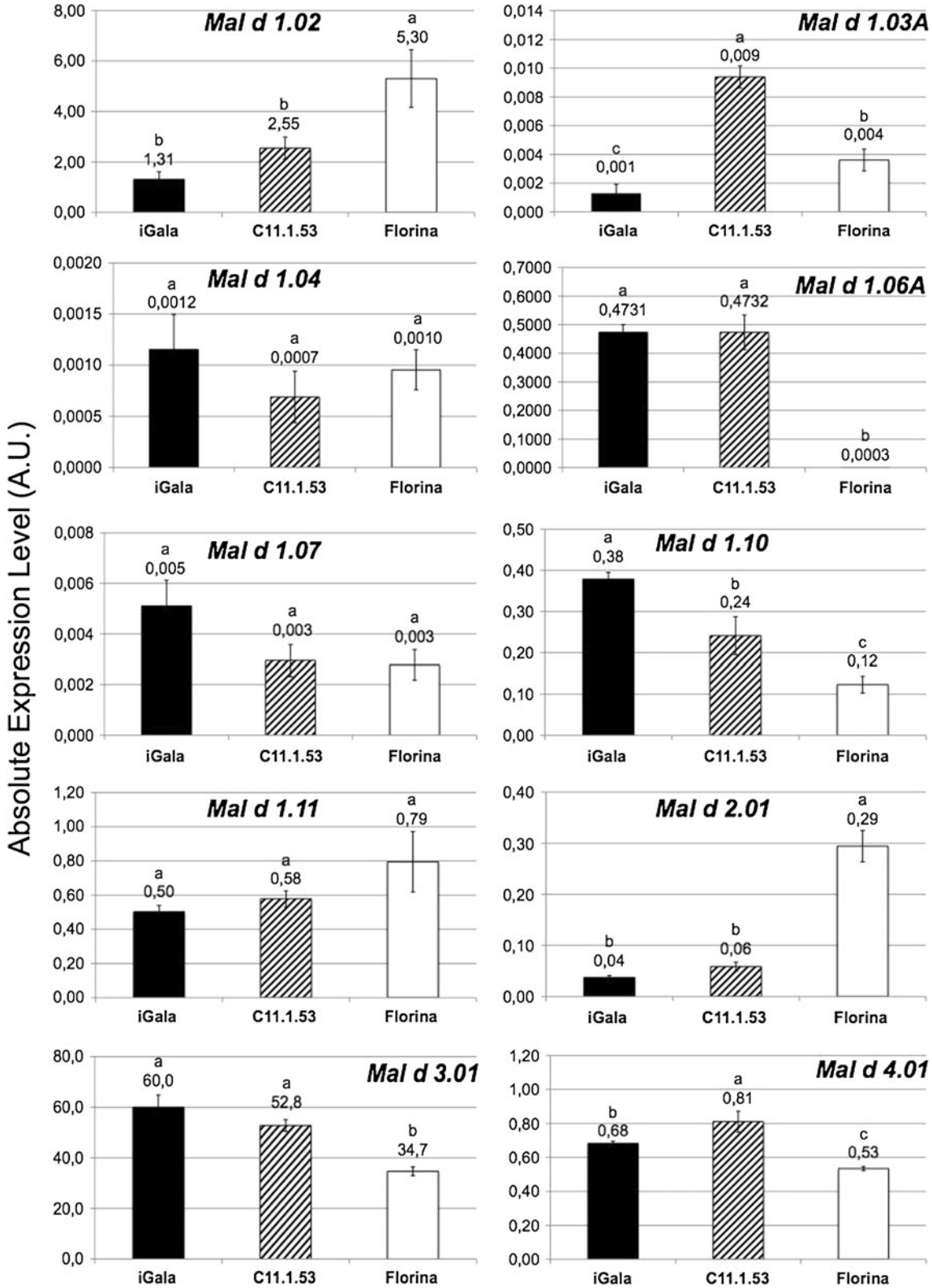

Fig. 3 Absolute expression levels of ten different apple allergen genes in iGala, the cisgenic C11.1.53 line and Florina. The expression level is normalized for the *UBC* reference gene, and the average among four biological replicates is reported in A.U. The *bars* refer to the SEM. Means with the same *letter* are not significantly different

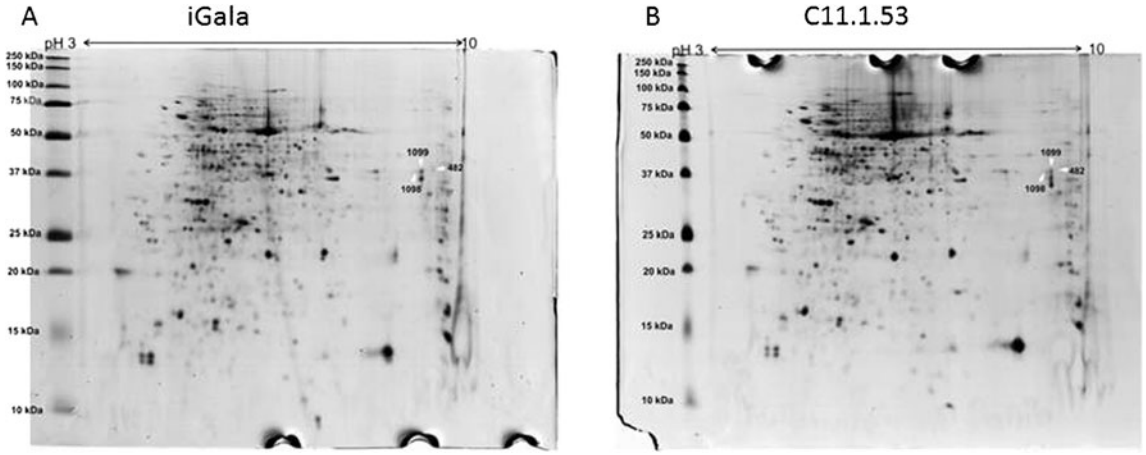

Fig. 4 Representative raw 2D-PAGE gel scans (uncropped) of **a** iGala and **b** C11.1.53 indicating the three differentially expressed proteins (spots 482, 1,098 and 1,099) with p and q values <0.05 at statistical power >0.8. Spots 482, 1,098 and 1,099 were up-regulated in iGala with expression levels below the threshold of 2, i.e. 1.55-, 1.45- and 1.64-fold, respectively

multigene families (Radauer and Hoffmann-Sommergruber 2004). Mal d 1, 2 and 3 are pathogenesis-related (PR) proteins, belonging to class PR-10 (ribonuclease-like proteins), PR-5 (thaumatin-like proteins (TLPs)) and PR-14 (non-specific lipid transfer proteins (nsLTPs)), respectively. Mal d 4 is a profilin, with a putative actin-binding role.

The cisgenic line C11.1.53 carries the resistance gene *Rvi6* together with its natural regulatory sequences. As the inserted and expressed sequence is already present in traditionally bred scab-resistant varieties like Florina and 'Santana', the cisgenic line should not represent a cause for concern regarding modified allergenicity compared to the non-transformed cultivar (Davies 2005). Moreover, compared to conventional breeding, cisgenesis does not introduce additional genes/nucleotide sequences associated with linkage drag and therefore avoids the introgression of unwanted traits and potential hazards associated with these traits. However, the introgression of a resistance gene will activate a cascade of events downstream the recognition of the pathogen, which lead to decreased susceptibility. This involves, for example, modulation of the expression of apple defence genes such as *Mal d* genes coding for PR proteins (Paris et al. 2009, 2012).

Therefore, we investigated the expression of some selected apple allergen genes in apple leaves as fruits are yet not available for the C11.1.53 line. In fact, since these genes are expressed both in leaves and in fruits, it is possible to investigate if they change their expression level in cisgenic plants at all developmental stages (Gilissen et al. 2005).

In our experiment, significant differences in the foliar expression of three (*Mal d 1.03A*, *Mal d 1.10* and *Mal d 4.01*) out of the ten *Mal d* genes analyzed were observed in C11.1.53 compared to iGala and Florina plants. *Mal d 1.03A* and *Mal d 1.10* are genes coding for PR-10 proteins that are putatively involved in defence mechanisms (van Loon et al. 2006), but for which the actual biological function is still unclear. *Mal d 1.03A* has previously been reported to be significantly up-regulated in Florina leaves following inoculation with *V. inaequalis*, and this suggests a possible role in plant defence response, following activation by *Rvi6* (Paris et al. 2012). In the same work, the authors found *Mal d 1.10* to be strongly down-regulated in Florina following pathogen challenge, suggesting a different role in the *Rvi6*-mediated response pathway. Finally, *Mal d 4.01* codes for a putative profilin, which is an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton. Therefore, variability in *Mal d 4.01* expression might be due to slightly different growth rates or due to differences in developmental status between the three genotypes.

As the expression of *Mal d 1.10* was lower in C11.1.53 than in iGala, C11.1.53 can be considered to have a lower potential allergenic risk compared to iGala, under these growth conditions. However, the expression levels of both *Mal d 1.03A* and *Mal d 4.01* was significantly ($p \leq 0.05$) higher in C11.1.53 than either iGala or Florina (even if within the same order of magnitude) and could therefore actually represent an increased allergenic risk of the cisgenic line compared to iGala if this trend is also observed in fruits. While these results still need to be confirmed in fruit tissues of the cisgenic line, it is interesting to note that differences in expression levels were not reflected in significant differences in leaf protein abundance (see 2D-PAGE results). Of course, it is impossible to distinguish different protein isoforms belonging to large protein families by proteome analysis, unless isoform-specific antibodies are available (this is not the case). For this reason, qRT-PCR with specific primers is the only method nowadays available for a detailed analysis of gene families,

Table 3 Overview of the 37 differentially expressed proteins ($p < 0.05$) with a power >0.8

Spot #	ANOVA (p)	Fold	Max CV (%)	Highest mean	Lowest mean	Area	pI	MW
1,099	5.6E−06	1.64	12.09	iGala	C11.1.53	852	9.26	37
113	4.0E−05	3.06	36.33	iGala	C11.1.53	94	3.47	73
482	1.2E−04	1.55	17.24	iGala	C11.1.53	1,027	9.45	38
1,098	1.3E−04	1.45	12.08	iGala	C11.1.53	1,126	9.14	37
942	7.5E−04	1.4	14.57	C11.1.53	iGala	654	5.35	15
562	9.8E−04	1.16	5.57	iGala	C11.1.53	1,099	5.1	34
418	1.4E−03	1.51	19.19	iGala	C11.1.53	448	8.03	43
927	1.5E−03	1.28	12.05	C11.1.53	iGala	799	5.12	16
706	1.5E−03	1.63	21.37	iGala	C11.1.53	1,204	3.69	26
292	1.7E−03	1.75	22.63	C11.1.53	iGala	422	5.28	52
233	1.8E−03	1.32	13.74	C11.1.53	iGala	317	5.12	60
955	2.2E−03	1.81	29.15	C11.1.53	iGala	337	9.37	14
419	2.4E−03	1.41	18.95	iGala	C11.1.53	189	7.66	43
464	2.7E−03	1.54	21.73	iGala	C11.1.53	1,661	9.73	40
225	2.8E−03	1.16	8.83	C11.1.53	iGala	1,695	4.92	62
1,064	3.0E−03	1.75	30.34	iGala	C11.1.53	235	4.69	11
510	3.2E−03	1.46	23.41	iGala	C11.1.53	999	9.68	36
1,018	3.3E−03	1.65	22.95	iGala	C11.1.53	430	8.95	12
672	3.4E−03	1.35	17.84	iGala	C11.1.53	1,694	9.69	28
107	0.0044566	1.59	22.71	C11.1.53	iGala	297	8.82	74
922	4.5E−03	1.26	12.52	C11.1.53	iGala	1,421	5.33	16
665	5.0E−03	1.53	21.22	C11.1.53	iGala	697	4.69	28
329	5.1E−03	1.37	16.9	C11.1.53	iGala	268	6.57	49
34	5.1E−03	1.44	18.27	C11.1.53	iGala	135	7.47	90
700	5.6E−03	1.34	16.49	iGala	C11.1.53	425	5.72	26
938	5.6E−03	1.5	20.96	C11.1.53	iGala	866	8.26	15
4	5.6E−03	1.96	35.25	C11.1.53	iGala	143	6.45	108
114	6.3E−03	3.99	111.78	iGala	C11.1.53	458	3.57	73
143	9.2E−03	1.25	16.26	C11.1.53	iGala	521	5.42	71
497	1.0E−02	1.36	17.49	iGala	C11.1.53	1,307	8.43	37
267	2.2E−02	2.1	54.82	iGala	C11.1.53	1,866	3.83	55
180	2.4E−02	4.15	131.65	iGala	C11.1.53	68	3.82	67
279	3.0E−02	2.77	100.1	iGala	C11.1.53	320	3.57	53
129	3.0E−02	3.81	132.42	iGala	C11.1.53	243	3.65	72
1,080	3.7E−02	2.18	66.72	C11.1.53	iGala	127	3.7	11
1,087	4.1E−02	2.12	54	C11.1.53	iGala	70	3.72	11
995	4.3E−02	8.19	179.74	iGala	C11.1.53	34	4.77	13

Bold: proteins with FDR<0.05; italics: proteins with expression values >2-fold

assuming that there is a correlation between protein and transcript abundance (Baerenfaller et al. 2008). If differences in the expression levels of these genes in fruits of C11.1.53 and Gala are identified, it will still be necessary to verify whether these differences are within the naturally occurring range of expression present in other apple cultivars.

2D-PAGE

The 2D-PAGE analysis performed here allowed us to study for the first time the soluble leaf proteome of a cisgenic line and to compare it with its parental background. In total, 1,064 protein spots were identified, but there were no statistically significant differences in foliar leaf protein expression patterns between C11.1.53 and iGala based on the mean cutoff value of ±2-fold difference in abundance. This indicates that the insertion event did not significantly influence the expression of metabolic genes and that no significant reprogramming of metabolism, at least in leaf tissues, took place. We did not observe any differentially expressed proteins in the regions of the gel where Mal d proteins would be expected.

Conclusions

The results presented in this paper are to the best of our knowledge the first phenotypic and molecular–biochemical evaluation of a cisgenic line expressing the natural *Rvi6* scab-resistance gene. As such, they provide a first insight into the potential impact of the commercial use of this cisgenic line as food crop, but should neither be considered as a general discussion on cisgenesis nor a formal risk assessment of the approach. All the data produced, with the possible exception of few differences in the expression of two *Mal d* transcripts (*Mal d 1.03A* and *Mal d 4.01*), support the hypothesis formulated by Schouten et al. (2006b) and the conclusions of the EFSA GMO Panel (Anonymus 2012) that cisgenic plants are equivalent to conventionally bred cultivars.

Acknowledgments The authors wish to acknowledge the financial support by the Swiss National Science Foundation NRP59 and Dr Jörg Samietz and Didier Socquet-Juglard for the help in the statistical analysis of the data.

References

Anonymus (2012) EFSA Panel on Genetically Modified Organisms (GMO); Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. EFSA J 10:2561

Baerenfaller K, Grossmann J, Grobei MA, Hull R, Hirsch-Hoffmann M, Yalovsky S, Zimmermann P, Grossniklaus U, Gruissem W, Baginsky S (2008) Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science 320: 938–941

Belfanti E, Silfverberg-Dilworth E, Tartarini S, Patocchi A, Barbier M, Zhu J, Vinatzer BA, Gianfranceschi L, Gessler C, Sansavini S (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc Natl Acad Sci U S A 101: 886–890

Bolhaar ST, van de Weg WE, van Ree R, Gonzalez-Mancebo E, Zuidmeer L, Bruijnzeel-Koomen CA, Fernandez-Rivas M, Jansen J, Hoffmann-Sommergruber K, Knulst AC, Gilissen LJ (2005) In vivo assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars. J Allergy Clin Immunol 116:1080–1086

Bringe K, Schumacher CFA, Schmitz-Eiberger M, Steiner U, Oerke E-C (2006) Ontogenetic variation in chemical and physical characteristics of adaxial apple leaf surfaces. Phytochemistry 67:161–170

Davies HV (2005) GM organisms and the EU regulatory environment: allergenicity as a risk component. Proc Nutr Soc 64:481–486

Fernandez-Rivas M, Bolhaar S, Gonzalez-Mancebo E, Asero R, van Leeuwen A, Bohle B, Ma Y, Ebner C, Rigby N, Sancho AI, Miles S, Zuidmeer L, Knulst A, Breiteneder H, Mills C, Hoffmann-Sommergruber K, van Ree R (2006) Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods. J Allergy Clin Immunol 118:481–488

Gao Z, van de Weg E, Matos C, Arens P, Bolhaar STHP, Knulst AC, Yinghui L, Hoffmann-Sommergruber K, Gilissen LJWJ (2008) Assessment of allelic diversity in intron-containing *Mal d 1* genes and their association to apple allergenicity. BMC Plant Biol 8:116

Gilissen LJ, Bolhaar ST, Matos CI, Rouwendal GJ, Boone MJ, Krens FA, Zuidmeer L, Van Leeuwen A, Akkerdaas J, Hoffmann-Sommergruber K, Knulst AC, Bosch D, van de Weg WE, Van Ree R (2005) Silencing the major apple allergen Mal d 1 by using the RNA interference approach. J Allergy Clin Immunol 115:364–369

Joshi SG, Schaart JG, Groenwold R, Jacobsen E, Schouten HJ, Krens FA (2011) Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. Plant Mol Biol 75:579–591

Larionov A, Krause A, Miller WA (2005) Standard curve based method for relative real time PCR data processing. BMC Bioinforma 6:62

Neuhoff V, Stamm R, Eibi H (1985) Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis 6:427–448

Pagliarani G, Paris R, Tartarini S, Sansavini S (2009) Cloning and expression of the major allergen genes in apple fruit. J Hortic Sci Biotechnol, ISAFRUIT Special Issue 84:176–181

Pagliarani G, Paris R, Arens P, Tartarini S, Ricci G, Smulders MMJ, van de Weg E (2013) A qRT-PCR assay for the expression of all *Mal d 1* isoallergen genes. BMC Plant Biol 13:51

Paris R, Cova V, Pagliarani G, Tartarini S, Komjanc M, Sansavini S (2009) Expression profiling in *HcrVf2*-transformed apple plants in response to *Venturia inaequalis*. Tree Genet Genomes 5:81–91

Paris R, Pagliarani G, Tartarini S, Sansavini S, Gessler C, van de Weg E (2012) Allergen expression in control and transgenic apple plants. Acta Hortic (ISHS) 929:135–142

Patocchi A, Bigler B, Koller B, Kellerhals M, Gessler C (2004) *Vr2*: a new apple scab resistance gene. Theor Appl Genet 109: 1087–1092

Radauer C, Hoffmann-Sommergruber K (2004) Profilins. In: Mills ENC, Shewry PR (eds) Plant food allergens. Blackwell, Oxford, pp 105–124

Ricci G, Dondi A, Belotti T, Baldi E, Tartarini S, Paris R, Pagliarani G, Serafini-Fracassini D, Casadio R, Giannetti A, Masi M (2010) Allergenicity of different apple cultivars assessed by means of skin prick test and sensitisation to recombinant allergens Mal d 1 and Mal d 3 in a group of Italian apple-allergic patients. Int J Food Sci Technol 45:1517–1523

Schaart JG, Krens FA, Pelgrom KTB, Mendes O, Rouwendal GJA (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J 2:233–240

Schouten HJ, Krens FA, Jacobsen E (2006a) Do cisgenic plant warrant less stringent oversight? Nat Biotechnol 24:9

Schouten HJ, Krens FA, Jacobsen E (2006b) Cisgenic plants are similar to traditionally bred plants. EMBO Rep 7:750–753

Silfverberg-Dilworth E, Besse S, Paris R, Belfanti E, Tartarini S, Sansavini S, Patocchi A, Gessler C (2005) Identification of functional apple scab resistance gene promoters. Theor Appl Genet 110: 1119–1126

Szankowski I, Waidmann S, Degenhardt J, Patocchi A, Paris R, Silfverberg-Dilworth E, Broggini GAL, Gessler C (2009) Highly scab-resistant transgenic apple lines achieved by introgression of *HcrVf2* controlled by different native promoter lengths. Tree Genet Genomes 5:349–358

van Loon LC, Rep M, Pieterse CM (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135–162

Vanblaere T, Szankowski I, Schaart J, Schouten H, Flachowsky H, Broggini GAL, Gessler C (2011) The development of a cisgenic apple plant. J Biotechnol 154:304–311

Vanblaere T, Flachowsky H, Gessler C, Broggini GAL (2013) Molecular characterization of cisgenic apple lines of the cultivar 'Gala'

carrying the *HcrVf2* scab resistance gene. Plant Biotechnol J. doi:10.1111/pbi.12110

Vinatzer BA, Patocchi A, Gianfranceschi L, Tartarini S, Zhang HB, Gessler C, Sansavini S (2001) Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. Mol Plant Microbe Interact 14:508–515

Vlieg-Boerstra BJ, van de Weg WE, van der Heide S, Kerkhof M, Arens P, Heijerman-Peppelman G, Dubois AEJ (2011) Identification of low allergenic apple cultivars using skin prick tests and oral food challenges. Allergy 66:491–498