Current Progress in Trans- and Cisgenic Apple and Strawberry Breeding

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Abstract

A summary is presented of the state-of-the-art in apple and strawberry biotechnological research going on in the department of Plant Breeding at Wageningen University and Research Centre. In apple, the research directed towards the introduction of scab resistance by inserting a barley gene has reached the stage of a field trial where performance of transgenic lines could be tested in an orchard situation. The development of a marker-free system allowing the removal of undesired sequences when desired will lead to the generation of cisgenic apples carrying only newly introduced apple genes. Knocking out the major allergen in apple by RNAi technology has decreased the allergenic reaction in sensitive patients. In strawberry, progress was made in studying firmness and flavor using genetic modification and antisense technology and introducing resistance to *Botrytis* following an intragenic approach where a strawberry promoter is used to provide a new expression pattern of a strawberry gene.

INTRODUCTION

At Wageningen-UR Plant Breeding breeding programmes in apple and strawberry have been running now for many decades resulting in some excellent cultivars grown predominantly in north-western Europe. In the recent past the breeding programme was extended with modern biotechnological research, e.g., on the development of molecular markers for marker assisted breeding, on gene function analysis and genetic modification. The ultimate goal of breeding through genetic modification (GM) entails that future GM cultivars should be put on the market. Therefore, our research in apple and strawberry, but also in potato, provided the basis for a discussion and a view on the application of GM technology in plants at Wageningen University and Research Centre (Wageningen-UR). This goal also led to collaboration with other faculties and universities on topics such as public perception, transparency and on communication on new technologies. Wageningen-UR has also dialogues with the Ministry of the Environment on new technologies, rules and regulations and implementation. Amongst others this led to the formulation of a classification scheme of GM plant categories related to potential environmental risks and the concept of cisgenesis. Here, the emphasis will be on the technological progress that was made with apple and strawberry in our Plant Breeding Group.

APPLE

Scab Resistance

About one third of the amount of chemical crop protectants applied every year in The Netherlands is used in apple cultivation. 80% of this is applied to avoid damage and yield losses caused by apple scab (data from CBS). The fungal causal agent of this disease is *Venturia inaequalis*. The number of applications (chemical sprays) required to ensure the desired yields can be as high as 30 per growing season; even in organic apple

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cultivation up to 30 times spraying with sulphur is needed to reduce apple scab incidence. In 2001 the Dutch agricultural sector together with the government formulated as a target the reduction of the use of agrochemical protectants in 2010 to a level of 5% of that of 1998. This target is a very ambitious one that will not be reached in apple, unless resistant high quality cultivars become available on a short-term. Classical breeding for scab resistance made use of resistance to *Venturia* found in the crab apple *Malus floribunda* 821 in the USA in 1953 (Hough et al., 1953). It took some 45 years before cultivars reached the market that showed resistance and were of sufficient fruit quality. Unfortunately, already after approximately 5 years of cultivation, this resistance was recently broken by the fungus (Parisi et al., 1993; Parisi et al., 2006). Because breeding through crossings in apple is slow and monogenic resistance can be easily broken, other techniques and strategies are desperately required.

In 1995 hordothionin, a small protein isolated from the endosperm of barley, was found to inhibit the in vitro growth of *Venturia inaequalis*. The gene coding for this protein was isolated and transferred in 1996 to three apple cultivars; 'Elstar', 'Gala' and 'Golden Delicious' by genetic modification. GM shoots were rooted and transferred to the greenhouse where they were assayed for resistance against apple scab. This assay was repeated with graft-propagated promising lines (10-15 repetitions). Six lines proved to be significantly less susceptible compared to the controls (non-GM individuals of the same cultivar or GM individuals lacking the hordothionin gene). The difference was either in scab incidence (n=5) or in delayed incubation period (n=1) (Table 1). These results were obtained with greenhouse assays.

Next questions were how these more resistant lines (Table 1, bold printed lines) would behave in an actual field or orchard situation and how stable the trait would be over multiple years of cultivation. In order to determine this, a permit for a field trial was applied for with the appropriate Ministry (the Ministry of Housing, Spatial Planning and the Environment, VROM) in the year 2000. After coping with several hurdles, the trees that were multiplied by grafting on non-GM rootstocks could be planted legally in 2003. The permit for the field trial was finalized in 2005 after being challenged several times by a.o. Greenpeace. Despite damage inflicted by activists in the years 2004 and 2005, results on scab resistance could be obtained over a period of 4 years. The summarized data of three years are presented in Table 2.

Four of the original six lines were found to be consistently and significantly less scab susceptible than the controls (Table 2 and Fig. 1). The best performing GM-lines showed 60% reduction in symptom development. The best performing line in the trial was cultivar 'Santana', the resistant control, with 0% symptoms. This cultivar, that was generated by classical breeding, carries the *M. floribunda Vf* gene. In conclusion, the barley *hth*-gene has a significant effect, but is on its own not enough to avoid scab totally. The apple *Vf*-gene works best, but unfortunately the *Vf*-gene is broken by some isolates of the fungus. Therefore, there still is a need for new resistance genes, either from other crops or from apple itself. Stacking of such genes should result in durable resistance which can be achieved much more rapidly by genetic modification than by classical breeding in apple.

Functional genes against apple scab that are to be stacked, preferably originate from plant genomes. A study of Lusk and Sullivan (2002) has shown that only 20% of the respondents when asked for their preference indicated that they would eat GM-food carrying genes of bacterial origin, which happen to be all of the present-day, conventional GMOs. The percentage willing to eat GM-food increased to 80% when the extra genes would be derived from the original species to be modified. This picture was confirmed in informal discussions that were organized by Plant Research International (PRI, Wageningen) with different stakeholders. Based on this and on Article 4 paragraph 2 of Directive 2001/18/EC of the EU, which states that the use of antibiotic resistance markers for selection have to be phased out completely by 31 December 2008, led to the formulation of a new strategy in GMO production for food and feed by PRI. GM products meant to enter the market and the environment at some point in time made by PRI would

be ‘marker-free’ and would be preferentially equipped with plant-derived, species-own genes regulated by their own regulatory sequences (promoter-terminator) or by plant-derived expression signals.

For resistance to apple scab the available genes were hordothionin (from barley), linalool synthase (from strawberry), *Vf*-genes (from apple) and other new apple-derived resistance genes. Expression signals that can be used are the Rubisco small subunit gene promoter (and terminator) of chrysanthemum, that drives gene expression in green tissues, ibid. from apple and the native promoters of the apple resistance genes. The use in apple of *Vf* or other apple-derived resistance genes with their own natural promoters in GM-plants without undesired genes, such as selection markers, would lead to a class of GM-plants that are very similar to scab resistant genotypes obtained by classical plant breeding. We have named this form of GM ‘cisgenesis’. Cisgenesis is defined as genetic modification of a recipient plant with a natural gene from a crossable - sexually compatible - plant. Such a gene includes its introns and is flanked by its natural promoter and terminator in the normal sense orientation. Schouten et al. (2006a,b) have argued that because of the similarity to products of classical breeding this type of GMOs could be exempted from Directive 2001/18/EC. If this were the case, implementation of cisgenesis in the production of food and feed GM plant varieties within Europe might result in an increase in acceptance of GM technology altogether by EU consumers and would become affordable for small and medium sized enterprises. Cisgenesis does in no way imply that transgenesis is dirty or unsafe. It is a powerful strategy to break through the European status-quo with respect to GM plant regulations and public perception.

Marker-Free Technology

For the generation of GM end products that are free of antibiotic resistance genes or any other undesired genes, several technical approaches can be followed (Krens et al., 2004). Firstly, only the genes-of-interest can be integrated into the T-DNA transferred to the plants, without the use of any selectable markers. This approach requires high gene transfer and regeneration frequencies. Success has been reported in just a few cases in particular cultivars in potato and apple (Vetten et al., 2003; Malnoy et al., 2007). A second approach is cotransformation and subsequent segregation by sexual crossing. In this approach the genes-of-interest are offered to recipient plant cells on a separate T-DNA than the selection gene(s). When the two T-DNAs are integrated on separate loci or chromosomes, they can be separated by crosses and segregation in the subsequent progeny. For vegetatively propagated crops or crops with a long life cycle this route is not convenient. For the latter, a DNA-excision system based on induced recombination followed by negative selection, has been developed in our group.

Our DNA-excision system employs a gene coding for a site-specific recombinase (R) from *Schizosaccharomyces rouxii* that recognizes two recombination sites (Rs) for excision. In order to keep the recombinase inactivated at initial selection stages it is linked to the ligand binding domain (LBD) from a glucocorticoid receptor. The LBD forms complexes with cytoplasmic factors preventing the enzyme-LBD combination from passing the nuclear envelope. The complex will stay within the cytosol. Treatment with dexamethasone (Dex) will result in binding of Dex to the LBD and in subsequent release of the cytoplasmic factors, thereby allowing movement of the recombinase into the nucleus where it will exert its function. A dual positive-negative selection cassette allows selection of transformed cells on antibiotics first (positive selection) and after the Dex treatment successful recombination events can be selected for by the use of fluorocytosin (FC) (negative selection). This non-toxic compound is converted into the cytotoxic fluorouracil (FU) by cytosine deaminase (*cod*A; negative selection). Cells containing the non-recombined T-DNA will still carry the *cod*A gene and, therefore, cannot survive growth on FC medium; cells that carry a recombined T-DNA and thus lack the *cod*A gene will survive FC selection. A model vector was prepared (Fig. 2) in which the 35S promoter driving expression of the R gene was placed outside the Rs sites, as was a promoter-less GUS coding sequence. In this way, the 35S promoter is combined with the

GUS coding sequence after recombination and successful recombination can be visualized by GUS staining. This system was developed in tobacco and potato and was positively tested on apple and strawberry (Schaart et al., 2004). A new vector was constructed with all undesired gene and regulator sequences between the Rs sites and with a multiple cloning site between the borders but outside the Rs sites allowing easy insertion of the gene(s)-of-interest. After recombination all that is left in the GM plant are the remnants of the T-DNA borders, one Rs site and the gene(s)-of-interest (Fig. 2).

For our work in apple we used as gene-of-interest the hth-gene under regulation by the chrysanthemum or apple Rubisco promoter/terminator (all plant-transgenic) and the *Vf*-genes regulated by their own promoter/terminator (all apple-cisgenic). The transcription factor MYB10 stimulating the enhanced generation of health-promoting flavonoids have been isolated from a specific apple cultivar leading to red flesh (Espley et al., 2007) and will be used in a cisgenic approach, as well.

Allergenicity

Apple contains proteins homologous to the birch pollen allergen Bet v 1. This apple protein, designated Mal d 1, is recognized by Bet v 1-specific IgE antibodies that are present in the blood of patients allergic to birch pollen. This cross-reactivity can cause adverse reactions to apples in 70% of such patients. Because the Mal d 1 represents a gene family consisting of approximately 18 members and because the contribution of each member to the sensitivity is unknown, breeding for hypoallergenicity is very complex. Reduction of overall Mal d 1 expression levels by RNA interference and genetic modification seems so much faster with a higher chance for success. For this, an intron-containing Mal d 1 gene was isolated from the cultivar 'Gala' and used for the construction by PCR of a sequence coding for an intron-spliced hairpin RNA. The coding sequence was under expression control by the 35S promoter and introduced into the cultivar 'Elstar' by transformation. Transgenic plants showing a normal phenotype were selected (n=17) and some were used for further characterization (n=9). Six (out of 9) proved to contain the construct and of those six, five showed a strongly reduced expression of Mal d 1 in their leaves. Immunoblotting and skin prick tests with three patients showed significantly reduced cross-reactivity (Fig. 3) and reduced allergenicity respectively. Producing hypoallergenic apple cultivars by genetic modification using RNAi seems feasible (Gilissen et al., 2005).

STRAWBERRY

Fruit Firmness

In strawberry fruit firmness is an important quality trait in the fresh market as well as in the food processing market. Controlling fruit firmness by modifying expression levels of genes coding for cell wall degradation enzymes seems obvious, but might be a difficult task. Many enzymes and genes are involved in modifying the cell wall during fruit development and softening of fruits. They can act in a synergistic fashion and they can take over each other's role to some extent.

In our group DNA microarray technology was used to investigate differential expression of genes that might be involved in determining fruit firmness. For this, a firm cultivar, 'Holiday', and a soft cultivar, 'Gorella', were selected and mRNA was isolated from them at the ripe stage of fruit development. DNA microarrays carrying 1701 cDNAs isolated from ripe 'Elsanta' fruits were used to screen for genes that were differentially expressed between the two chosen cultivars (Salentijn et al., 2003). Results from the microarrays were confirmed by Northerns. Several genes associated to cell wall metabolism were found to be differentially expressed. They encompassed genes involved in lignin biosynthesis, e.g., cinnamoyl CoA reductase (CCR) and genes coding proteins involved in cell wall degradation such as polygalacturonase (PG), endo-β-1,4-glucanase (Cel1), pectinesterase-like and expansin (Fig. 4). To determine their role in fruit firmness an antisense (AS) approach was set up.

For testing the applicability of the AS approach in strawberry, the gene coding for the chalcone synthase (CHS) was used as a target. CHS is a key enzyme involved in the flavonoid biosynthesis pathway, leading to the formation of anthocyanins, responsible for the red pigmentation of ripe strawberry fruits. Reduction of the CHS mRNA production to levels ranging from 2 to 43% of the control could be achieved in cultivar 'Calypso' by introducing a 440 bp antisense fragment to the 5'-end of the CHS gene (Lunkenbein et al., 2006a). The phenotype was stable for more than 4 years in 5% of the transgenic plants. The reduced level of CHS activity led to lower levels of anthocyanins, flavonols and other flavonoids. Instead of entering the flavonoid biosynthesis pathway, the precursors were shunted to the phenylpropanoid pathway. Pigmentation was only affected when CHS transcript levels were below 5% of the control expression levels.

One of the first candidates for application of AS strategy to improve fruit firmness was Cel1 which was differentially expressed between a soft and a firm cultivar, with the highest expression found in the firm cultivar 'Holiday'. In order to understand the role of endo-β-1,4-glucanases (Cel1 and Cel2) in fruit ripening, AS constructs were prepared and introduced into cultivar 'Calypso', either alone or combined (Palomer et al., 2006). Only transgenic plants with a significantly reduced level of Cel1 protein accumulation could be obtained. In those plants the extractable level of EG-ase activity was down to 40% of that of the controls, however, fruit firmness was not affected. Hence, Cel1 is not the main determinant of fruit firmness and its reduction in activity was possibly compensated for by other cell wall modifying enzymes.

In a second series of experiments a multiple AS approach was chosen. AS fragments of genes coding for expansin (*FaExp2*), polygalacturonase (*Fa-sPG* and *FaPG-like*), pectate lyases (*FaPel1* and *FaPel2*) were combined and placed together under control of a single 35S promoter and introduced in strawberry cultivar 'Calypso' (E.M.J. Salentijn, unpublished). From the transgenic plants produced, individual lines were selected that showed a reduction in expression levels of one or several enzymes of more than 40%. One line showed a significant increase in firmness that was also stable and uniform (Fig. 5). In this line, CW34, the expression was reduced to levels of 4-11% for *Fa-sPG*, 21-33% for *FaPG-like*, 3-29% for *FaPel2* and 10-11% for *FaExp2*. Only for *FaPel1* it was not possible to reduce the expression to levels below 40% of the control.

In order to study the relationship between firmness and aroma, the aroma of fruits from different transgenic lines was compared by a panel test: 11 out of 12 panelists marked fruits of the two different transgenic lines with increased fimness as having a recognizable different aroma (E.M.J. Salentijn and W. Schwab, unpublished). GC-MS analysis of these fruits has to be performed to characterize differences in volatile composition in transgenic fruits. In our breeding programs it has been suggested that a negative correlation might exist between firmness and flavor. Our results with the multiple AS cell wall modifying genes might be a first indication for such a correlation, but more research is needed on this topic.

Flavor

More than 300 compounds determine the aroma of the strawberry fruit. One of the major determinants is the volatile flavor component furaneol (4-hydroxy-2,5-dimethyl-3(2H)-furanone, HDMF). HDMF is methylated by a O-methyltransferase into 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF). This odorous compound is disliked by flavorists. The effect of antisense downregulation of the *Fragaria* × *ananassa* O-methyltransferase (*FaOMT*) gene on HDMF and DMMF concentrations has been studied (Lunkenbein et al., 2006b). In a series of AS-FaOMT-lines the *FaOMT* gene expression was down regulated to mRNA levels ranging from 75 to 3% of the control. The ratio between HDMF and DMMF did not change when the level of FaOMT mRNA was reduced to 45% of the control, but in three lines that had levels down to 8% or less of the control the ratio of HDMF to DMMF changed from 68:32 in the control to 99:1 in transgenic fruit.

Hence, this study shows the possibility to modify the ratio of odorous furanones

and provides a basis for the improvement of strawberry flavor through a GM approach.

Botrytis cinerea Resistance

As part of an EU project entitled "Sustainable production of transgenic strawberry plants. Ethical consequences and potential effect on producers, environment and consumers", QLK5-CT-1999-01479, genetic modification was used as a way to engineer fruit rot resistant strawberries. As a candidate gene for conferring *Botrytis* resistance the gene coding for the Polygalacturonase Inhibiting Protein (PGIP) was selected. This choice was based on in vitro inhibition studies by the protein of polygalacturonases of fungal origin. In addition, introduction of the PGIP isolated from pear and introduced into tomato gave reduced *Botrytis* colonization (Powell et al., 2000). The *FaPGIP* gene was isolated from strawberry and expression was checked during strawberry fruit ripening. Highest expression was found during the red, ripe stage and this was further enhanced after exposure to *Botrytis*. However, the expression levels reached apparently were insufficient for resistance. The *FaPGIP* coding sequence was combined with the 35S promoter in order to overexpress *FaPGIP*. When leaves of overexpressing plants were tested for resistance against *Botrytis*, an enhancement of this trait was observed (Fig. 7).

The next step was to put the coding sequence under control of an endogenous strawberry gene that is very active in red, ripe fruits. For this, the promoter from a fruit-specific expansin gene (*FaExp2)* was chosen. The performance of these transgenic plants with all strawberry-derived gene coding and regulatory sequences in disease assays is presently under investigation (L. Mehli, NTNU, Trondheim, Norway, pers. commun.).

Cisgenesis

Within the framework of the same EU project mentioned in the previous paragraph sociological studies were performed by interviewing 720 consumers in Great Britain, Norway and Denmark and by probing their perception of GM in general, of GM-food and of GM-strawberries. Up till 60% of the respondents indicated that they would buy GM strawberries when they would be grown using less pesticides or when they would be more health beneficial. More than 70% could completely or at least partly agree with the statement that 'it is more acceptable that one moves genes inside a species rather than moving them between different species' (Fig. 8).

In our concept of removing selectable marker genes, using preferentially genes and regulatory sequences from the species itself, exemplified by the strawberry with the *FaPGIP* gene regulated by the *FaExp2* promoter, one of the partners involved in the EU project first used the term cisgenic for this type of GMOs rather than transgenic. The present definition as used by us (see paragraph on scab resistance) is a bit narrower because of the aim to exempt this type of GMOs from EU regulations under Directive 2001/18/EC, referring to the similarity of cisgenic plants to conventionally bred plants.

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Tables

Table 1. Representation of greenhouse scab resistance tests with eleven transgenic (*hth)* and four control apple clones.

Clone	Scab incidence (%)	P(a)	P(b)	Incubation period (days)	P(a)	P(b)	N
First series							
Elstar-hth 4	47.2	***		10.6	*		13
Elstar-hth 6	**55.3**	***		**10.4**			**16**
Elstar non-tr.	69.7			9.9			13
Gala-hth 7	71.7			10.5			13
Gala-hth 13	69.6			10.2			15
Gala-hth 15	**58.0**			**11.3**	***	***	**12**
Gala non-tr.	67.3			10.5			9
Gala-21 (nptII GUS)	63.8			10.4			14
Santana non-tr.	0.0						10
Second series							
Gala-hth 53	80.0			9.2			10
Gala-hth 56[1]	**44.7**	***	***	**10.4**		*	**14**
Gala-hth 61	71.9	**	*	9.7			9
Gala-hth 64	79.9			9.6			14
Gala-hth 65	**67.7**	***	***	**10.2**		**	**12**
Gala-hth 68	**59.6**	***	***	**10.3**		***	**13**
Gala-hth 69	82.5			9.4			12
Gala-hth 78	80.1			9.4			13
Gala non-tr.	83.3			10.0			6
Gala-21 (nptII GUS)	80.4			9.5			15
Santana non-tr.	0.0						12

Significance of difference according to Student's t-test: P(a)=compared to non-transformed control, P(b) compared to transformed control containing nptII and Gus. * P<0.05; ** P<0.01; *** P<0.005. [1] Same as **Gala-hth 60**, also taken for the field trial, as all the **bold**-printed ones.

Table 2. Field scab resistance data from three years of scoring.

Line	Scab incidence 2004/2005/2006	Difference	%-age relative to the mean of the controls
Elstar-hth 6	3.43	bc	100.0
Elstar control	3.41	bc	-
Gala-hth 15	2.55	ab	67.5
Gala-hth 56	1.50	a	40.0
Gala-hth 60	1.45	a	39.5
Gala-hth 65	3.46	bc	92.0
Gala-hth 68	2.02	a	53.5
Gala controls	2.70	c	-

The resistant cultivar 'Santana' showed no symptoms. Pairwise testing, homogeneous groups in t-probability of 5%.

Figures

Fig. 1. Field performance of transgenic (left) and non-transgenic (right) apple lines.

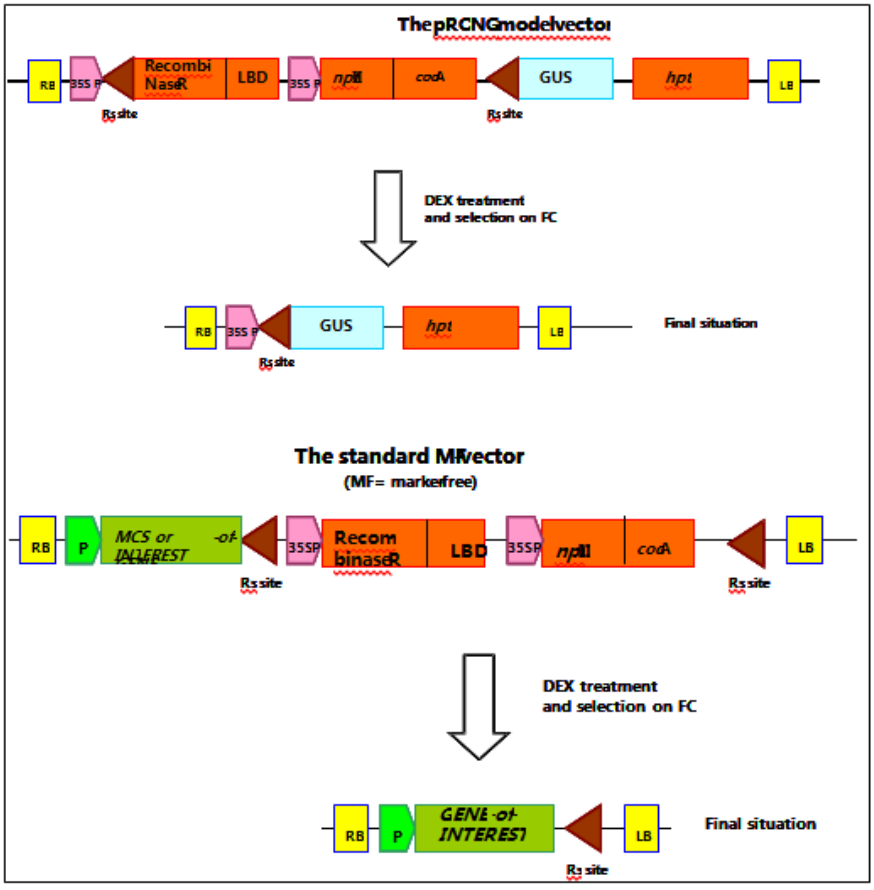

Fig. 2. Binary vectors for the generation of marker-free plants by excision.

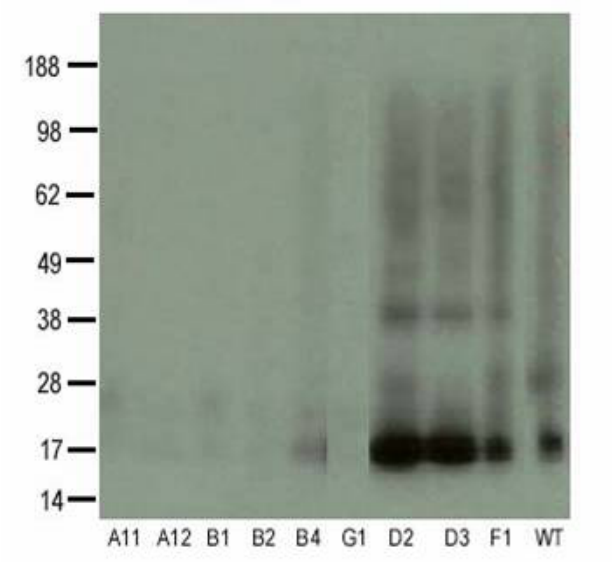

Fig. 3. Cross-reactivity of the monoclonal antibody 5H8 (directed to Bet v 1) in 6 transformants (A11-G1) and 4 controls (D2-WT). Reprinted with permission of Elsevier Ltd.

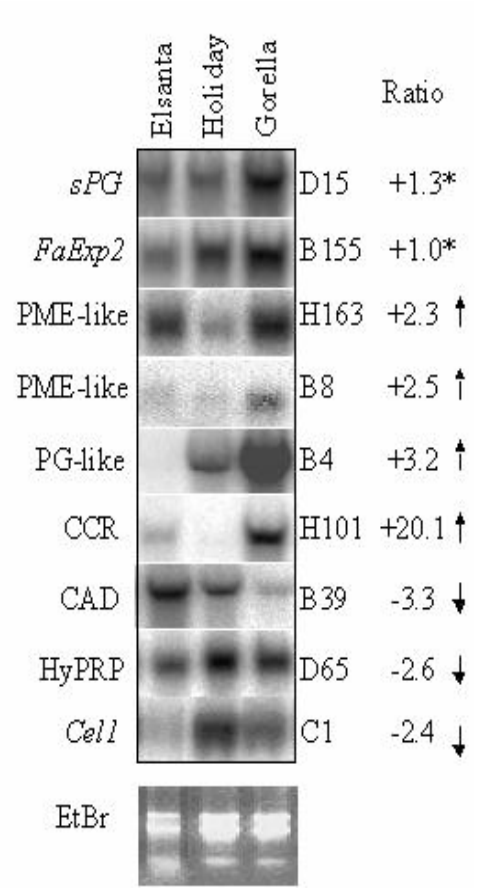

Fig. 4. Differential expression of genes involved in cell wall metabolism. Left, Northern; right, ratio of 'Gorella' (soft):'Holiday' (firm) obtained from cDNA microarray analysis. Reprinted with permission of Blackwell Publishing.

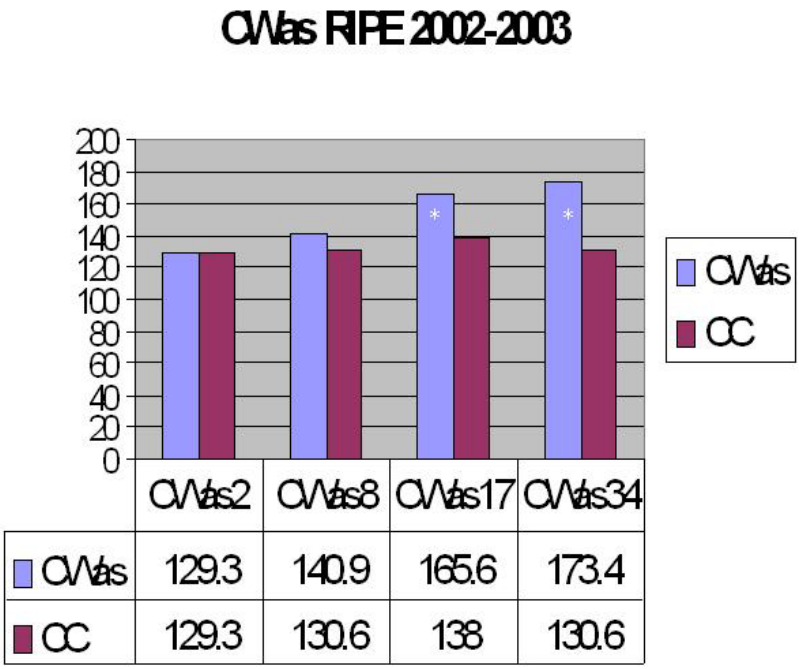

Fig. 5. Firmness measured by penetrometer for controls (CC) and 4 transgenic lines carrying the multiple AS construct (CWas). * means the difference is significant.

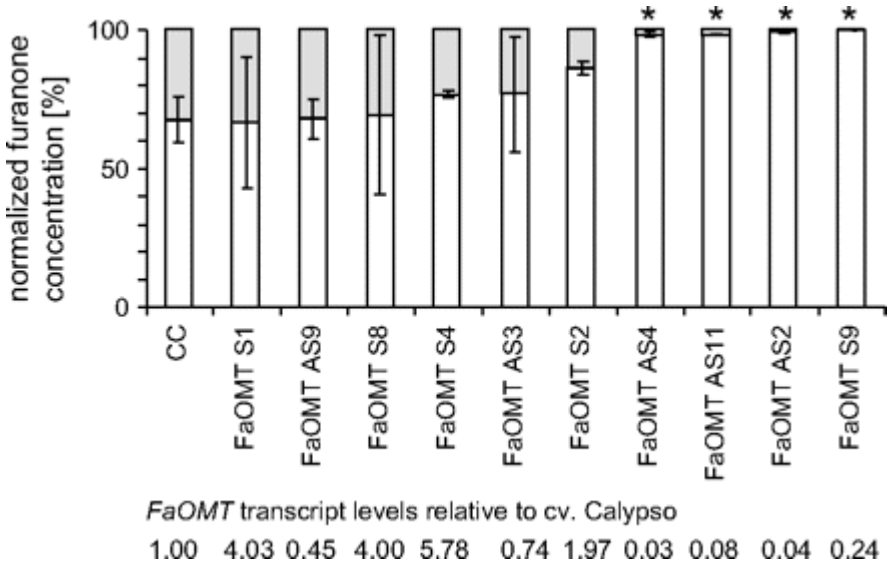

Fig. 6. Normalized concentration of HDMF (white bar) and DMMF (grey bar) in control fruit (CC) and fruit of plants transformed with the sense (FaOMT S) and antisense (FaOMT AS) constructs. Asterisks indicate that the data are significantly different from the data of control fruit (P<0.01). Reprinted with permission of Oxford University Press.

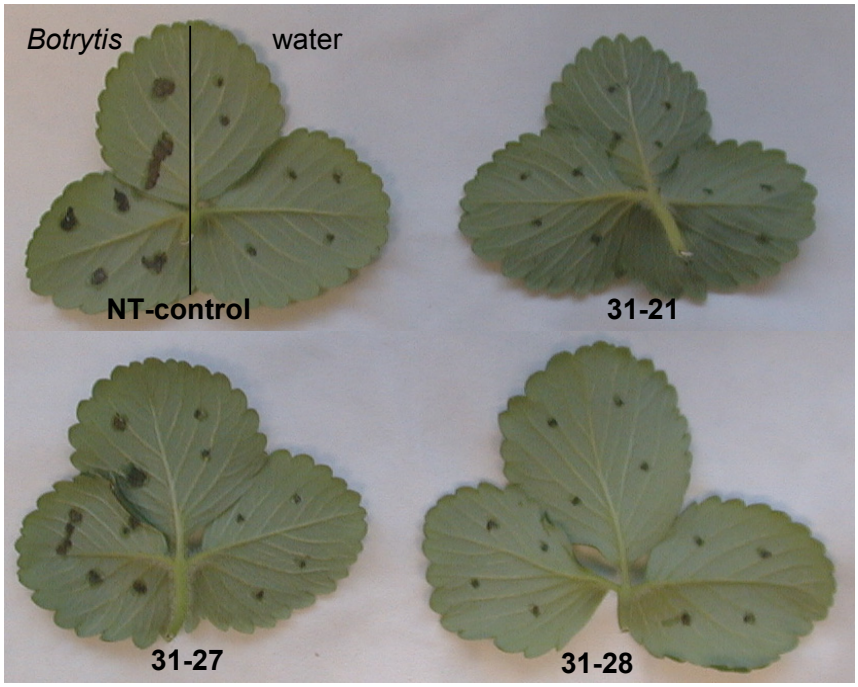

Fig. 7. Disease response on strawberry leaves of a control and 3 transgenic lines with the PGIP gene under control of the 35S promoter after inoculation with *Botrytis*.

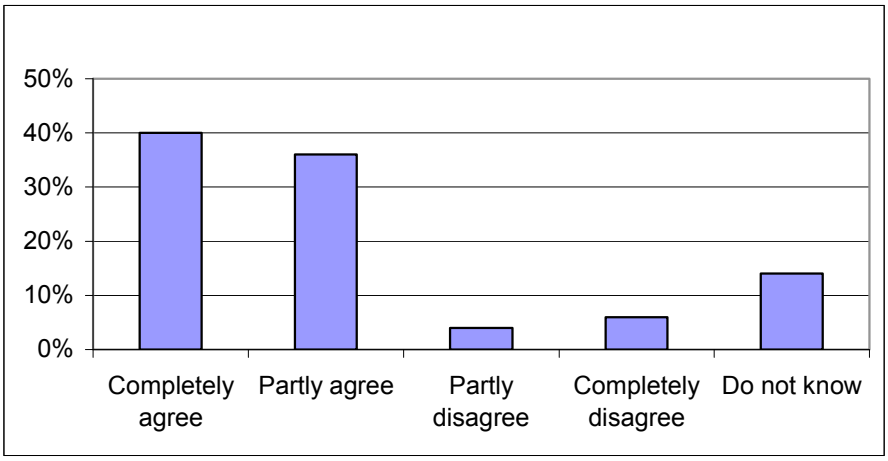

Fig. 8. Consumer's response to statement on acceptability (see text).