Towards the production of genetically modified strawberries which are acceptable to consumers

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Towards the Production of Genetically Modified Strawberries which are Acceptable to Consumers

Jan G. Schaart[1*] • Trygve D. Kjellsen[2] • Lisbeth Mehli[3] • Reidun Heggem[4] • Tor-Henning Iversen[5] • Henk J. Schouten[1] • Frans A. Krens[1]

[1] Wageningen UR Plant Breeding, Wageningen University and Research Centre. P.O. Box 16, 6700 AA Wageningen, the Netherlands
[2] Department of Biotechnology, Norwegian University of Science and Technology, 7491 Trondheim, Norway
[3] Faculty of Technology, Sør-Trøndelag University College, 7004 Trondheim, Norway
[4] Centre for Rural Research, 7491 Trondheim, Norway
[5] Department of Biology, Norwegian University of Science and Technology, 7491 Trondheim, Norway

Corresponding author: * **jan.schaart@wur.nl**

ABSTRACT

This manuscript discusses different aspects that are relevant to genetically modified strawberry plants with improved characteristics and 'acceptable' to consumers and growers of strawberry. It starts with a consumer acceptance survey, held in Norway, Denmark and the UK, studying public perception of genetic modification in general and specifically of genetically modified strawberries with altered properties. This study revealed that genetically modified plants are better accepted by consumers if only genes from the species itself are used for the genetic modification. Subsequently, the results of a functional analysis of the strawberry polygalacturonase inhibiting protein gene (*FaPGIP*) are described. This indicates that this gene is a possible candidate to induce resistance to *Botrytis cinerea* when upregulated in strawberry fruits. For this analysis, the *FaPGIP* gene was overexpressed in transgenic strawberry plants using the cauliflower mosaic virus 35S (*CaMV35S*) promoter. This showed that *FaPGIP* overexpression led to resistance to *Botrytis* in transgenic leaves. For the generation of intragenic (i.e. genetically modification using native genetic elements only) strawberry plants, a transformation vector was constructed in which *FaPGIP* was combined with a strawberry fruit-specific promoter and terminator that were isolated from a strawberry expansin gene (*FaExp2*). This vector also included elements that allow the elimination of (foreign) selectable marker genes after genetically modified plant lines have been established. Using this vector, genetically modified strawberry plants were produced that contained only genes from the species itself, and therefore these plants were called intragenic, rather than transgenic. Unfortunately, further evaluations of the intragenic strawberry plants could not demonstrate any enhanced level of resistance to *Botrytis* in fruits.

Keywords: genetic modification, intragenic, consumer acceptance, polygalacturonase inhibiting protein gene (*FaPGIP*), soft rot resistance, *Botrytis cinerea*
Abbreviations: ***FaPGIP***, strawberry polygalacturonase inhibiting protein gene; ***FaExp2***, strawberry expansin gene; ***CaMV35S***, cauliflower mosaic virus 35S

INTRODUCTION

Breeding for improvement of strawberry is difficult. Many traits, such as disease resistances, firmness and vulnerability of the fruit, productivity and of course its taste, have to be considered in the selection of a successful strawberry cultivar. In addition, genetic variation in *Fragaria. x ananassa* is very limited, while genetic variation is a prerequisite for progress in conventional breeding. Furthermore, breeding is hampered because strawberry is an octoploid, hybrid species, originating from a rather recent cross between two wild octoploid *Fragaria* species, *F. virginiana* and *F. chiloensis* (Darrow 1966). The complicated genetic constitution of the strawberry genome has kept most researchers from investing in the development of methods that could improve breeding of strawberry. Only a few years ago, the first results towards the production of a genetic map for strawberry have been published (Haymes *et al*. 2000; Lerceteau-Kohler *et al.* 2003), opening up possibilities for molecular marker-assisted breeding.

Another example of modern breeding technologies is genetic modification. In strawberry, the first genetic modification protocols were developed in the early 90ties (James *et al*. 1990; Nehra *et al*. 1990a, 1990b) and this approach has gained increasing interest over the last decade (Debnath and Teixeira da Silva 2007). In principle, genetic modification allows a relatively quick improvement of existing important strawberry cultivars, for example, by the introduction of disease resistance genes. However, the availability of suitable genes and specific regulatory sequences that will result in desired improvements has been the rate-limiting step until recently. Identification and isolation of such genes and sequences still requires specific investments, but comes more and more within our reach with the ever increasing power of DNA sequencing techniques. Furthermore, the public attitude toward genetically modified crops in general is, at least in Europe, still sceptic, hampering the introduction of genetically modified strawberries in the immediate future. In addition to this, strict regulations, like the EU Directive 2001/18/EC, require very expensive testing to warrant environmental and food safety, and thereby limit the use of this modern technology by small and medium-sized enterprises. Nevertheless, for many important crops transformation methods have been developed, many genetically improved lines have been produced and several transgenic crops have been commercialized and are grown on a world-wide scale (ISAAA 2008).

CONSUMER ACCEPTANCE OF INTRAGENIC CROPS

In the multidisciplinary EU-project entitled '*Sustainable production of transgenic strawberry plants. Ethical consequences and potential effect on producers, environment and*

Received: 27 February, 2010. Accepted: 21 August, 2010.

Original Research Paper

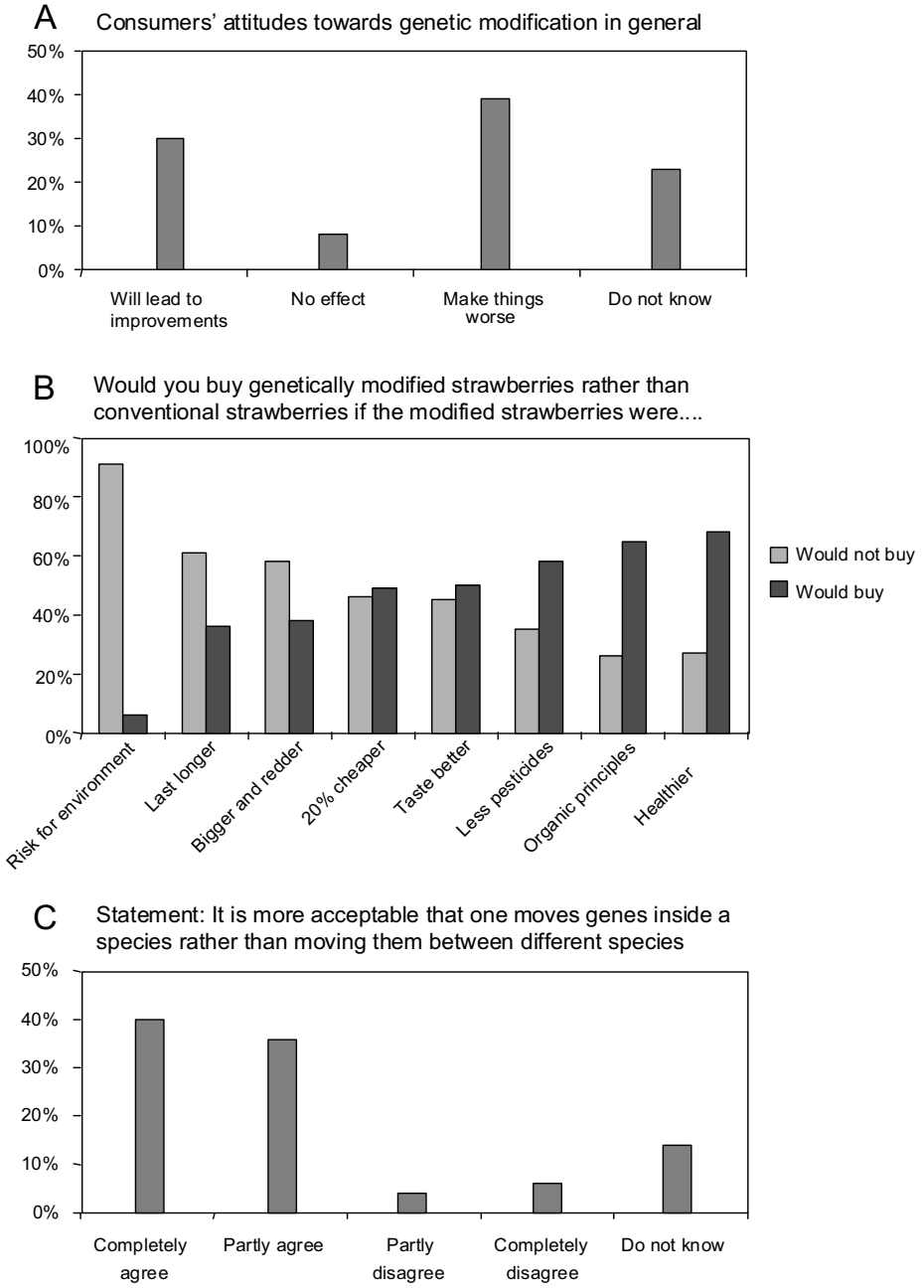

Fig. 1 Sociological inquiry among 720 consumers in Norway, Denmark and the UK.

consumers' (QLK5-CT-1999-01479) one of the aims was to produce genetically modified strawberry plants with enhanced levels of resistance towards *B. cinerea.* This would be attained by enhancing the expression level of the *PGIP* (polygalacturonase inhibiting protein) gene which was known to give resistance towards *Botrytis* in transgenic tomato plants in which a *PGIP* gene from pear was introduced (Powell *et al*. 2000). To enhance consumer and producer acceptance of genetically modified strawberry plants, it was considered desirable that only genes and regulatory elements from strawberry itself were used for the improvement and that the ultimate genetically modified strawberry plants were completely free of any foreign regulatory and coding DNA sequences. Nielsen (2003) introduced the term intragenesis for this condition. In case solely species-own DNA is used for the genetic modification of a plant, he proposed to call such plants intragenic rather than transgenic. Rommens (2004) and Rommens *et al*. (2004, 2007) elaborated on this topic in several articles in which they reviewed crop improvement using the plants own DNA only. In the EU-project mentioned above, also the attitude of consumers toward genetic modification in general, and particularly towards genetically modified strawberries, was monitored (study performed in 2002-2003). In this survey it was shown that the attitude of consumers in Norway, Denmark and the UK towards genetic modification in general was rather negative (**Fig. 1A**), but in more specific cases, regarding genetically modified strawberry plants that had undergone different hypothetical modifications, consumer acceptance increased when traits beneficial to consumers could be introduced (**Fig. 1B**). Furthermore, it was shown that modifications involving the use of strawberry-own DNA exclusively (**Fig. 1C**). This latter finding was confirmed by a consumer's survey in the USA, which showed that the majority of the respondents would eat vegetables with an extra gene from the same species or from another vegetable species, while this was only a minority in case viral genes had been used (Lusk and Sullivan 2002; Lusk and Rozan 2006).

GENETIC MODIFICATION USING SPECIES-OWN DNA SEQUENCES

The above mentioned sociological studies suggested rela-

tively high levels of public acceptance of genetically modified crop plants that have only genes from the species itself or from a cross-compatible species. In such genetically modified crop plants the introduction of native DNA sequences is referred to as intragenesis or cisgenesis. In cisgenesis the newly introduced DNA is a natural genome fragment, containing a gene of interest together with its own introns, 5′- and 3′-untranslated regions and regulatory elements (promoter and terminator) (Schouten *et al*. 2006). Like cisgenesis, intragenesis also uses donor gene sequences from the species itself or from a natural crossable donor species, but in intragenesis new genes can be created by combining functional genetic elements such as promoters, coding parts (with or without introns) and terminators of different natural genes, and insert this new chimeric gene into existing varieties (Rommens 2004; Rommens *et al*. 2004; Rommens 2007; Schouten and Jacobsen 2008).

ISOLATION AND CHARACTERISATION OF STRAWBERRY *PGIP*

For the ultimate production of intragenic or cisgenic crops the availability of specific genes and regulatory sequences within a species is a prerequisite. Up to date, for a number of plant species the complete genome sequence is available or will become available soon, which facilitates identification and isolation of the required gene and promoter sequences. However, for most crop species up till now, only limited information on genes and regulatory sequences is available and approaches like amplification using degenerated primers for the isolation of new genes and genome walking for the isolation of desired promoter and terminator sequences have to be employed (Agius *et al*. 2005). After isolation of species-specific gene and regulatory sequences, accurate functional characterisation of the sequences needs to be performed, in order to be able to anticipate the effects of the envisaged modification.

For the aimed introduction of *B. cinerea* resistance in strawberry, we focussed on the *FaPGIP* gene sequences from strawberry. Plant-pathogenic fungi, like *Botrytis*, produce cell wall degrading enzymes with which they attack the plant. Studies have shown that PGIP from a variety of origins is able to inhibit *B. cinerea* polygalacturonase (a cell wall degrading enzyme) activity *in vitro* (Sharrock and Labavitch 1994; Yao *et al.* 1995). It was also shown that introduction of a *PGIP* from pear into transgenic tomato plants resulted in an enhanced level of resistance towards *B. cinerea* (Powell *et al.* 2000). Richter *et al*. (2006) and Janni *et al*. (2008) also showed that overexpression from *PGIP* of raspberry or bean in transgenic pea and wheat, respectively, increased resistance to infections by fungal pathogens. Finally, the important role of PGIP in conferring resistance to *Botrytis* was demonstrated by antisense expression of *PGIP* in Arabidopsis, which reduced accumulation of PGIP and subsequently resulted in an enhanced susceptibility to *Botrytis* (Ferrari *et al.* 2006). This information suggested that for strawberry, overexpression of the *PGIP* gene would be a suitable option to achieve an enhanced *Botrytis* resistance level.

We isolated and characterised a *PGIP* gene from strawberry (Mehli *et al*. 2004; Schaart *et al.* 2005) and showed that in the natural situation this *FaPGIP* was expressed at relatively low level in leaves and immature fruit tissue, but that it was upregulated during strawberry fruit ripening. Inoculation of fruits with *B. cinerea* spores led to a rapid upregulation of *FaPGIP* expression to a level that, depending on the strawberry cultivar tested, was 4-40 times higher than found for the control red fruits. This upregulation was however transient and *FaPGIP* was downregulated again two days after inoculation. These observations prompted us to aim at modifying *FaPGIP* gene expression in such a way that sufficient FaPGIP activity would be present in *B. cinerea* susceptible tissues and stay present.

For functional analysis of *FaPGIP* in strawberry, we produced transgenic strawberry plants in which *FaPGIP*

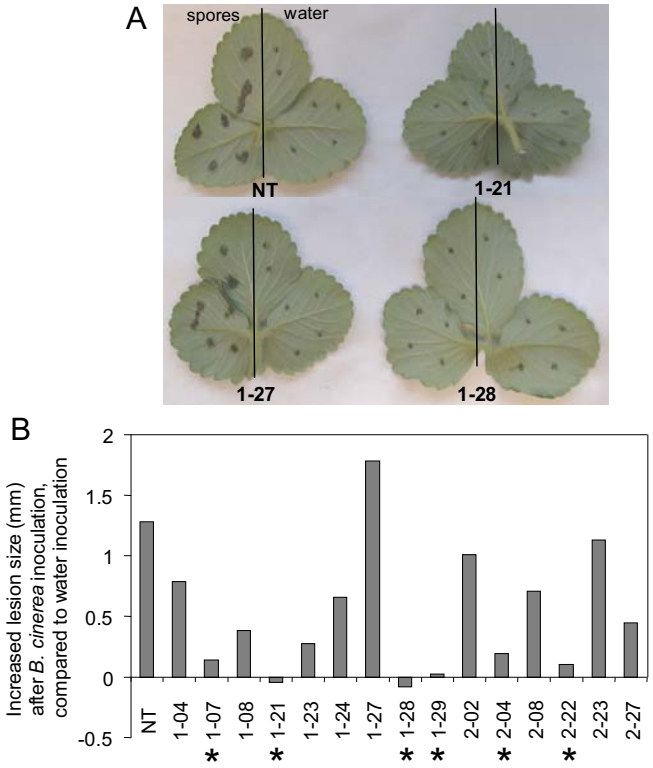

Fig. 2 *B. cinerea* colonisation test on detached leaves of non-transgenic control (NT) and genetically modified strawberry plants transformed with a construct containing *FaPGIP* under the regulation of the *CaMV 35S* promoter. Detached leaves were wounded with a needle, giving an approximately 1 mm diameter lesion. Two µl (10^5 spores/ml) of germinating *B. cinerea* spores (line BCNL) were pipetted on each wound. The left half of the leaf was inoculated with spores, while the right half was inoculated with water. For each transgenic line 3 leaves were inoculated at six positions per inoculum (spores vs. water) per leaf. Leaves were incubated in separate containers for 7 days, after which the diameter of each lesion was measured. (**A**) Example of *B. cinerea*-inoculated leaves, 7 days after inoculation. NT = non-transgenic control; 1-21, 1-27 and 1-28 are leaves from three different transgenic lines. (**B**) Average differences in lesion size (mm) of non-transgenic control (NT) and several transgenic lines. Difference is calculated with respect to the average of all water control lesion diameters (1.95 mm; SD= 0.54). Statistical analysis was done with two-way ANOVA. Transgenic lines marked with an asterisk differ significantly from the non-transgenic control at *P*-values < 0.05.

was overexpressed using the constitutive *CaMV35S* promoter. Because this promoter provides strong expression in strawberry leaf tissue (Schaart *et al*. 2011), its use allows early screening of *B. cinerea* resistance in transgenic strawberry leaf tissue. Inoculation of detached leaves of strawberry plants with *B. cinerea* showed that for a certain number of these transgenic plants, inoculation did not result in a significantly different reaction as compared to control (water) inoculations on the same leaf (**Fig. 2**), indicative for enhanced resistance. For non-transgenic control plants as well as for some of the transgenic plants, inoculation with *B. cinerea* resulted in a clear destruction of leaf tissue giving significantly larger lesions than the control (water) inoculations. These results indicated that overexpression of *FaPGIP* was able to confer resistance to *B. cinerea* in transgenic strawberry plants, at least in leaf tissue. The correlation between the level of resistance to *B. cinerea* and expression pattern and levels of *FaPGIP* was not investigated in these plants.

Because our ultimate aim was to achieve intragenic rather than transgenic strawberry lines, we did not induce flowering and fruiting of the transgenic plants in which the *CaMV35*S promoter was used to drive *FaPGIP* expression.

SELECTION OF SUITABLE STRAWBERRY GENE PROMOTER

In strawberry, primary *B. cinerea* infections take place through the flower after which the fungus remains latent in immature fruits. Once the strawberry fruit ripens, *B. cinerea* causes fruit rot which subsequently can lead to secondary infections of the so far unaffected other ripe and unripe fruits. In order to restrain *B. cinerea* in an effective way, *FaPGIP* upregulated expression should be extended at least into the ripe fruit stage, but preferentially also in flowers and immature fruits. In order to achieve an effective *FaPGIP* expression pattern, specific promoter sequences had to be identified. Initially, for a transgenic approach we focussed on the heterologous *CaMV35S* and the petunia *fbp7*-promoter sequences that were already available, and we tested these promoter sequences for their expression pattern in transgenic strawberry plants (Schaart *et al*. 2002). Both promoter sequences seemed to be able to direct expression of the β-glucuronidase reporter gene in flowers as well in different developmental fruit stages, and are, therefore, suitable to induce the intended upregulation of *FaPGIP*. However, to follow the intragenic approach, suitable promoter sequences have to be isolated from strawberry itself. For this purpose, a strawberry expansin gene, *FaExp2*, that showed fruit ripening-specific expression (Civello *et al.* 1999; Aharoni *et al.* 2002; Salentijn *et al.* 2003) was selected and its promoter was isolated and characterized using transgenic plants in which the promoter was fused to a *gus* reporter gene (Schaart *et al*. 2011). It was shown that the *FaExp2* promoter fragments regulated *gus* expression in a fruit-specific way, which was in agreement with the described *FaExp2* expression pattern. Interestingly, plants with the 1.6 Kb *FaExp2*-promoter fragment showed a much higher *gus* expression than a shorter 0.7 Kb *FaExp2*-promoter fragment. In order to achieve high levels of *FaPGIP* expression for inhibition of *B. cinerea* in the ultimate intragenic strawberry plants, the 1.6p*FaExp2*-fragment was considered to be most suitable and was subsequently chosen for further experimentation.

USE OF SELECTABLE MARKER-REMOVAL SYSTEM

For the efficient production of genetically modified plants the use of selectable marker genes is a prerequisite. In many transformation protocols either herbicide or antibiotic resistance genes have been shown to act as very effective selectable markers for genetically modified tissue and they have found wide application. However, public debate concerning health and environmental risks has focused particularly on such resistance genes, which make them undesirable in the final products. The public concerns have resulted in the development of selection methods which make use of alternative, less objectionable selectable marker genes. Such genes are mostly genes of bacterial origin, like the phosphomannose-isomerase gene which enables transgenic plants to proliferate on mannose, which cannot be metabolised by many plant species (Joersbo *et al.* 1998).

Next to the use of alternative selectable marker genes, systems have been developed which allow the elimination of selectable marker genes after they have been used. Such a marker removal system is especially valuable for vegetatively propagated crops, like strawberry, and for crops with long reproductive cycles. In view of the higher level of acceptance of genetically modified plants which are devoid of foreign gene sequences, the use of elimination systems is preferable to the use of alternative selectable marker genes. We therefore developed and tested a recombinase based system for elimination of undesired DNA sequences in strawberry (Schaart *et al.* 2005, 2010). We demonstrated that this method could be applied effectively using our standard strawberry transformation protocol and that by marker removal, marker-free plants could effectively be produced.

PRODUCTION OF INTRAGENIC STRAWBERRY PLANTS

In the end, the combined use of all aspects described above, the strawberry *PGIP* gene to confer resistance to *Botrytis,* the strawberry fruit-specific promoter from the *FaExp2* gene to direct gene expression to high levels in strawberry fruits and a marker-removal system for elimination of foreign DNA sequences from the predestined intragenic plants, enables the production of genetically modified plants which contain only gene and promoter sequences from strawberry itself. To demonstrate the possibility of producing such intragenic plants, we constructed a transformation vector in which *FaPGIP* was combined with regulatory sequences of *FaExp2.* For this, next to the 1.6 kb promoter also a 500 bp sequence fragment which is flanking the 3′-end of *FaExp2* was isolated and was used as terminator sequence (t*Exp2*). The 1.6p*FaExp2-FaPGIP*-t*FaExp2* chimeric gene was then introduced in the binary vector pMF1 for production of marker-free genetically modified plants (Schaart *et al.* 2011) (**Fig. 3**). In this binary vector an inducible recombinase gene and the bifunctional selectable marker gene are flanked by recombination sites. Chemical induction of recombinase activity enables recombination mediated removal of undesired gene sequences at the desired point in time. For a detailed description of the pMF1 vector and of the marker removal protocol, see Schaart *et al*. (2004, 2010). Using this vector for transformation of strawberry and for successive removal of the selectable marker and recombinase gene from the transgenic plants that were obtained, resulted in 14 putative intragenic strawberry plants. PCR analysis showed that in 11 out of 14 of these plants the new 1.6p*FaExp2-FaPGIP*-t*FaExp2* gene combination was present and that the selectable marker gene was successfully removed (data not shown) and that these plants could be labelled as intragenic. The presence of binary vector DNA (which is of foreign origin) was not checked in these putative intragenic plants. In similar experiments using a pMF1-based vector in strawberry transformation demonstrated however, that in a considerable number of transformed plants (up to 50%) pMF1 vector backbone sequences were

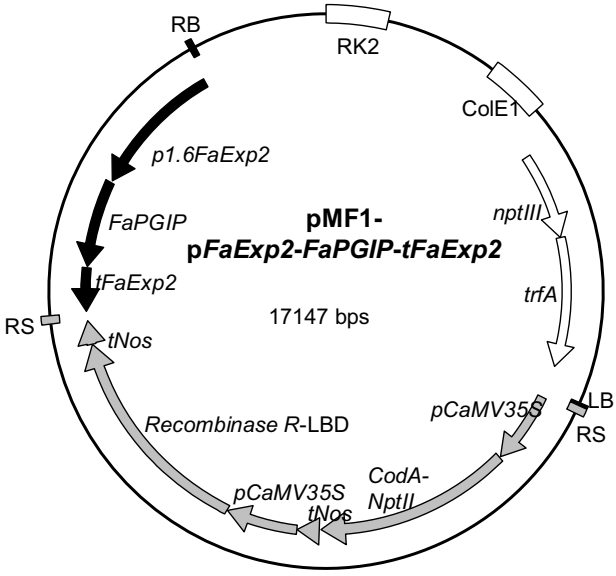

Fig. 3 pMF1 binary vector with the intragene 1.6p*FaExp2-FaPGIP*-t*FaExp2* for obtaining marker-free GM plants that overexpress *FaPGIP* in a fruit-specific way. White boxed sequences are located on the binary vector backbone. The black and grey boxed sequences are located on the T-DNA, which is flanked by RB and LB (right and left T-DNA border sequences, respectively) and which is transferred to the plants cell and incorporated into the plant genome. The grey boxed sequences are flanked by RS, Recombination sites, and these sequences will be removed after induction of recombinase activity (see Schaart *et al.* 2011 for detailed explanation).

Fig. 4 Greenhouse with intragenic strawberry plants transformed with the pMF1- 1.6p*FaExp2-FaPGIP*-t*FaExp2*..

co-integrated with the gene of interest. This result indicates that the number of true intragenic (marker- and vector backbone-free) plants obtained described here is likely to be lower. Although the aim of the EU project was just to demonstrate the possibility to produce intragenic strawberry plants, we obviously were interested in the performance of the newly introduced *FaPGIP* gene under the regulation of the *FaExp2* promoter and terminator. For this the intragenic strawberry plants were transferred to the greenhouse (**Fig. 4**) for production of fruits for further characterisation. For evaluation of the level of *Botrytis* resistance in ripening fruits, *Botrytis* spores were injected (50 µl of conidial suspension of 10^5 spores.ml^{-1} in fruits at different developmental stages and fruit rot incidence was monitored one week after injection of the fruits. Unfortunately, this assay could not demonstrate any increase in *Botrytis* resistance in the intragenic fruits as compared to control fruits. Because we have not quantified *FaPGIP* transcript or FaPGIP protein levels in the intragenic fruits, we cannot conclude whether the lack of improved resistance was due to poor *FaPGIP* expression in the fruits tested or that PGIP alone was insufficient to stop *Botrytis* colonisation in the intragenic strawberry fruits or that the number of spores that were injected was too high to discriminate between resistant and susceptible.

CONCLUSION

In this short communication different steps have been described to come to genetically modified plants in which only gene sequences from the species itself have been introduced. To demonstrate the successful production of intragenic strawberry plants, an intragene was constructed by combining the regulatory properties of the strawberry *FaExp2* gene with the functional gene properties of the strawberry *FaPGIP* gene. This new gene combination was successfully introduced into strawberry plants after which the undesired selectable marker genes, that were essential for the production of the genetically modified strawberry plants, were removed. This resulted ultimately in the production of intragenic strawberry plants.

Because the intragenic strawberry plant did not show the expected phenotype, i.e. enhanced resistance to *Botrytis*, other intragenes should be constructed and tested to ultimately reach the goal of producing *Botrytis* resistant intragenic strawberry lines. Cultivating such intragenic strawberries will result in reduction of fungicide applications, which will be favourable to producers, consumers and environment, and because of its intragenic nature, it is envisaged that such a particular intragenic strawberry will find good acceptance by producers and consumers of strawberries. In the end, the use of intragenic strawberry plants may lead to a new way of sustainable crop production practices.

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