Towards the production of genetically modified strawberries which are acceptable to consumers
Towards the Production of Genetically Modified Strawberries which are Acceptable to Consumers

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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 × 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 step until recently. Identification and isolation of such genes and sequences still requires specific investments, but comes.

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...
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 cispogenesis. 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 cispogenesis, 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 cispigenic crops the availability of specific genes and regulatory sequences within a species is a prerequisite. Up to date, for a number of crop 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 degenerate 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 pea 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; Schaar 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* was overexpressed using the constitutive *CaMV35S* promoter. Because this promoter provides strong expression in strawberry leaf tissue (Schaar 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 *CaMV35S* 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 infections in the flower, suitable to induce the intended upregulation of *FaPGIP*, 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 (*tExp2*). The 1.6p*FaExp2-*FaPGIP-*tFaExp2* 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 Schaar 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-*tFaExp2* 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 removed after induction of recombinase activity (see Schaar et al. 2011 for detailed explanation).
Intragenic strawberries. Schaart et al.

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 105 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 intragenate 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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