

Genotyping of red and white fruited strawberry (*Fragaria* L.) accessions and hybrids based on microsatellite markers and on the genetic diversity in the allergen genes *fra a 1* and *fra a 3*

René Kaiser · Vera Mageney · Konrad Schwefel · Daniela Vollmers ·
Aenne Krüger · Renate Horn

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Abstract Despite the beneficial nutritional value of strawberries, there is a potential risk of allergic reactions in consuming strawberries, especially for people with Bet v 1 allergy. Allergen content varies in the fruits depending on environmental and genetic backgrounds, also involving the fruit color. Diploid strawberry varieties on the European market, bearing white and red colored fruits ('Blanc Amélioré', 'Weiße Hagmann', 'Weiße Solemacher', 'Yellow Wonder', 'Rote Baron Solemacher', 'Walderdbeere') in addition to a new accession VM2012 were characterized by microsatellite analyses. Three strawberry varieties with white colored fruits, 'Weiße Hagmann', 'Weiße Solemacher', 'Yellow Wonder', were very similar to each other, but 'Blanc Amélioré' and VM2012 could be distinguished from the other varieties. The allergen genes *fra a 1* and *fra a 3* of diploid red and white colored strawberry varieties and VM2012 were identical on genomic level. Of the eight gene variants described for *fra a 1* in octoploid

strawberries (*Fragaria* × *ananassa*) only the gene variant *fra a 1* A2 was highly conserved present in the diploid varieties of *Fragaria vesca*, indicating a role of *F. vesca* in the phylogeny of octoploid strawberries. For *fra a 3*, the sequence differed only in a single nucleotide exchange T503C from the octoploid sequence of the variety 'Camarosa', with no effect on the protein sequence. No complementation with regard to the fruit color was observed in crosses between VM2012 and the white colored diploid varieties 'Weiße Hagmann' and 'Weiße Solemacher' indicating that the genetic cause behind the white fruit color might be similar.

Keywords Allergen genes · Flow cytometry · *Fragaria* · Phylogeny · Simple sequence repeats · White fruit color

Introduction

Most commercially available strawberry varieties (*Fragaria* × *ananassa* [Duchesne ex Weston] Duchesne ex Rozier) are octoploid ($2n = 8x = 56$). These cultivated strawberries can be propagated both sexually and asexually via stolons, also called runners (Slovin and Michael 2011). Due to the polyploidy level *F.* × *ananassa* has an interesting, not yet fully resolved phylogeny of which only the last step is well documented. *F.* × *ananassa* was accidentally produced in the early 1700s by the hybridization of the

R. Kaiser · V. Mageney · K. Schwefel ·
D. Vollmers · A. Krüger · R. Horn (✉)
Institut für Biowissenschaften, Abteilung Pflanzengenetik,
Universität Rostock, Albert-Einstein-Str. 3,
18059 Rostock, Germany
e-mail: rene.horn@uni-rostock.de

Present Address:

V. Mageney
Carl-von-Ossietzky Universität Oldenburg, Carl-von-
Ossietzky-Straße 9-11, 26129 Oldenburg, Germany

white-fruited *Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* with pollen of *Fragaria virginiana* Mill. subsp. *virginiana*, both representing octoploid species (Darrow 1966; Staudt 1961, 1989). Octoploid varieties show higher yields and higher vigor than diploid ones, but diploid strawberry varieties ($2n = 2x = 14$) are commercially available and are preferable for genetic studies due to the simpler genetic constitution e.g. for obtaining the whole genome sequence (Shulaev et al. 2011). Simple sequence repeat (SSR) markers, also called microsatellites, have been developed for genotyping different accessions within the genus *Fragaria* (Bassil et al. 2006; Chambers et al. 2013; Gil-Azira et al. 2006; Govan et al. 2008; Monfort et al. 2006; Njuguna et al. 2011; Zorrilla-Fontanesi et al. 2010). Due to the reliability, the good transferability and the high variation detectable with this marker technology, SSR markers have been applied to describe the genetic diversity in strawberry cultivars as well as in diploid and octoploid *Fragaria* species. In addition, SSR markers were used to construct linkage maps, especially a reference genetic map for diploid strawberry based on the interspecific cross *Fragaria vesca* L. $\times$ *Fragaria bucharica* Losinsk. (FV $\times$ FB) (Sargent et al. 2006, 2008, 2009). Recently, an additional set of 152 SSR primer pairs has been developed for a better resolution of the *Fragaria* spp. reference genetic map and to improve the assembly of the scaffolds into pseudochromosomes for the diploid *F. vesca* genome sequence (Sargent et al. 2011). The diploid strawberry genome with 240 Mb is relatively small (Shulaev et al. 2011).

The strawberry represents an accessory fruit, consisting of an enlarged receptacle, which carries the true fruits in form of achenes (Perkins-Veazie 1995). The strawberry fruit can vary in its size and color. The red color of strawberries is created by three major anthocyanins, pelargonidin 3-glucoside, pelargonidin 3-rutinoside and cyanidine 3-glucoside (Gil et al. 1997; Kosar et al. 2004; Simirgiotis et al. 2009; Tulipani et al. 2008) that are produced via the flavonoid pathway during fruit ripening (Almeida et al. 2007; Fait et al. 2008). In white colored strawberries, these anthocyanins are reduced in the receptacle and in most cases also in the achenes (Cheel et al. 2005; Salvatierra et al. 2014; Simirgiotis and Schmeda-Hirschmann 2010). A number of white colored strawberry varieties are commercially available, e.g. the octoploid strawberry variety ‘Weisse

Ananas’ ($2n = 8x = 56$) or the diploid varieties ($2n = 2x = 14$), ‘Blanc Amélioré’, ‘Weiße Hagmann’, ‘Weiße Solemacher’, and ‘Yellow Wonder’. Interestingly, white strawberries are supposed to be lower in allergen content than red varieties and to be tolerated by allergy affected people (Alm et al. 2007; Hjærnø et al. 2006). In a study of six red and three white strawberry varieties the allergen content in the white fruits was always lower than in the red fruits (Alm et al. 2007). Food allergies against strawberries have been observed due to the IgE-cross reactivity between the birch-pollen allergen Bet v 1 and strawberry allergens (Musidlowska-Persson et al. 2007). Bet v 1, a protein of 17 kDa in birch (*Betula pendula* Roth), is the major allergen responsible for tree pollen allergy in humans affecting more than 100 million people worldwide, especially in North America and Europe (Mogensen et al. 2002; Vrtala et al. 2001). About 70 % of the patients with birch pollen allergy demonstrate IgE mediated food allergies against fruits like peach (*Prunus persica* [Batsch] L.), apple (*Malus domestica* Borkh.), strawberry (*F. $\times$ ananassa*) or vegetables like celery (*Apium graveolens* L.) and carrots (*Daucus carota* L.) (Guhsl et al. 2015; Hauser et al. 2010; Karlsson et al. 2004; Marzban et al. 2005). Allergy reactions to strawberries are mostly restricted to the oral cavity, so called oral allergy symptoms (OAS), and urticaria (Grattan and Harman 1985; Rodriguez et al. 2000; Taylor et al. 1996; Zuidmeer et al. 2006). First protein sequence information of a strawberry allergen, named Fra a 1, and additional isoforms came from Hjærnø et al. (2006). For octoploid strawberries (*F. $\times$ ananassa*), the genomic sequences of the allergen gene *fra a 1* were subsequently cloned for the varieties ‘Korona’, ‘Honeoye’ and an unknown, locally available variety (Musidlowska-Persson et al. 2007). Eight gene variants of *fra a 1* were identified resulting in five isoforms of the protein (Fra a 1A–Fra a 1E). In addition, Fra a 2 and Fra a 3 have been identified in the variety ‘Camarosa’, which showed 79 and 76 % homology to Fra a 1, respectively (Munoz et al. 2010). RNA silencing experiments demonstrated a direct link between *fra a* expression and the flavonoid biosynthesis (Munoz et al. 2010). Hairpin constructs of *fra a 1e* and *fra a 3* in the octoploid *F. ananassa* ‘Camarosa’ resulted in white fruits. Down-regulation of *fra a 1e* and *fra a 3* was accompanied with reduced transcript levels of phenylalanine ammonium lyase (PAL) and

chalcone synthase (CHS) (Munoz et al. 2010). On protein level, Hjærnø et al. (2006) had also observed a down-regulation of the allergen Fra a 1 in concert with enzymes of the flavonoid pathway in white strawberries. The hypothesis that Fra a proteins might be involved in the transfer of intermediates from one enzyme to the other in the flavonoid biosynthesis (Munoz et al. 2010) could be confirmed by affinity binding assays showing that intermediates of the flavonoid pathway have different affinities to Fra a proteins (Casanal et al. 2013). Quercetin-3-*O*-glucuronide binds to Fra a 1E, myricetin to Fra a 2 and (+) catechin to Fra a 3 with dissociation constants (K_d) in low micromolar ranges. However, it is so far unknown whether sequence differences in the *fra a* genes between white and red colored strawberries might be responsible for the white fruit color and/or reduced allergic reactions.

Other hypotheses are available regarding the cause of the missing anthocyanin pigmentation in white colored strawberries. Differences in the expression of structural (enzymes) or regulatory genes (MYB transcription factors) of the flavonoid biosynthesis pathway have been investigated to explain the white fruit color. The fruit color locus *c* was mapped together with one of the six investigated candidate genes, the flavanone 3-hydroxylase (F3H), on linkage group 1 using a F₂-population of the cross *F. vesca* L. ‘Yellow Wonder’ (YW, Europe) and *Fragaria nubicola* (Lindl. ex Hook.f.) Lacaita FRA520 by Deng and Davis (2001). This led to the conclusion that the wild type flavanone 3-hydroxylase might be essential for the red fruit color (Deng and Davis 2001). On the other hand, down-regulation of the anthocyanidin glucosyltransferase also led to reduced anthocyanin contents in ripened fruits (Griesser et al. 2008). Transcriptome analyses of flavonoid biosynthesis genes in *Fragaria chiloensis* subsp. *chiloensis*, which has a white colored fruit form *chiloensis* and a red colored fruit form *patagonica*, revealed a clear down-regulation of the flavonoid biosynthesis genes during the whole fruit development in the white colored fruits of *F. chiloensis* subsp. *chiloensis* (Salvatierra et al. 2010; Saud et al. 2009). Especially, low transcript levels of the UDP-glucose:flavonoid 3-*O*-glucosyltransferase may play a role in the missing pigmentation. In addition, reduced expression levels of MYB1 were detected in *F. × ananassa* (Saud et al. 2009) and correspondingly down-regulation of *FaMYB1* led to more anthocyanins

in *F. chiloensis* fruits (Salvatierra et al. 2013). Transient silencing of *FaMYB10* resulted in white strawberries (Medina-Puche et al. 2014). Even though artificial down-regulation experiments have shown the importance of single components of the flavonoid pathway for the development of the red fruit color, the molecular basis for the naturally occurring white fruit color in strawberries is still not well understood.

The role of *fra a 1* and *fra a 3* in the flavonoid biosynthesis and the involvement in allergic reactions against strawberries makes it important to look into the genetic diversity within these genes in *Fragaria*, especially in white and red colored fruit accessions. In this study, we used SSR markers to characterize commercially available diploid strawberry varieties bearing red and white colored fruits as well as a new accession VM2012 with white colored strawberries in order to distinguish the varieties and to apply specific SSR primer combinations for the verification of successful hybridizations between accessions, taking advantage of the co-dominant inheritance of SSR markers. In addition, regarding the mechanism(s) and the gene(s) involved in the white colored fruits in diploid strawberries, hybridizations of VM2012 and two varieties, ‘Weiße Hagmann’ and ‘Weiße Solemacher’, were made to see if complementation of the white colored fruits can be observed. In this case, different genes would be involved in the white fruit color. Finally, the genetic diversity in the *fra a 1* and *fra a 3* genes between red and white colored diploid strawberry accessions in comparison to red colored octoploid strawberry varieties was analyzed to get an insight into the evolution of octoploid strawberry (*F. × ananassa*) and into the role of *fra a 1* and *fra a 3* in the white fruit color as well as the allergen composition in strawberries.

Materials and methods

Plant material and DNA extraction

Commercial varieties, diploid as well as octoploid and a new accession VM2012, which had been family-owned for several decades and is locally available in Germany (Table 1), were cultivated in the green house (20 °C, 90–400 µE/m²s light intensity, 16 h/8 h day/night, 40 % humidity). In addition to the molecular analyses, the plant material was also characterized

Table 1 Overview of the strawberry varieties and the new accession VM2012

Variety/accession	Species	Fruit color	Runners	Origin/company
'Blanc Amélioré'	<i>Fragaria vesca</i> var. <i>vesca</i> L.	White	Yes	Gartenfachhandel König-Riesner, Rastede, Germany
'Mieze Schindler'	<i>Fragaria</i> × <i>ananassa</i> (Duchesne ex Weston) Duchesne ex Rozier	Red	Yes	Gardenandmore, Memmingen, Germany
New Accession VM2012	<i>Fragaria vesca</i> L.	White	Yes	Family owned Carola Mageney
'Rote Baron Solemacher'	<i>Fragaria vesca</i> var. <i>vesca</i> L.	Red	No	Deaflora, Werder, Germany
'Walderdbeere'	<i>Fragaria vesca</i> var. <i>vesca</i> L.	Red	Yes	Gartenfachhandel König-Riesner, Rastede, Germany
'Weiße Ananas'	<i>Fragaria</i> × <i>ananassa</i> (Duchesne ex Weston) Duchesne ex Rozier	White	Yes	Deaflora, Werder, Germany
'Weiße Hagmann'	<i>Fragaria vesca</i> var. <i>semperflorens</i> (Duchesne ex Rozier) Staudt	White	Yes	Deaflora, Werder, Germany
'Weiße Solemacher'	<i>Fragaria vesca</i> var. <i>semperflorens</i> (Duchesne ex Rozier) Staudt	White	No	Family Gaiser, Rot am See, Germany; Deaflora, Werder, Germany
'Yellow Wonder'	<i>Fragaria vesca</i> var. <i>semperflorens</i> (Duchesne ex Rozier) Staudt	White	No	Alsagarden, France

regarding fruit color and formation or absence of runners. DNA from the commercial varieties and the new accession VM2012 was extracted from very young strawberry leaves (less than 1 cm), harvested early in the morning to reduce the starch content, using the protocol of Porebski et al. (1997). DNA was finally dissolved in TE buffer and stored in the refrigerator or freezer (−20 °C).

Flow cytometry

Very young leaves (less than 1 cm large) were harvested and analyzed by Plant Cytometry Services, Schijndel, The Netherlands. DNA of *Ilex crenata* Thunb. 'Fastigiata' was used as internal standard.

SSR analyses

For the SSR analyses primer combinations published for strawberry by Govan et al. (2008) and Sargent et al. (2006) were used (Table 2). All forward primers carried an additional M13 tail (5'-TTTCCCAGTCAC-GACGTT-3') at the 5' end to apply the M13 tailing procedure (Oetting et al. 1995) for fluorescence labeling the PCR amplification products. For the PCR, 4 µL DNA (50 ng/µL) were added to 11 µL master mix. The master mix contained 0.2 mM

dNTPs, 1× PCR buffer (for FIREPol® DNA polymerase), 0.05 µM M13-IRD800 primer for labeling, 0.25 U FIREPol DNA polymerase (Solis Biodyne, Tartu, Estonia), and 0.05 µM each of forward and reverse primer. The PCR program was performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Darmstadt, Germany) starting with a denaturation at 95 °C for 5 min, followed by 36 cycles of 95 °C for 20 s, 50 °C for 20 s and 72 °C for 30 s; a final elongation step at 72 °C for 5 min. The PCR products were mixed with an equal volume (15 µL) of loading buffer (98 mL formamide, 2 mL of 0.5 M EDTA pH 8.0 and 37.5 mg bromophenol blue), denatured for 2 min at 94 °C and transferred to ice before loading 0.5 µL into each well for separation. The PCR products were separated on 6 % denaturing polyacrylamide gels using the DNA Analyzer Model 4300 (LI-COR Biosciences, NE, USA).

Cloning of *fra a 1* and *fra a 3* genes

The gene sequences for *fra a 1* and *fra a 3* were amplified by PCR using gene-specific primers (Table 3). The PCR mix for 25 µL STS-PCR reaction contained 82.5 ng DNA, 0.2 mM dNTP, 1× PCR-reaction buffer with MgSO₄, 2 U Taq Polymerase (NEB), 0.4 µM forward and reverse primer each. All

Table 2 SSR primer combinations used for the molecular analysis

SSR marker	Primer sequence ^a 5'-3'	Expected size in bp	LG	References
EMFn123	Forward: CATTTCCGGGCACACTTCC Reverse: AGACGGCAAAGAGACTCACC	219	1	Sargent et al. (2006)
EMFn125	Forward: CCAACCTAAACCATACCC Reverse: ATGGTTGCCTTTGATTACAG	222	1	Sargent et al. (2006)
EMFn136	Forward: TTTCTCTTTTGTCCATAGTTCC Reverse: TTCATCAGGATCCAGAAGTCC	242	2	Sargent et al. (2006)
EMFn148	Forward: TTACCTGCACAGAAACAACG Reverse: CAACTTCCTCCTCACTCACC	239	2	Sargent et al. (2006)
EMFn170	Forward: CAGTTTGCCCAACAACAAGG Reverse: TTGATGGCAACAAATCACG	188–238	3	Govan et al. (2008)
ChFaM023	Forward: AGGAGAAGACCGGCTGTGTA Reverse: TGCCTATAGCTGTGGCTGTG	161–200	4	Govan et al. (2008)
EMFvi136	Forward: GAGCCTGCTACGCTTTTCTATG Reverse: CCTCTGATTCGATGATTGCT	111–188	4	Govan et al. (2008)
EMFn152	Forward: GGGCCAAAATGAGTATCTTGC Reverse: TTAGAGCGAGGTGGTAATGC	155	5	Sargent et al. (2006)
EMFn238	Forward: TTTACTACAGAGCTGAAGCTACCC Reverse: GAAGAAGCCCATTATCAGAAGC	296	7	Sargent et al. (2006)
ARSFL11	Forward: GCGAAGCATAACTGGCAGTATCTG Reverse: GCGGGCCTAGGTGATCTTGGA	257–321	7	Govan et al. (2008)

^a Each forward primer contained in addition the sequence 5'-TTTCCCAGTCACGACGTT-3' for the M13 tailing

Table 3 Primer combinations used for the gene specific amplification of *fra a 1* and *fra a 3*

Gene	Primer sequence 5'-3'	NCBI accession no./Reference	Annealing temperature (°C)	Expected PCR product (bp)
<i>fra a 1</i>	Forward: ATGGGTGTTTACACTTATG Reverse: GTTGTATTGCTGGGGTG	AM236320.1	55	581
<i>fra a 3</i>	Forward: ATGGGTGTGTTACATACG Reverse: GTTGTATTCTCAGGATG	GQ148819.1	55	596

reactions were performed with the PCR cyclor program: 2 min, 94 °C denaturing, followed by 35 cycles (1 min 94 °C, 2 min 55 °C, 1 min 72 °C) and finished with 5 min of extension. PCR reactions were separated on 2 % agarose gels using 100 bp ladder (NEB Corporation, Ipswich, MA, USA) as marker.

PCR products were cloned using the pGEM®-T Easy Vector System I (Promega, Madison, WI, USA). Cloning was performed according to the manufacturer's instruction. Successful cloning of the PCR fragments was confirmed by PCR amplification using

T7 and SP6 primers. At least four, in most cases eight positive clones for each gene and each investigated variety were sequenced using T7 and SP6 primers by GATC or SeqLab.

The strawberry genome sequence (Shulaev et al. 2011) was accessed via the Genome Database for Rosaceae (GDR, <http://www.rosaceae.org>, Horn et al. 2005; Jung et al. 2014) or Phytozome (<http://phytozome.net>). BLAST (Altschul et al. 1997) was used for sequence alignment of the *fra a 1* and *fra a 3* genes with the *Fragaria vesca* pseudomolecules v1.1.

Results

Molecular characterization of strawberry varieties bearing red and white colored fruits as well as a new accession VM2012 using SSR markers

Ten selected SSR primer combinations (ChFaM023, EMFn123, EMFn125, EMFn136, EMFn148, EMFn152, EMFn170, EMFn238, EMFvi136, ARSFL11) covering six of the seven chromosomes were applied for the molecular characterization. Of special interest was whether the new accession VM2012 represents a unique accession or resembles one of the commercially available white and red colored fruit varieties. As no further information about VM2012 was accessible, a broad spectrum of commercial varieties from the European market was included in the study: two octoploid varieties ‘Mieze Schindler’ (red fruits) and ‘Weiße Ananas’ (white fruits) as well as four diploid varieties with white strawberries (‘Blanc Ameliore’, ‘Weiße Hagmann’, ‘Weiße Solemacher’, ‘Yellow Wonder’) and two diploid varieties ‘Rote Baron Solemacher’ and ‘Walderdbeere’ bearing red strawberries. All 10 SSR primer combinations gave polymorphic banding patterns for the strawberry varieties and the new accession (Fig. 1). The octoploid varieties were easily recognizable due to the, in most cases, more complex banding patterns. Three SSR markers ChFaM023, EMFn148 and ARSFL11 gave a unique banding pattern for VM2012. The SSR analyses demonstrated that VM2012 is different and not represented by the investigated commercial varieties. The white strawberry varieties ‘Yellow Wonder’, ‘Weiße Solemacher’ and ‘Weiße Hagmann’ showed very similar SSR patterns to each other. However, the white colored variety ‘Blanc Ameliore’ could be distinguished from the other white ones by seven out of 10 SSR primer combinations. Unique patterns for ‘Blanc Ameliore’ were obtained for EMFn123, EMFn170 and ARSFL11, which would allow identification of this variety.

Estimation of the ploidy level of the new accession VM2012 and testing of the cross ability with diploid and octoploid strawberry varieties

As the ploidy level of VM2012 was unknown, flow cytometry analyses were performed (Table 4). The

results identified VM2012 as a diploid species. In addition, the cross ability of the new variety with octoploid and diploid varieties was tested. None of the crosses with the octoploid strawberry varieties resulted in any seed set. However, VM2012 could be successfully crossed with diploid white strawberry varieties (‘Weiße Hagmann’, ‘Weiße Solemacher’) as well as with diploid red strawberry varieties (‘Rote Baron Solemacher’, ‘Walderdbeere’) (Table 5). To verify the crosses on the molecular level the progenies, mostly six plants, were analyzed using SSR primer combinations as shown in Fig. 2a. Plant no. 5 of the progenies VM2012 × ‘Rote Baron Solemacher’ proved to be a selfed progeny, but all others represented hybrids. All progenies of the cross VM2012 × ‘Weiße Solemacher’ were verified as hybrids (Fig. 2b). Table 5 gives an overview about the SSR primer combinations used for the verification of the crosses.

Fruit development and color

Regarding the fruit color, the combinations between VM2012 and ‘Weiße Solemacher’ as well as ‘Weiße Hagmann’ resulted in F₁-hybrids bearing white colored fruits indicating that the white fruit color cannot be complemented by the genetic constitution of the other two white colored fruit varieties. However, crosses with the red colored fruit varieties ‘Rote Baron Solemacher’ and ‘Walderdbeere’ resulted in F₁-hybrids with red colored fruits showing that the fruit color in the new accession is recessively inherited. The fruit development of VM2012 in comparison to ‘Rote Baron Solemacher’ is shown as well as the progenies of the successful hybridization of VM2012 with ‘Rote Baron Solemacher’ (Fig. 3). In combination, the results indicate that the molecular cause for the white fruit color in the new accession VM2012 and the varieties ‘Weiße Solemacher’ and ‘Weiße Hagmann’ might be similar as no complementation of the fruit color was observed.

Comparison of the sequences of the allergen genes *fra a 1* and *fra a 3*

Comparing the gene sequences of *fra a 1* (581 bp) from the diploid white fruit colored varieties ‘Blanc Ameliore’, ‘Weiße Hagmann’, ‘Weiße Solemacher’, ‘Yellow Wonder’, and VM2012 as well as the diploid

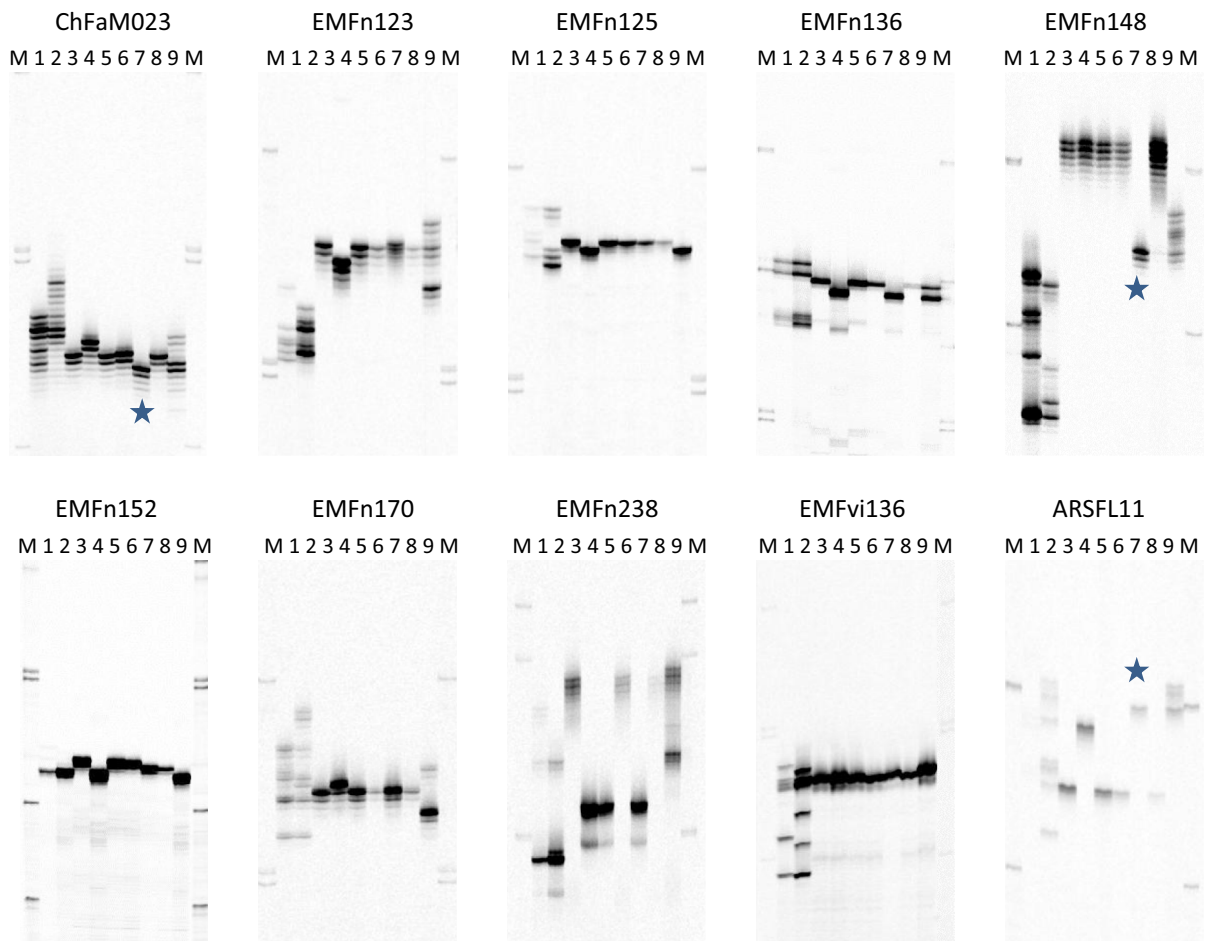


Fig. 1 Comparison of the 10 SSR patterns of the new accession VM2012 and six commercial varieties with red and white fruits. 1 ‘Mieze Schindler’, 2 ‘Weiße Ananas’, 3 ‘Yellow Wonder’, 4 ‘Blanc Amélioré’, 5 ‘Weiße Hagmann’, 6, ‘Weiße Solemacher’,

7 VM2012, 8 ‘Rote Baron Solemacher’, 9 ‘Walderdbeere’, M 50–350 bp IRD-Marker. Asterisks indicate unique banding patterns for the new accession VM2012

red colored fruit varieties ‘Rote Baron Solemacher’ and ‘Walderdbeere’ with the known gene variants of the octoploid *F. × ananassa* (Musidłowska-Persson et al. 2007), it could be demonstrated that only the gene variant *fra a 1 A2* was present (Fig. 4), independent of the fruit color. This gene variant is characterized by two distinct SNPs at position 117 and 323 that differentiate it from the gene variant *fra a 1 A1* and a deletion of 10 bp in the intron region that, apart from other SNPs, distinguishes *fra a 1 A1* and *fra a 1 A2* from all other gene variants *fra a 1 B-E*. The gene sequence of *fra a 1 A2* present in all investigated diploid varieties as well as VM2012 results in an amino acid sequence corresponding to Fra a 1 A (160 aa) as described by Musidłowska-Persson et al. (2007). The allergen gene *fra a 1* showed 100 %

homology (identities 581/581) to *gene07080* on LG4 at position 20343368..20342788 using BLAST against the *Fragaria vesca* pseudomolecules v1.1.

Analyzing *fra a 3* of ‘Rote Baron Solemacher’, ‘Walderdbeere’, ‘Blanc Amélioré’, ‘Weiße Hagmann’, ‘Weiße Solemacher’, ‘Yellow Wonder’ as well as of VM2012 on genomic level we amplified a gene of 596 bp that contained an intron of 119 bp at a conserved position (185–303) (Fig. 5). The gene encodes a protein of 159 amino acids. The allergen gene *fra a 3* showed 100 % homology (identities 596/596) to *gene07082* on LG4 at position 20349804..20350399 using BLAST against the *Fragaria vesca* pseudomolecules v1.1. However, in all investigated diploid accessions a unique SNP at position 503 (T to C) was present in comparison to the cDNA clone of *fra a 3* of the octoploid variety

Table 4 Ploidy analysis of very young *Fragaria* leave samples of the new accession VM2012 in comparison to commercial varieties (Plant Cytometry Services, Schijndel, The Netherlands)

Variety/accession	DNA ratio ^a (±standard deviation)	Ploidy
‘Blanc Amelioré’	0.226 ± 0.01	Diploid
‘Rote Baron Solemacher’	0.226 ± 0.01	Diploid
‘Walderdbeere’	0.226 ± 0.01	Diploid
‘Weiße Hagmann’	0.230 ± 0.00	Diploid
‘Weiße Solemacher’	0.226 ± 0.01	Diploid
‘Yellow Wonder’	0.220 ± 0.02	Diploid
‘Mieze Schindler’	0.787 ± 0.01	Octoploid
‘Weiße Ananas’	0.783 ± 0.01	Octoploid
New accession VM2012	0.226 ± 0.01	Diploid

^a n = 3, Based on internal standard of *Ilex crenata* Thunb. ‘Fastigiata’

‘Camarosa’ (Munoz et al. 2010), which is so far the only sequence available for *fra a 3* for octoploid varieties in the NCBI database (Accession GQ148819.1). This SNP has no influence on the protein sequence, which is the same as in the variety ‘Camarosa’.

Discussion

Microsatellite markers for distinction of varieties and identification of hybrids

Genotyping strawberry accessions bearing red and white colored fruits using SSR markers revealed that most of the white colored diploid cultivars (‘Weiße

Hagmann’, ‘Weiße Solemacher’ and ‘Yellow Wonder’) were very similar. Only ‘Blanc Amelioré’ and the new accession VM2012 showed unique SSR patterns. SSR markers were successfully used to identify hybrids produced between VM2012 and ‘Weiße Hagmann’, ‘Weiße Solemacher’, ‘Rote Solemacher’ or ‘Walderdbeere’ in order to study the genetics of the white fruit color. No complementation of the white fruit color could be observed in the hybrids of VM2012 with ‘Weiße Hagmann’ and ‘Weiße Solemacher’ indicating a similar genetic mechanism behind this recessively inherited trait. As breeding for yield and fruit size has narrowed the genetic basis of the cultivated strawberry (Hancock et al. 2010; Olbricht et al. 2008), the new accession VM2012 might offer a possibility to introduce new traits into the breeding pool. Flow cytometry analyses and the successful crosses confirmed the diploid level of VM2012. Flow cytometry had previously also proven useful in comparing different ploidy levels of *Fragaria* species and in characterizing *F. iturupensis* at the NCRG (National Clonal Germplasm Repository, Corvallis, Oregon) as decaploid species (Hummer et al. 2009). The complex SSR patterns observed in the octoploid varieties ‘Mieze Schindler’ and ‘Weisse Ananas’ pose a challenge for further genetic analyses. For research with octoploid strawberries, the application of allele dose based assessments of microsatellites is recommended (van Dijk et al. 2014). Nevertheless, the high transferability of SSR markers in the *Rosaceae* family has made microsatellites a valuable tool for genotyping in this family (Bassil et al. 2006; Chambers et al. 2013; Dantec et al. 2010; Gianfranceschi et al. 1998; Sajer et al. 2012).

Table 5 Successful hybridizations of VM2012 with other diploid varieties

Trait	Progeny			
	VM2012 × RBS ^a	VM2012 × FV ^b	VM2012 × WH ^c	VM2012 × WS ^d
Fruit color	Red	Red	White	White
Runners	Yes	Yes	Yes	Yes
Molecular verification	EMFn136	EMFn152	EMFn152	EMFn136
	EMFn148			EMFn148
	ChaFaM023			ChaFaM023

^a RBS ‘Rote Baron Solemacher’

^b FV ‘Walderdbeere’

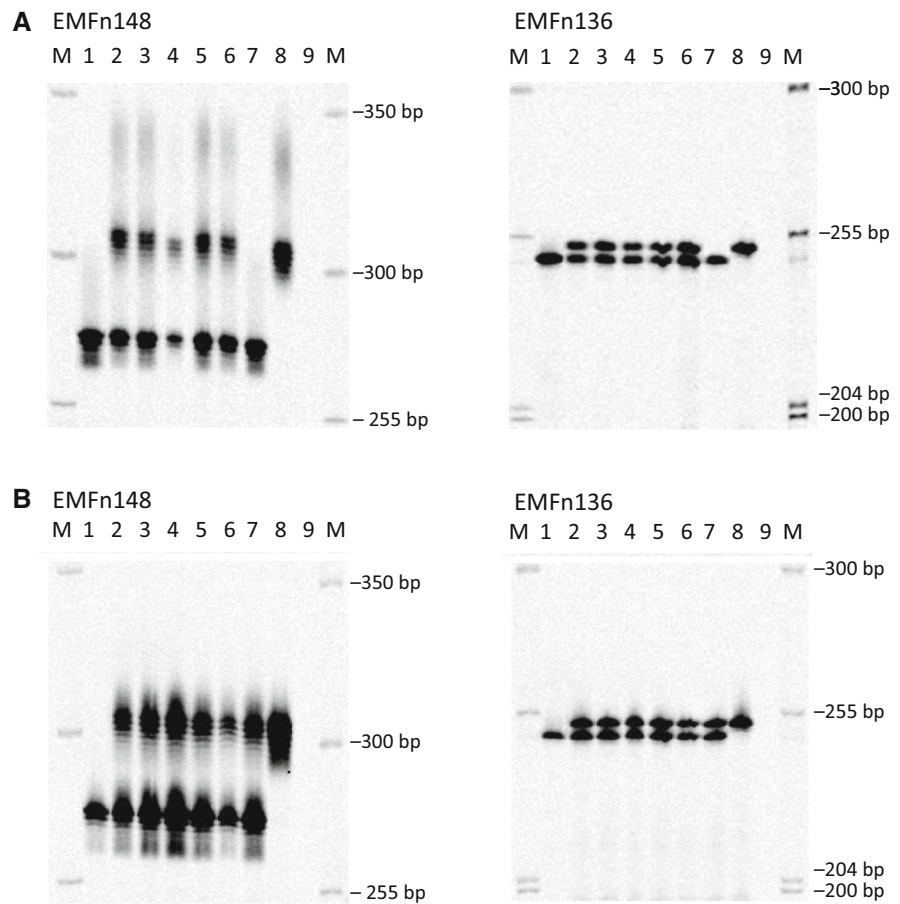
^c WH ‘Weiße Hagmann’

^d WS ‘Weiße Solemacher’

Fig. 2 Molecular verification of the cross combinations with SSR markers EMFn136 and EMFn148.

a VM2012 × ‘Rote Baron Solemacher’,

b VM2012 × ‘Weiße Solemacher’. Lanes: 1 VM2012, 2–7 six progenies, 8 ‘Rote Baron Solemacher’, 9 H₂O, M 50–350 bp IRD-Marker



Genetic diversity in the *fra a 1* and *fra a 3* genes and its implication for the phylogeny of octoploid strawberries

To analyze the genetic diversity in the *fra a 1* and *fra a 3* gene, we sequenced the genes from diploid red and white colored fruit varieties as well as VM2012 and compared the sequences to published sequences of octoploid strawberries (*F. × ananassa*). From the eight gene variants of *fra a 1* present in octoploid strawberries as described by Musidlowska-Persson et al. (2007) only one gene variant *fra a 1* A2 exists in the investigated diploid varieties as well as in VM2012. This means that Fra a 1 A as potential allergen would be produced in diploid strawberries. Fra a 3 is also identical in diploid strawberries and ‘Camarosa’. However, as there are no differences in the coding sequence between red and white fruited diploid strawberry varieties there are no indications

that would justify the assumption that white colored strawberries would produce less or no allergen encoded by *fra a 1* or *fra a 3*. This does not exclude that differences in the expression levels of these genes might play a role in the white color of the strawberries. Knock-downs of the allergen genes *fra a 1* and *fra a 3* have led to white colored fruits with reduced allergen content (Munoz et al. 2010). The tight connection between the Fra a proteins and the control of the flavonoid biosynthesis pathway (Casanal et al. 2013; Hjærnø et al. 2006; Munoz et al. 2010) means that breeding for strawberries with no Fra a 1 and Fra a 3 would result in white colored fruits. However, it is noteworthy to mention that *fra a 1* (*gene07080*) and *fra a 3* (*gene07082*) are located on linkage group 4, whereas the fruit color locus *c* had been mapped on linkage group 1 (Deng and Davis 2001).

From the evolutionary standpoint, it is interesting that the highly conserved gene variant of *fra a 1*



Fig. 3 New accession VM2012, ‘Rote Baron Solemacher’ as well as the cross combination of the two. **a** Fruit development of ‘Rote Baron Solemacher’, **b** fruit development of VM2012,

c fruits of the cross VM2012 × ‘Rote Baron Solemacher’, **d** VM2012, **e** successful cross combinations VM2012 × ‘Rote Baron Solemacher’

present in the diploid *F. vesca* varieties is identical to *fra a 1 A2* in octoploid strawberries (*F. × ananassa*). Phylogenetic studies grouped diploid strawberries into three main clades, named X, Y and Z (Rousseau-Gueutin et al. 2009). *F. vesca* L. and *F. mandshurica* Staudt were located as close relatives in subclade Y1, whereas *F. viridis* Weston was placed into subclade Y2. Clade X included the six diploid species *F. daltoniana* J. Gay, *F. nilgerrensis* Schltdl. Ex J. Gay, *F. nipponica* Makino, *F. nubicola* (Lindl. Ex Hook.f.) Lacaita, *F. pentaphylla* Losinsk. and *F. yezoensis* (H. Hara) Kitam. Finally, clade Z contains one Japanese species, *F. iinumae* Makino. These clades with slight variations correspond to A (Y), B (Z), and C (X) based on chloroplast DNA and rDNA, respectively (Njuguna et al. 2013; Potter et al. 2000). Regarding the phylogeny of the octoploid genome the postulated models AAA'A'BBB'B' for *F. × ananassa*, *F. virginiana* and *F. chiloensis* (Bringhurst

1990) or the more recently published Y1'Y1'Y1''Y1''ZZZZ or Y1Y1Y1Y1ZZZZ for *F. iturupensis* Staudt, *F. virginiana* and *F. chiloensis* (Rousseau-Gueutin et al. 2009) indicate the contribution of two to four diploid sources to the phylogeny of octoploid strawberries (Hummer et al. 2011). There exists evidence for the contribution of *F. vesca* in combination with *F. mandshurica* as maternal donor and *F. iinumae* as paternal parent (Rousseau-Gueutin et al. 2009; Staudt 2009). The tetraploid *F. orientalis* Losinsk., as a potential ancestral candidate of the octoploids, could be Y1Y1Y1Y1 or Y1'Y1'Y1''Y1'' depending on an auto- or allopolyploid hypothesis, in which Y1 would represent the *F. vesca* or the *F. manshurica* genome and Y1' and Y1'' would correspond to *F. vesca* and *F. mandshurica* genomes (Rousseau-Gueutin et al. 2009). In addition, a combination of *F. vesca* with *F. nubicola* and *F. orientalis* has been discussed (Davis et al. 2007; Potter et al.

A1	ATGGGTGTTTACACTTATGAAAACGAGTTCACCTCCGATATCCAGCACCTAAGTTGTTCAAGGCCTTCGTCTCGATGCCGACAACTCATCCCCAAGATTGCACCTCAGGCAGTAAAG	120
A2	T.....	120
B1	A.T.....	120
B2	A.T.....	120
C	A.T.....	120
D	A.T.....	120
E1	T.....	120
E2	T.....	120
A1	TGGCCGAGATCCTCGAAGGAGATGGCGGCCCGGAACCATCAAGAAGATCACCTTTGGTGAAGGTATAACT-----CTCAATCCATTTTACGTATATTTCAATTTGGTAATT	230
A2	C.....GATTTACCCT.....	230
B1	C.....GATTTACCCT.....	240
B2	C.....GATTTACCCT.....	240
C	C.....GATTTACCCT.....	240
D	C.....GATTTACCCT.....	240
E1	C.....GATTTACCCT.....	240
E2	C.....GATTTACCCT.....	240
A1	TTGGACCGTAGTAAATATATGCTCAGTCCCTTTTTTACATTGGAACGACAGGCAGCCACTACGGGTACGTCAGCACAAGATCCACTCTATTGACAAAGAGAACCACACCTACAGC	350
A2	C.....	350
B1	C.....	360
B2	C.....	360
C	C.....	360
D	C.....	360
E1	C.....	360
E2	C.....	360
A1	TACAGTTTGATCGAGGGAGATGCCTTGTGACACAACATCGAGAAGATCGACTACGAGACCAAGTTGGTATCAGCTCCTCAGGAGGAACCGTCATCAGACCACCAGTACACACAC	470
A2	A.....	470
B1	A.....	480
B2	A.....	480
C	A.....	477
D	A.....	480
E1	T.....	480
E2	T.....	480
A1	AAGGGAGATGTCGAGATCAAGGAAGAGCACGTCAGGCCGCAAGAGAAGGCCTCGACCTCTTCAAGCTCATTGAGGGATACCTCAAGGACACCCCGCGAATACAACCTAA	584
A2	A.....	584
B1	A.....	594
B2	A.....	594
C	A.....	591
D	A.....	594
E1	C.....	594
E2	C.....	594

Fig. 4 Comparison of the sequences of allergen gene *fra a 1* in the varieties ‘Rote Baron Solemacher’, ‘Walderdbeere’, ‘Blanc Amélioré’, ‘Weiße Hagmann’, ‘Weiße Solemacher’, ‘Yellow Wonder’ as well as of the new accession VM2012 with the

known sequences of the octoploid *F. × ananassa* (Musidowska-Persson et al. 2007). The boxed sequence represents the only gene variant *fra a 1* A2 present in all investigated diploid accessions

CA	ATGGGTGTTTACATACGAATCCGAGTTCACCTCAGTCATCCACCACCTAAGTTGTTCAAGGCCTTCGTCTCGATGCCGACAACTCATCCCCAAGATTGCCCTCAGGCTGTGAAG	120
FV		120
RBS		120
BA		120
WH		120
WS		120
YW		120
SX		120
CA	AGTGCTGAAATCATTGAAGGAGATGGAGGTGTTGGAACCATCAAGAAGATCCATCTTGGTGAAG-----GTTAGTCAAATTAAGTAAATATTATCGTCATTAGTAAGTAGTAAGTTGTTGAAT	185
FV		240
RBS		240
BA		240
WH		240
WS		240
YW		240
SX		240
CA	-----GAAGTGAATACAGCTACGTGAAGCATAAGATTGATGGAATCGACAAAGACAACCTCG	241
FV		360
RBS		360
BA		360
WH		360
WS		360
YW		360
SX		360
CA	TGTACAGCTACAGTATCATCGAGGAGATGCTATCGGAGACAAGATTGAGAAGATCTCCTATGAGATTAAGTTGGTAGCATCCGGTGGAGGTCCATCATCAAGAGCACCAAGTCACTACC	361
FV		480
RBS		480
BA		480
WH		480
WS		480
YW		480
SX		480
CA	ACACCAAGGGTGAGTTCGAGATTAAAGGAAGACATGTTAAGGCCGAAAGAAAGAGCCGCTGGTCTGTTCAGATCATTGAGAACCCTCTTGCCCATCCTGAGGAATACAAC	477
FV		596
RBS		596
BA		596
WH		596
WS		596
YW		596
SX		596

Fig. 5 Comparison of the sequences of the allergen gene *fra a 3* of the varieties ‘Rote Baron Solemacher’ (RBS), ‘Walderdbeere’ (FV), ‘Blanc Amélioré’ (BA), ‘Weiße Hagmann’ (WH), ‘Weiße Solemacher’ (WS), ‘Yellow Wonder’ (YW) as well as of the new

accession VM2012 (VM) with the published cDNA sequence of the octoploid variety ‘Camarosa’ (CA) (*F. × ananassa*, accession GQ148819.1)

2000). *F. vesca* is widely regarded as maternal donor of the A/Y genome of the octoploid strawberry (Davis et al. 2009; Njuguna et al. 2013; Rousseau-Gueutin et al. 2009), whereas *F. iinumae* could be the donor of the Z genome. Our investigations show that the gene variant *fra a 1* A2 of the octoploid varieties (*F. × ananassa*) might have its origin in the diploid *F. vesca* as this unique sequence for *fra a 1* was present in all investigated diploid varieties of *F. vesca*. It would be interesting to see whether the gene variants *fra a 1* B–E, present in *F. × ananassa*, originate from *F. iinumae* and/or other *Fragaria* species.

Dissection of the octoploid strawberry genome by deep sequencing in comparison with the genomes of *F. iinumae*, *F. nipponica*, *F. nubicola*, *F. orientalis* and the already available *F. vesca* genome sequence might further elucidate the origin of the octoploid genome of *F. × ananassa* (Hirakawa et al. 2014). Using dense targeted capture linkage maps, Tennessen et al. (2014) studied the evolution and dynamics of octoploid strawberry subgenomes. In contradiction to the existing theories that postulate two *F. vesca*-like subgenomes (Rousseau-Gueutin et al. 2009; Sargent et al. 2012), only one *F. vesca* subgenome (Av) and three subgenome of *F. iinumae* (Bi, B1, and B2) were postulated (Tennessen et al. 2014). The hypothesis here is that a *F. vesca*-like diploid hybridized with a *F. iinumae*-like diploid forming an allotetraploid (AvAv-BiBi). This form then hybridized with a not yet identified *F. iinumae*-like autotetraploid (B1B1B2B2) to form the octoploid ancestor of *F. chiloensis* and *F. virginiana* (Tennessen et al. 2014).

Additional allergene genes in strawberry and implications for breeding

Regarding the allergen genes in strawberries, the availability of the whole genome sequence of *F. vesca* (Shulaev et al. 2011) proved that the situation is even more complex. Hyun and Kim (2011) identified by an in silico approach 15 putative allergen genes (*Fra v1* family) representing PR-10 proteins, one of these *gene07082* (*Fra v1.08*) corresponds to *fra a 3* as described by Munoz et al. (2010). However, Hyun and Kim (2011) missed in the in silico approach one allergen gene, *gene07080*, probably because of the incorrect gene structure given in the *F. vesca* pseudo-molecule v1.1 assembly. This *gene07080* corresponds

to the only gene variant of *fra a 1* present in *F. vesca*, which is identical to *fra a 1* A2 in octoploid strawberries (Musidlowska-Persson et al. 2007). In addition to the 16 allergen genes of the PR10 family (named *Fra v1.x*), five allergen genes (named *Fra v3.x*) belonging to the nonspecific lipid transfer protein (nsLTP) family and another five allergen genes (named *Fra v4.x*) as part of the profilin group are present in the *F. vesca* genome sequence (Hyun and Kim 2011). It will be interesting to see how all putative allergen genes will fit into the picture and how these genes evolved in octoploid strawberries. Whole genome and transcriptome analysis between low allergen and high allergen containing strawberry fruits will be necessary to unravel the whole story of the allergen genes and their role in the allergic reaction of patients as well as the link to the white fruit color. There still remain several open questions regarding the allergen potential of strawberries and the connection with the white fruit color. However, recent advances in strawberry research have provided tools to study gene functions directly in strawberry (Folta 2015).

Breeding strawberries with high nutritional value for human health (Giampieri et al. 2015; Tulipani et al. 2009) and favorable aroma compounds (Olbricht et al. 2011) should also have an eye on low allergen content. As *Fra a 1* and *Fra a 3* are linked to the flavonoid biosynthesis, total elimination of these gene products would lead to white strawberries. However, considerable variation in the *Fra a 1* content has been described for red colored strawberries depending on the variety and growing conditions (Tulipani et al. 2011) indicating that *Fra a 1* and *Fra a 3* might not be limiting in the flavonoid biosynthesis, which would allow a reduction of both proteins without losing the red fruit color. Screening for strawberry accessions with low content of *Fra a 1* and *Fra a 3* by immunological methods would be necessary to identify and breed strawberries with reduced potential for allergic reactions.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical standards The authors comply with the ethical standards.

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