Towards consumer-friendly cisgenic strawberries which are less susceptible to *Botrytis cinerea*

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Towards consumer-friendly cisgenic strawberries which are less susceptible to *Botrytis cinerea*

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Voor Carin

Voor Doortje, Teun en Daan

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Chapter 1

Introduction to the thesis

Limitations and opportunities in strawberry breeding

Strawberry (*Fragaria x ananassa*) is in many aspects a remarkable fruit species. What generally is regarded as the strawberry fruit, is in fact a swollen part of the strawberry flower, called flower base or receptacle, and the true strawberry fruits are the achenes, which are embedded in the outer layer of the receptacle and which we generally denote as seeds. The unique aromatic flavour and sweet taste of strawberry is highly appreciated by many people, and, therefore, strawberry is widely produced for fresh fruit consumption. In addition, strawberry has found a wide application in many processed products like in conserved forms as jams, syrups and juices, as flavours in sweets, pastry and dairy products, and as fragrance for perfumes.

The production of strawberry relies on a limited number of genotypes, which are initially selected for adapted growth characteristics to different geographic and climatic conditions. For example in North-West Europe for 60-95% of the strawberry production the cultivar Elsanta is used, while this cultivar is less suitable for commercial production in either Scandinavia or in the South of Europe. For these regions other cultivars have been selected for strawberry production.

Breeding for improvement of strawberry cultivars is difficult. Many traits, like 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 *F. x ananassa* has found to be 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*. The complicated genetic constitution of the strawberry genome has kept most researchers from investing in the development of methods that can assist breeding of strawberry. Only recently, the first results towards the production of a genetic map for strawberry have been published (Haymes et al., 2000; Lerceteau-Kohler et al., 2003). Moreover, reliable methods for screening for disease resistances in strawberry are becoming more available.

Next to classical breeding, the last decade genetic modification of strawberry has gained increasing interest. 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 regulatory sequences that will result in desired improvements is limited at the moment, and identification of such information still requires large investments. Furthermore, the public attitude toward genetically modified crops in general is, at least in Europe, still rather sceptic, hampering the introduction of genetically modified strawberries in the immediate future.

Strawberry fruit rot

Strawberry is susceptible to several fungal diseases. Among others *Botrytis cinerea, Verticillium dahlia, Phytophthora cactorum, P. fragariae* and *Colletotrichum acutatum* are important fungi causing losses in strawberry production. *B. cinerea*, the fungal agent causing gray mould or fruit rot, can also lead to a very limited postharvest life of strawberries, leading to losses in strawberry production at the end of the production chain. Because of the high value of the end product, economic losses can, therefore, be considerable.

Depending on the weather conditions, growers have to use at least a weekly application of fungicides for effective *B. cinerea* disease management, and these applications will have to be continued until just before harvest of the fruits (MacKenzie et al., 2003). High production costs and concern about fungicide residues in fruits has initiated the search for alternative disease control strategies. One such strategy might involve the introduction of resistance genes in strawberry by a genetic modification approach, aiming at reducing susceptibility towards *B. cinerea* or other pathogens.

Polygalacturonase inhibiting protein

Plants have developed many strategies to deal with the continuous attack by pathogenic organisms. First of all, the plant cell wall plays an important role in resistance by presenting a physical barrier to the infecting pathogens. Moreover, enzymes and proteins involved in host defence mechanisms are located in the cell wall (Karr and Albersheim, 1970).

Many pathogenic fungi make use of cell wall degrading enzymes, in order to cross the plant cell wall barrier. For example, like many other phytopathogenic fungi, *B. cinerea* produces several polygalacturonases (PGs) (Wubben et al., 1999), a family of enzymes involved in degradation of the pectin fraction of the host cell wall.

PG inhibiting proteins (PGIPs) have been found in cell walls of a wide range of plants. PGIPs exhibit a high-affinity binding to fungal PGs, but not to plant PGs, resulting in inhibition of the fungal enzymes (Cervone et al., 1989, 1990), thus presenting a potential defence mechanism of

the plant. Different PGIPs from a single plant source are capable of differentially inhibiting PGs from several fungal species, or different PGs from a single fungal pathogen (Leckie et al., 1999). In addition to variation in recognition specificity, different *PGIP* genes show also diversity in regulation of gene expression. For example, in apple, wounding and fungal inoculation induced *PGIP* gene expression (Yao et al., 1999). The expression of a *PGIP* gene of *Phaseolus vulgaris* however, is activated by wounding only and not by pathogen infection (Devoto et al., 1998), whereas for strawberry fungal inoculations, but not wounding, induced *PGIP* expression (Mehli et al., 2004). Moreover, apple, raspberry and strawberry fruits show variation in developmentally regulated *PGIP* expression (Yao et al., 1999; Ramanathan et al., 1997, Mehli et al., 2004).

Despite the diversity in recognition and expression patterns of *PGIP*s and other defence related genes, the action of these genes appears to be insufficient to completely prevent colonisation by *B. cinerea* in many cases.

Genetic modification for improvement of fungal disease resistance

For many plant species, including crop species like strawberry, transformation protocols have been developed, which allow the introduction of new gene sequences into the plant genome. Genetic modification involving pathogenesis-related genes may lead to improvement of existing high quality crops with enhanced levels of resistance to fungal diseases. In order to substantiate the potential of this basic principle, many different plants species have been genetically modified, involving introduction of a variety of pathogenesis-related genes (see review by Punja, 2001). Many of these studies have shown that the development of fungal pathogens can be significantly reduced, but disease control has never been complete. However, new strategies involving the introduction of combinations of pathogenesis-related genes and the use of more specific promoters, may result in useful disease resistances for commercial application.

Scope of this thesis

In this thesis different aspects are described that are important for the ultimate production of genetically modified strawberry plants which will be acceptable for consumers and which will have added-value for both growers as well as consumers.

The genetic modification involves the introduction of a *PGIP* gene which has been isolated from strawberry itself and from which the natural expression levels have been investigated (Chapter 2 and 3). Although the strawberry *PGIP* (*FaPGIP*) gene is naturally present in the strawberry genome, endogenous expression levels are expected to be too low for efficient

inhibition of *B. cinerea*. In order to be able to achieve the desired *FaPGIP* expression level and pattern, we have selected alternative promoters from different origins, including strawberry, and studied the activity of these promoters in different tissues and several developmental stages of strawberry fruits (Chapter 4 and 5). The subsequent combination of such new promoters with the *FaPGIP* gene might allow changes in timing, specificity and level of *FaPGIP* expression, finally resulting in improved containment of fungal pathogens.

In order to increase acceptance of the final product, i.e. genetically modified strawberries, by consumers, it should be envisaged that the transgenic strawberry plants are produced according to the 'clean gene' approach, which demands that transgenic plants are free of foreign coding and regulatory DNA sequences. In addition to this criterion, the strict uses of strawberry-own DNA sequences as target gene and as promoter was considered as extra beneficial conditions for this concept. For the 'clean gene' approach a selectable marker removal method has been developed (Chapter 6) for the elimination of marker genes. Selectable marker genes are required for initial steps in the production of transgenic strawberry plants. Since these genes are usually of bacterial origin and mostly based on antibiotic resistance, presence of marker genes is undesired in the genetically modified end-products.

Finally, the combined use of all aspects described allows the production of transgenic strawberry plants of which the genome contains exclusively genes and regulatory sequences of strawberry origin. It is, therefore, proposed (General discussion) to call these genetically modified strawberry plants cisgenic, rather than transgenic.

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Chapter 2

Cloning and characterisation of a strawberry (*Fragaria x ananassa*) polygalacturonase inhibiting protein (*FaPGIP*) gene

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Abstract

A full-length cDNA encoding a polygalacturonase-inhibiting protein (*FaPGIP*) was cloned from strawberry (*Fragaria* x *ananassa*) fruit. It showed a high degree of homology to fruit *PGIP* cDNA's of related species such as raspberry, apple and pear. The genomic *FaPGIP* sequence contains an intron of 167 bp at a conserved position compared to other intron-containing *PGIP* genes. The open reading frame encodes a polypeptide of 332 amino acids, in which the 24 amino acids at the N-terminus code for a signal peptide. Furthermore, the strawberry *PGIP* contains ten loosely conserved leucine-rich repeats, averaging 24 amino acids in length. Compared to other tissues, mature fruit showed the highest constitutive *FaPGIP* expression, suggesting that the gene is developmentally regulated. The strawberry cultivar Polka showed higher constitutive *FaPGIP* expression than the cultivars Elsanta, Korona, Senga sengana, and Tenira. *FaPGIP* was highly induced by *Botrytis cinerea* infection in the five cultivars, whereas wounding did not have an impact on the transcript level. The implications for disease resistance are discussed.

Introduction

The plant cell wall, composed of complex polysaccharides, phenolics and structural proteins, acts as the initial physical barrier to pathogens. In order to penetrate the cell wall, most microbes produce an array of cell-wall degrading enzymes, among them exo- and endo-polygalacturonases and pectin methylesterases that are involved in pectin degradation (Walton, 1994; Hadfield and Bennett, 1998). The polygalacturonases (PGs) have been demonstrated as being an important pathogenicity factor for soft-rot fungi (ten Have et al., 1998). In response, plant species have evolved polygalacturonase inhibitor proteins (PGIPs) that specifically recognise and inhibit fungal PGs (Powell et al., 1994; De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002).

In strawberry (*Fragaria* x *ananassa*) production, the polyphagous grey mould fungus (*Botrytis cinerea*) is undoubtedly the most important pathogen causing fruit rot. In organic or unsprayed fields, the losses due to fruit rot can be up to 55% (Daugaard, 1999). In strawberry, the primary infection of *B. cinerea* takes place by conidia, commonly through the open flowers. The secondary infection takes place by mycelial growth through the intact ripening fruit skin. The infected berries act then as a source of inoculum in the field, even after harvest (Stensvand et

al., 1999). No existing strawberry cultivar has been reported to be completely resistant to *B. cinerea*, but cultivars do vary in susceptibility (Daugaard, 1999). Cell wall degrading enzymes, such as pectinases, are essential in the pathogenesis of *B. cinerea*. PGs catalyse the degradation of pectic chains into galacturonic acid residues, and are among the first enzymes secreted by *B. cinerea* upon infection. They have also been shown to be active in all other steps of the infection process (van der Cruyssen et al., 1994). Six members of the PG gene family from *B. cinerea* have been cloned thus far (Wubben et al., 1999). These PGs show differential expression depending on host tissue, infection stage and temperature (ten Have et al., 2001). The elimination of a single PG gene, *Bcpg1*, from *B. cinerea* by partial gene replacement (ten Have et al., 1998) resulted in reduced virulence of the mutated strain, which indicated the specific functions for *B. cinerea* PGs.

PGIPs responding to fungal PGs have been isolated and purified from cell wall extracts from a wide range of plant species. They are related to plant resistance genes by sharing a key motif with leucine-rich repeats (LRR) for protein-protein interaction (Jones and Jones, 1997). The proposed mode for PG/PGIP interaction is that the inhibiting protein partly covers the active site of PGs, thus preventing substrate binding (Frederici et al., 2001). Different PGIPs show variation in recognition abilities and specificity. Stotz et al. (2000) reported a purified pear PGIP that completely inhibited endo-PG II from *Aspergillus niger* and a mix of PGs from *B. cinerea*, but did not inhibit a PG from *Fusarium moniliforme*, whereas a purified apple PGIP showed differential inhibition towards five different PG's from *B. cinerea* (Yao et al., 1995). In the study of Leckie et al. (1999), two *PGIP* genes from field bean (*Phaseolus vulgaris*) were separately expressed in *Nicotiana benthamiana.* The first PGIP-isoform did not inhibit a purified PG from *F. moniliforme,* and showed low affinity to crude preparations of PGs from *B. cinerea* and *Fusarium oxysporum*. The second PGIP showed high affinity to PGs from *B. cinerea*, and to purified preparations of PG from *F . moniliforme,* but lower affinity to the PGs from *F. oxysporum.* These data demonstrate that PGIPs differ in PG-target specificity, both within and between plant species.

PGIP genes are reported to be induced in response to fungal infection, wounding, and application of elicitors (Yao et al., 1999; Bergmann et al., 1994; Nuss et al., 1996; Machinandiarena et al., 2001; De Lorenzo et al., 2001). In addition to responding to external stimuli, *PGIP* genes can show developmental regulation. In apple (Yao et al., 1999), the expression of a monitored *PGIP* was at a higher level in mature, harvested apples relative to immature fruit. In raspberry, a low constitutive expression was observed from green to red fruit (Ramanathan et al., 1997).

In order to investigate the expression of *PGIP* in strawberry following infection by *B. cinerea*, a strawberry *PGIP* gene was cloned, and the transcript levels were monitored in response to infection by *B. cinerea* in five strawberry cultivars displaying variation in susceptibility. In addition, the gene's constitutive expression was studied in various tissues, including fruit at different maturities.

Material and methods

Experimental design

Flower-induced strawberry plants (*F*. x *ananassa*) were grown in a greenhouse at 18-20°C with a 16-hour photo period. To keep the plants healthy during the eight-week period preceding inoculation, they were treated with the biological control agent Spidex-T (Koppert B.V, Berkel en Rodenrijs, the Nederlands) against aphids and strawberry mites, and later with the fungicide Candit (BASF, Ludwigshafen, Germany) according to the manufacturers' recommendations. To prepare the fungal inoculum, conidia of *B. cinerea* (strain No. 700, IPO-DLO, Wageningen) were collected from potato dextrose agar (PDA) cultures in sterile water with Tween 20, and the number of spores was adjusted to 10^5 per ml. To find an efficient infection method for inducing *FaPGIP* expression, two methods were compared: syringe injection of 50 µl of conidial suspension into the berry, and inoculation by wounding the berry with a knife followed by dipping in spore solution. The former induced the highest *FaPGIP* gene expression (data not shown), and was thus chosen as the inoculation method. To secure humid conditions, the plants were covered with a plastic bag for 24 hours after treatment.

In order to follow constitutive *FaPGIP* gene expression during fruit development, flowers and fruits at small green, green, green-white, white, turning and red stages were harvested from Elsanta greenhouse plants. Young leaf material was used as a control. For RNA isolation, tissue was pooled from at least six different fruits at identical developmental stages, harvested from at least three different plants. Three samples were analysed for each tissue type. For the two inoculation experiments performed, the strawberry plants were completely randomised in three replicates. To obtain enough material for RNA isolation (5-10 berries), a replicate was composed of six plants. In the first experiment, the cv. Korona was used to monitor temporal *FaPGIP* expression upon infection in berries at four maturity stages, i.e. green, white, turning and red. Wounded berries (syringe-inoculated with sterile water) and untreated berries were used as controls. Incubation was continued for four days and samples, collected at one-day

intervals, were immediately frozen in liquid nitrogen and stored at -80°C until one replicate was processed for RNA isolation. In the second experiment, *FaPGIP* gene expression was monitored in white berries from five strawberry cultivars, *i.e.* Elsanta, Tenira, Polka, Korona and Senga sengana. White berries were inoculated with *B. cinerea*, and harvested after an incubation period of 24 hours. Non-treated strawberry plants were used as controls. All three replicates were analysed.

Isolation of nucleic acids

Total RNA was isolated as described by Schultz et al. (1994). Genomic DNA was isolated from young folded leaves from greenhouse plants according to the method described by Doyle & Doyle (1987), including 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer.

Isolation of strawberry-*PGIP* cDNA using degenerated primers

First strand cDNA was synthesised from DNaseI (Invitrogen, Carlsbad, CA, USA) treated total RNA of Elsanta strawberry fruit tissue, using the TaqMan Reverse Transcription Reagents (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) with oligo$(dT)_{16}$-anchor primers according to the manufacturer's instructions. A 800 bp PCR-product, obtained with the degenerated primer PGIP-N1, 5'-TGYAAYCCNGAYGAYAARAARGT-3' and PGIP-C2, 5'-CCNACNGGDATYTGNCCRCA-3' (Yao et al., 1999), was used as template for a second PCR with the degenerate primers 5'-GACTGYTGYGAYTGGTAC-3' and 5'-CCYGTRAGCTTRT-TNCKGTC-3' designed from internal conserved parts of *PGIP* sequences from apple (U77041) and apricot (AF020785). The obtained 400 bp PCR-product was gel-purified using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and subsequently cloned in a pGEM-T Easy cloning vector (Promega, Madison, WI, USA).

DNA sequencing and analysis

DNA sequencing of the cloned 400 bp PCR products was performed using the Applied Biosystems dye terminator cycle sequencing Ready Reaction kit and the Applied Biosystems 373 DNA sequencer. Comparison analysis of the sequences was conducted by using the advanced basic local alignment search tool, BLAST X server (Altschul et al., 1990) and the National Center for Biotechnological Information (www.ncbi.nlm.nih.gov) nonredundant protein database. DNA sequence analysis was performed with the DNASTAR program (DNASTAR, Madison, WI, USA).

5’- and 3’-RACE (Rapid Amplification of cDNA Ends) PCR to isolate the full length cDNA and genomic DNA of *FaPGIP*

Gene specific primers were designed, based on the sequence data of the cloned 400-bp PCR products, and 5’-RACE (primer 5’-TGAGGTGCTTGAGCTTGGCGATGGAGG-3') and 3’-RACE (5’-CCAGCTTAAGAACCTCACCTTCC-3') PCR were performed according to the SMART RACE cDNA Amplification kit protocol (Clontech Laboratories, Palo Alto, CA, USA) using strawberry (Elsanta) fruit cDNA as a template. The PCR products were purified, cloned and sequenced as described above. Several clones from the 5’- and 3’-RACE PCR were analysed and an overlapping consensus sequence was assembled. Based on this, two new primers, 5’-GGACTACCGACTTGTATATGCA -3’ (5’PGIP-1) and 5’-TATATCTAGCATC-CCTCCGT-3’ (3’PGIP-1), were designed to amplify a genomic PCR fragment containing the full-length coding sequence of *FaPGIP*. This 1.4-kb PCR fragment was cloned and several clones were sequenced as described above.

DNA gel-blot analysis

For DNA gel-blot analysis, 15 µg of genomic DNA from the different strawberry cultivars was digested with 5U/µg of the restriction enzymes *Eco*RI or *Bam*HI (Amersham Biosciences, Little Chalfont, UK). Within the genomic PGIP DNA sequence, *Eco*RI cuts at position 1049, while *Bam*HI does not have a restriction site within the sequence. After fragment separation on 1% agarose gel in 1×TAE for 16 hours at 25 V, the DNA was blotted onto a Hybond-N+ membrane (Amersham Biosciences) in 10×SSC. After UV-cross linking, the DNA blots were hybridised with the biotin-labelled PCR product (North2South™ Biotin Random Prime Kit; Pierce Biotechnology Inc., Rockford, IL, USA) hybridising to the region from base 361 to base 742 in the first exon. *FaPGIP* homologues were detected using chemiluminescence (North2South™ Chemiluminiscent Nucleic Acid Hybridisation and Detection Kit; Pierce Biotechnology Inc.) according to the manufacturer’s instructions, using high stringency washing conditions [2 × (1.0 SSC + 0.1 % SDS) + 1 × (0.1 SSC+0.1% SDS)]. The membranes were exposed to clear blue X-ray film (CL-Xposure™ Film; Pierce Cooperation Inc.), and the films were developed and fixed with Kodak X-ray developer (LX24) and Kodak X-ray fixer (AL4), respectively.

RT-PCR analysis

Real-time RT-PCR was performed using the ABI Prism7700 Sequence Detection System (Perkin Elmer, Applied Biosystems) as described by Schaart et al. (2002), but SYBR® Green

was used for detection of PCR products. For detection of *FaPGIP*, a forward 5'-TTCATCTAGACCGCAACCAGC-3'- and a reverse 5'-ACGGTGCCAACGAATTTCCC-3' *FaPGIP* primer was designed (product size: 67 bp). This primer pair enclosed the *FaPGIP* intron allowing gel electrophoresis detection of any contamination by genomic DNA. A strawberry gene with high homology to a putative DNA binding gene from *Arabidopsis thaliana*, was selected as a reference gene. This gene, indicated here as dbp (forward primer 5'-TTGGCAGCGGGACTTTACC-3', reverse primer 5'-CGGTTGTGTGACGCTGTCAT-3', product size: 98 bp), has shown a similar level of expression in several strawberry tissues (Schaart et al., 2002), and its expression level has not been influenced by wounding or inoculation. All PCR reactions were performed in triplicate. The target specificity of the *FaPGIP* primer set used for real-time RT-PCR was investigated by melting point analysis using genomic DNA from *B. cinerea* as a negative control and cDNA from the two inoculation experiments as a positive control. The thermal profile for melting point analysis, performed separately after real-time RT-PCR, was 95°C for 15s, 60°C for 20s followed by a melting step in which the temperature was raised from 60°C to 95°C with a ramping time of 20 min.

Statistical analyses

FaPGIP expression data from the inoculation experiment with white berries of the five cultivars was subjected to analysis of variance using the GLM procedure of SAS (SAS, 1990).

Results

Cloning, analysis and characterisation of the strawberry PGIP gene

The full length cDNA sequence of a strawberry *PGIP*, obtained with degenerate primers and specific primers designed for 5'- and 3'- RACE PCR, showed high homology at the DNA level to *PGIP* cDNA sequences from related species, especially from raspberry (85%), apple (80%) and pear (82%). The use of genomic DNA as template for a PCR reaction with the primers 5'PGIP-1 and 3'PGIP-1 revealed the presence of an intron of 167 bp (Fig. 1) with an AT content of 68 %, which is typical to introns of dicotyledonous plants. The presence of the intron was also expected from exon/intron splice sites predicted by NetPlantGene (Hebsgaard et al., 1996), and by sequence alignment with homologous plant *PGIP*s from other species. The strawberry *PGIP* gene is therefore structurally similar to *PGIP*s characterised from *Arabidopsis* (AB010697; Moj9.3), the flower snapdragon (X76995), apricot (AF020785), wild cherry (AF263465), and raspberry (AJ620355), which all contain an intron at a conserved position.

The intron sequence of these species is not conserved, and varies in length between 82 and 263 bp. For example, the 243 bp-intron in the raspberry *PGIP* (Ramanathan et al., 1997) shares a homology of only 29% with that of strawberry. Most of the fruit *PGIP*s characterised so far, including apple and pear *PGIP*, nevertheless, do not have an intron. Along with the coding sequence, the obtained *FaPGIP* sequence also contained a 5'- and a 3'- untranslated region of 194 bp and 27 bp, respectively. The complete 999 bp-long coding sequence of strawberry *PGIP* encodes a predicted protein of 332 amino acids. The theoretical molecular weight of the

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ggactaccgacttgtatatgcagcaaaccatgttaattcgtttctggatatccaacaagaccaaagcttccgtcactatatacatcaccccaaatcgcat : 100
.............................................................................................. :   -

ataatctttgacttacagacgcatgccaaacctataaatccagaacttcttcacatcaaatttctcatctcagttctcactgaaaacccaaaatcATGGA : 200
.....................................................................................M..D :   2

CCTAAAGCTCTCCACTCTCCTCTGCATTTCTCTGTTCTTCTCCACCATCTTAACCCCAACTCTCTCCGAGCTCTGTAACCCTACAGACAAAAAAGTTCTC : 300
..L..K..L..S..T..L..L..C..I..S..L..F..F..S..T..I..L..T..P..T..L..S..E..L..C..N..P..T..D..K..K..V..L.  :  35

TTCGAAATCAAGACAGCCTTCAATAACCCCTACATCTTGTCCTCATGGAAATCCGACGCCGACTGCTGTACCGACTGGTACAATGTTGAGTGTGATCCCA : 400
.F..E..I..K..T..A..F..N..N..P..Y..I..L..S..S..W..K..S..D..A..D..C..C..T..D..W..Y..N..V..E..C..D..P..  :  68

ACACCAACCGTATCAACTCCCTCACCATCTTTACTGACGTCCGCCTCACCGGCCAAATCCCCGCCCAAGTCGGAGACTTGCCCTACCTCGAAACCCTGGT : 500
N..T..N..R..I..N..S..L..T..I..F..T..D..V..R..L..T..G..Q..I..P..A..Q..V..G..E..L..P..Y..L..E..T..L..V  : 102

GCTCCGCAAGCTCCCCAATCTCACAGGTCCCATCCAGCCCTCCATCGCCAAGCTCAAGCACCTCAAGATGCTCAGGCTCAGCTGGAACGGCCTCTCTGGC : 600
..L..R..K..L..P..N..L..T..G..P..I..Q..P..S..I..A..K..L..H..K..L..K..M..L..R..L..S..W..N..G..L..S..G.  : 135

TCAGTCCCTGACTTCCTCAGCCAGCTTAAGAACCTCACCTTCCTTGAGCTTAACTATAACAACTTCACAGGCTCCGTTCCCAACTCGCTTTCAAAGCTAC : 700
.S..V..P..D..F..L..S..Q..L..K..N..L..T..F..L..E..L..N..Y..N..N..F..T..G..S..V..P..N..S..L..S..K..L..  : 168

CGAACTTGCTAGCTCTTCATCTAGACCGCAACCAGCTCACAGgtatacatcaaattaactctacagtttttcctcatttagttacagcttatactctacc : 800
P..N..L..L..A..L..H..L..D..R..N..Q..L..T..................................................... : 182

ggtcagaaaatgagtaggtaaatgacgttctagggatacatttagactaataagttaactgtgttagtaacagagttttgacgttgatatataataatgt : 900
.............................................................................................. : 182

gactgcagGTAATATTCCTAGCGCGTACGGGAAATTCGTTGGCACCGTTCCAGATCTCTTCCTCTCCCACAACAAGCTCACAGGCAAAATCCCAACCTCA :1000
........G..N..I..P..S..A..Y..G..K..F..V..G..T..V..P..D..L..F..L..S..H..N..K..L..T..G..K..I..P..T..S.  : 213

TTTGCTAATATGAACTTTGATCGGATAGACTTGTCACGCAACATGCTGGAAGGAGACGCGTCAATGATATTCGGGATGAACAAGACGACCCAGATTGTGG :1100
.F..A..N..M..N..F..D..R..I..D..L..S..R..N..M..L..E..G..D..A..S..M..I..F..G..M..N..K..T..T..Q..I..V..  : 246

ATTTGTCAAGGAACATGCTAGAATTCGATCTGTCCAAGGTGGTGTTTTCGACAAGCTTGATCTCACTGGACTTGAACCATAACAGGATGACAGGTAGTAT :1200
D..L..S..R..N..M..L..E..F..D..L..S..K..V..V..F..S..T..S..L..I..S..L..D..L..N..H..N..R..M..T..G..S..I  : 280

TCCGGAGCAGTTGACCCAATTGGATAATTTGCAGTTGTTCAATGTTAGCTACAACAGGTTGTGTGGTCAGATTCCGATTGGTGGGAAGTTGCAGAGCTTT :1300
..P..E..Q..L..T..Q..L..D..N..L..Q..L..F..N..V..S..Y..N..R..L..C..G..Q..I..P..I..G..G..K..L..Q..S..F.  : 313

GACACAACGTCATACTTCCATAACCGGYGTCTGTGCGGTGCTCCACTCCCAAGTTGCTAGtaatgggacggagggatgctagatata              :1387
.D..T..T..S..Y..F..H..N..R..C..L..C..G..A..P..L..P..S..C.............................              : 332
```

Figure 1. The nucleotide sequence and deduced amino acid sequence of the *F.* x *ananassa PGIP* gene. Intron and non-coding regions are indicated with lower-case letters, amino acids are indicated with single-letter abbreviation.

predicted mature protein is 34.8 kD, and the isoelectric point is calculated to be 7.63 (PeptideMass, Wilkins et al., 1997).

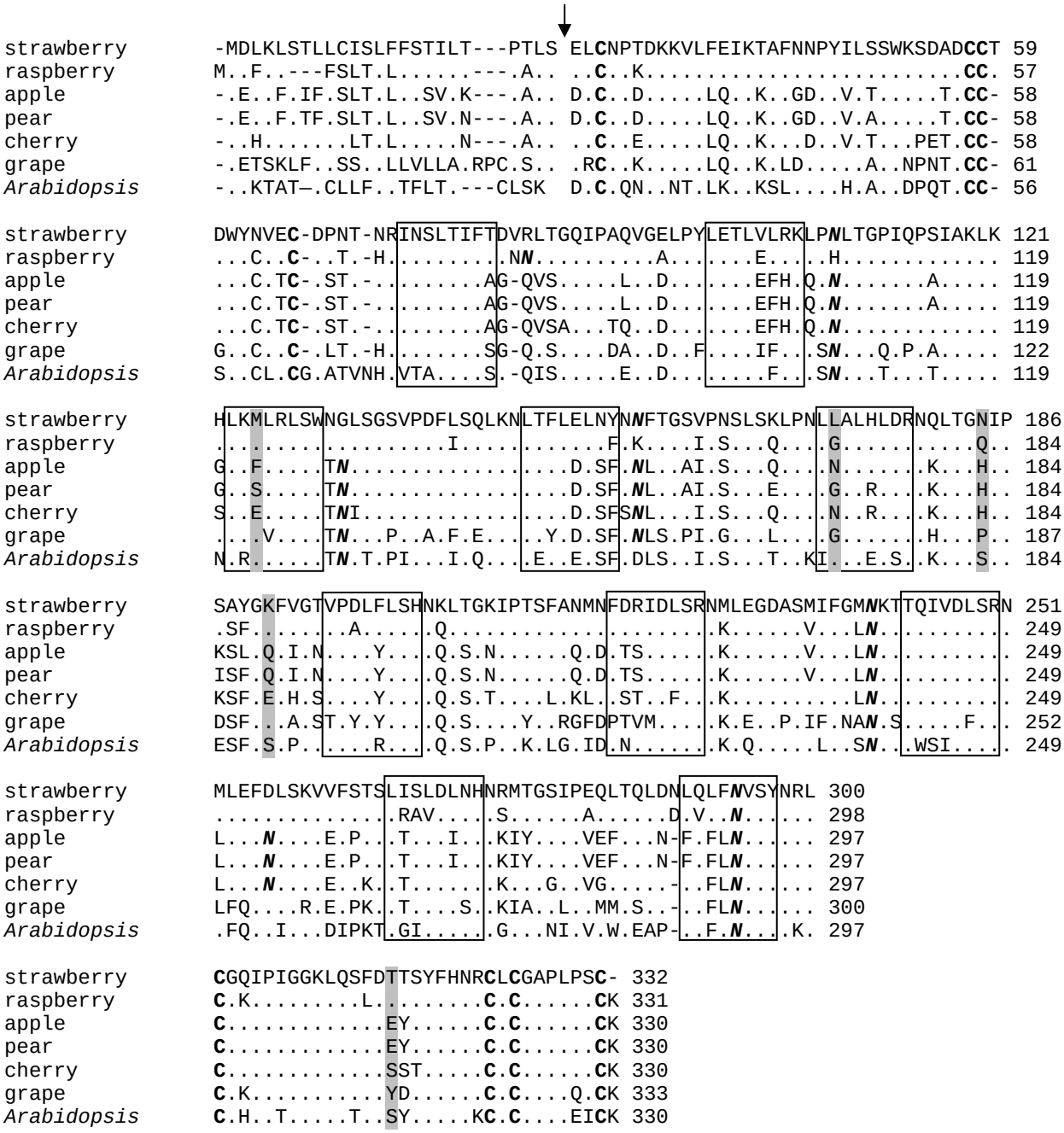
The predicted mature protein sequence of the strawberry *PGIP* gene showed a high degree of homology to the partial sequences of PGIP protein from the diploid *Fragaria* species, *F. vesca* (98% identity) and *F. iinumae* (97% identity). In comparison with other available fruit PGIP sequences, mature strawberry PGIP showed the highest homology at the protein level to raspberry, apple, pear, cherry, grape, kiwi, and *Citrus* spp., with identities of 86%, 74%, 74%, 73%, 66%, 67%, 67%, respectively. Excluding fruit plants, *Eucalyptus* spp. showed a high degree of homology (71% identity), while the homology of the strawberry PGIP to other non-fruit PGIPs was relatively low; e.g. *Arabidopsis* and field bean showed 63% and 47% similarity to strawberry PGIP, respectively.

The strawberry PGIP protein deduced from the predicted open reading frames contained all the characteristic domains found in other PGIP proteins, such as the N-terminal domain, motifs with leucine rich repeats (LRR) and the C-terminal domain (Fig. 2A). The first 24 amino acids in the N-terminal end of the strawberry PGIP protein sequence were identified as a signal peptide (SignalP V2.0; Nielsen et al., 1997). There are four potential N-glycosylation (N-X-S/T; where X is any amino acid except P) sites at the amino acid positions 108, 156, 240 and 294 in the protein. Two of these, at positions 240 and 294 in the strawberry PGIP, seem to be common for all fruit PGIPs (Fig. 2A). The position and number of consensus sites for N-linked glycosylation are not highly conserved among the PGIPs from different plants (Mattei et al., 2001). The N-terminal and the C-terminal region contain both four cysteine residues at highly conserved positions as compared to PGIPs from other fruit plants (Fig. 2A). These form four disulfide bridges important to maintain local structures and stabilize secondary structures in PGIP (Mattei et al., 2001; Di Matteo et al., 2003). The LRR sequences in the strawberry PGIP (Fig. 2B) have an average of 24 amino acids and follow the consensus sequence “xLxxLxLxxNxLTGxIPxxAxxLx” where “A” represents one of the hydrophobic amino acids (V, I, L, F or G), “x” represents any amino acid, and the named residue is present in the given position in at least 50% of the repeats.

Genomic organisation of the PGIP gene in strawberry

In the DNA gel-blot analysis, *Eco*RI- and *Bam*HI-digested genomic DNA from the five *F.* x *ananassa* cultivars (Korona, Elsanta, Tenira, Senga sengana and Polka) was hybridised with a biotin-labelled *FaPGIP* probe at high stringency conditions. *F.* x *ananassa* is an interspecific

A


strawberry -MDLKLSTLLCISLFFSTILT---PTLS ELCNPTDKKVLFEIKTAFNNPYILSSWKSDADCCT 59
raspberry M..F..---FSLT.L.....---.A.. ..C..K.....CC. 57
apple -.E..F.IF.SLT.L..SV.K---.A.. D.C..D.....LQ..K..GD..V.T.....T.CC- 58
pear -.E..F.TF.SLT.L..SV.N---.A.. D.C..D.....LQ..K..GD..V.A.....T.CC- 58
cherry -..H.....LT.L.....N---.A.. ..C..E.....LQ..K...D..V.T...PET.CC- 58
grape -.ETSKLF..SS..LLVLLA.RPC.S.. .RC..K.....LQ..K.LD.....A..NPNT.CC- 61
Arabidopsis -..KTAT—.CLLF..TFLT.---CLSK D.C.QN..NT.LK..KSL....H.A..DPQT.CC- 56
strawberry DWYNVEC-DPNT-NRINSLTIFTDVRLTGQIPAQVGELPYLETLVLRKLPNLTGPIQPSIAKLK 121
raspberry ...C..C-..T.-H.....N.....A.....E....H..... 119
apple ...C.TC-.ST.-.....AG-QVS.....L..D.....EFH.Q.....A..... 119
pear ...C.TC-.ST.-.....AG-QVS.....L..D.....EFH.Q.....A..... 119
cherry ...C.TC-.ST.-.....AG-QVSA...TQ..D.....EFH.Q..... 119
grape G..C..C-.LT.-H.....SG-Q.S....DA..D..F....IF...SN...Q.P.A..... 122
Arabidopsis S..CL.CG.ATVNH.VTA....S.-QIS.....E..D.....F..SN...T...T..... 119
strawberry HLKMLRLSWNGLSGSVPDFLSQLKNLTFLELNYNNFTGSVPNSLSKLPNLLALHLDRNQLTGNIP 186
raspberry .....I.....F.K....I.S...Q....G.....Q.. 184
apple G..F.....TN.....D.SFNL..AI.S...Q....N.....K...H.. 184
pear G..S.....TN.....D.SFNL..AI.S...E....G..R....K...H.. 184
cherry S..E.....TNI.....D.SFSNL...I.S...Q....N..R....K...H.. 184
grape ....V....TN...P..A.F.E....Y.D.SFNLS.PI.G...L....G.....H...P.. 187
Arabidopsis N.R.....TN.T.PI...I.Q...E..E.SF.DLS..I.S...T..KI...E.S..K...S.. 184
strawberry SAYGKFVGTVPDLFLSHNKLTGKIPTSFANMNFDRIDLSRNMLEGDASMIFGMNKTTQIVDLSRN 251
raspberry .SF.....A.....Q.....K.....V...LN..... 249
apple KSL.Q.I.N....Y...Q.S.N.....Q.D.TS.....K.....V...LN..... 249
pear ISF.Q.I.N....Y...Q.S.N.....Q.D.TS.....K.....V...LN..... 249
cherry KSF.E.H.S....Y...Q.S.T....L.KL..ST..F...K.....LN..... 249
grape DSF...A.ST.Y.Y...Q.S....Y..RGFDPTVM....K.E..P.IF.NAN.S.....F... 252
Arabidopsis ESF.S.P......R...Q.S.P..K.LG.ID.N.....K.Q.....L..SN...WSI.... 249
strawberry MLEFDLSKVVFSTSLISLDLNHNRMTGSIPEQLTQLDNLQLFNVSYNRL 300
raspberry .....RAV....S.....A.....D.V..N..... 298
apple L...N....E.P...T...I...KIY....VEF...N-F.FLN..... 297
pear L...N....E.P...T...I...KIY....VEF...N-F.FLN..... 297
cherry L...N....E..K..T.....K...G..VG.....-..FLN..... 297
grape LFQ....R.E.PK..T....S..KIA..L..MM.S..-..FLN..... 300
Arabidopsis .FQ..I...DIPKT.GI.....G...NI.V.W.EAP-..F.N....K. 297
strawberry CGQIPIGGKLQSFDTTSYFHNRCLCGAPLPSC- 332
raspberry C.K.....L.....C.C.....CK 331
apple C.....EY.....C.C.....CK 330
pear C.....EY.....C.C.....CK 330
cherry C.....SST.....C.C.....CK 330
grape C.K.....YD.....C.C....Q.CK 333
Arabidopsis C.H..T.....T..SY.....KC.C....EICK 330

B

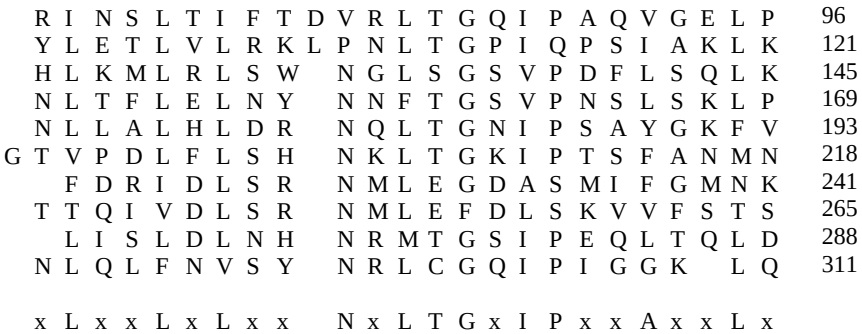

R I N S L T I F T D V R L T G Q I P A Q V G E L P 96
Y L E T L V L R K L P N L T G P I Q P S I A K L K 121
H L K M L R L S W N G L S G S V P D F L S Q L K 145
N L T F L E L N Y N N F T G S V P N S L S K L P 169
N L L A L H L D R N Q L T G N I P S A Y G K F V 193
G T V P D L F L S H N K L T G K I P T S F A N M N 218
F D R I D L S R N M L E G D A S M I F G M N K 241
T T Q I V D L S R N M L E F D L S K V V F S T S 265
L I S L D L N H N R M T G S I P E Q L T Q L D 288
N L Q L F N V S Y N R L C G Q I P I G G K L Q 311
x L x x L x L x x N x L T G x I P x x A x x L x

Figure 2. (previous page) **(A)** Multiple sequence alignment of deduced PGIP amino acid sequences from strawberry (*F.* x *ananassa*) and from five other fruit plants, raspberry (Ramanathan et al., 1997), apple (Yao et al., 1999), pear (Stotz et al., 1993), cherry (unpublished, accession number: AF263465), grape (Bézier et al., 2002), and from *Arabidopsis* (AB010697; Moj9.3). Dots indicate similarity, (-) an alignment gap. The predicted solvent exposed β-strand regions (leucine rich repeats; LRR), obtained with the Predict Protein program (Rost, 1996), are indicated in boxes. The conserved cysteins are marked in bold, potential N-glycosylation sites of the different proteins (N-X-S/T) are bold and italicised, and positively selected residues regarded as important for evolution of PGIP/PG interaction (Stotz et al., 2000) are shaded. The signal peptide cleavage site is indicated with an arrow. **(B)** Alignment of the LRR repeats of the strawberry PGIP. In the consensus sequence, "A" represents one of the hydrophobic amino acids (V, I, L, F or G), "x" represents any amino acid, and the named residue is present in the given position in at least 50% of the repeats.

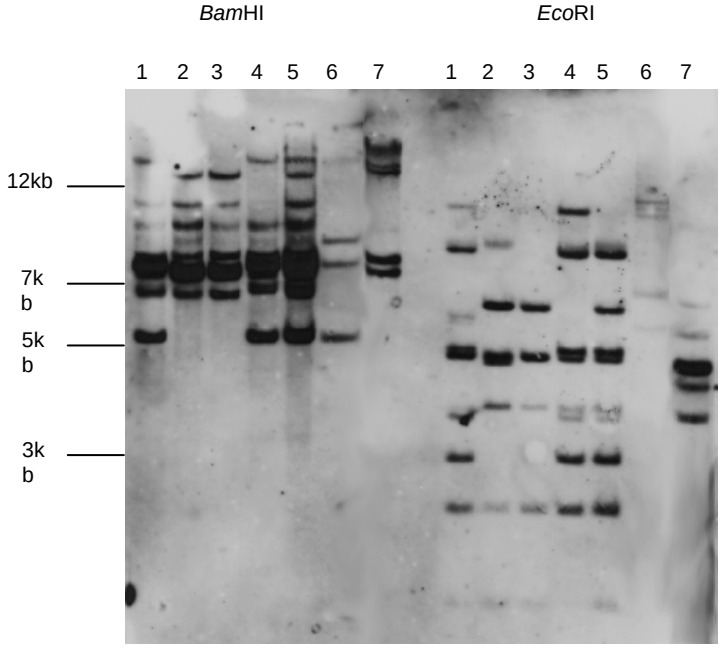


Figure 3. DNA-blot analysis of genomic DNA from *F.* x *ananassa* cultivars (Korona (1), Elsanta (2), Tenira (3), S. sengana (4) and Polka (5)) and different *Fragaria* species (*F. chiloensis* (6) and *F. vesca* (7). DNA (15 μg) was digested with 5 U/μg of *Bam*HI and *Eco*RI and hybridised with a biotin-labelled *FaPGIP* probe. Fragment size is indicated on the left.

hybrid of two other octoploid species, *F. chiloensis* and *F. virginiana;* the former and the diploid *F. vesca* were included as references in the analysis. The number of bands detected in *Eco*RI and *Bam*HI digested DNA was 4 and 5 in *F. vesca*, and 5 and 4 in *F. chiloensis*, respectively. Similarly, multiple bands were observed for both enzymes tested and restriction fragment length polymorphism was observed between the different *F.* x *ananassa* cultivars. Hybridisation of the *FaPGIP* probe to *Bam*HI-digested DNA from Korona, Elsanta, Tenira, S. Sengana and Polka resulted in 8, 7, 7, 9 and 10 bands ranging from 5-15 kb, respectively (Fig. 3). *Eco*RI digestion of the same samples gave 9, 7, 5, 8, 9 bands for the same cultivars, respectively, varying in size between 2 and 12 kb. The banding patterns of cultivars Elsanta and Tenira were almost identical with both restriction enzymes but differed from the other subgroup

composed of cultivars Korona, Senga sengana and Polka. The *Bam*HI restriction pattern of *F. chiloensis* appeared to be a subset of bands detected in *F.* x *ananassa* cultivars Korona, Senga sengana and Polka.

Expression of strawberry *PGIP* in different plant parts, during maturation and in response to wounding and infection

FaPGIP gene expression in different fruit developmental stages was monitored with quantitative RT-PCR in the strawberry cultivar Elsanta. There was a five- to ten-fold variation in *FaPGIP* transcript levels between leaves, flowers and fruit at different maturities, the highest level being observed in red mature berries (Fig. 4).

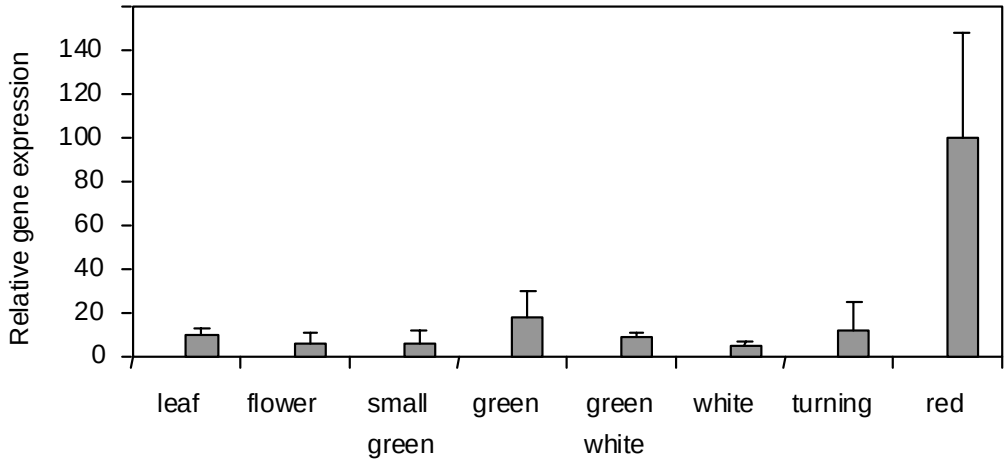


Figure 4. Monitoring *FaPGIP* expression in the strawberry cultivar Elsanta during development and fruit maturation using real-time RT-PCR with a putative DNA binding protein (dbp) as the reference gene. Three samples were analysed for each treatment, and all PCR reactions were performed in triplicate. Bars indicate standard deviation.

The expression of *FaPGIP* in response to wounding and *B. cinerea* infection was analysed from green, white, turning and red berries of *F.* x *ananassa* cv. Korona. The expression level in infected white berries one day after infection was used as the reference and referred to as 100%. The *FaPGIP* gene was highly induced in green, white and red berries one day after inoculation with *B. cinerea* spores, whereas in turning fruits, induction of *FaPGIP* by infection was postponed, the maximum peak occurring at day 2 (Fig. 5). In inoculated green and white berries, the level of the *FaPGIP* gene expression declined at day 2 and again showed an increase at days three and four, whereas in turning and red fruits the *FaPGIP* transcript levels returned to basal expression level two days after induction. No induction of *FaPGIP* was

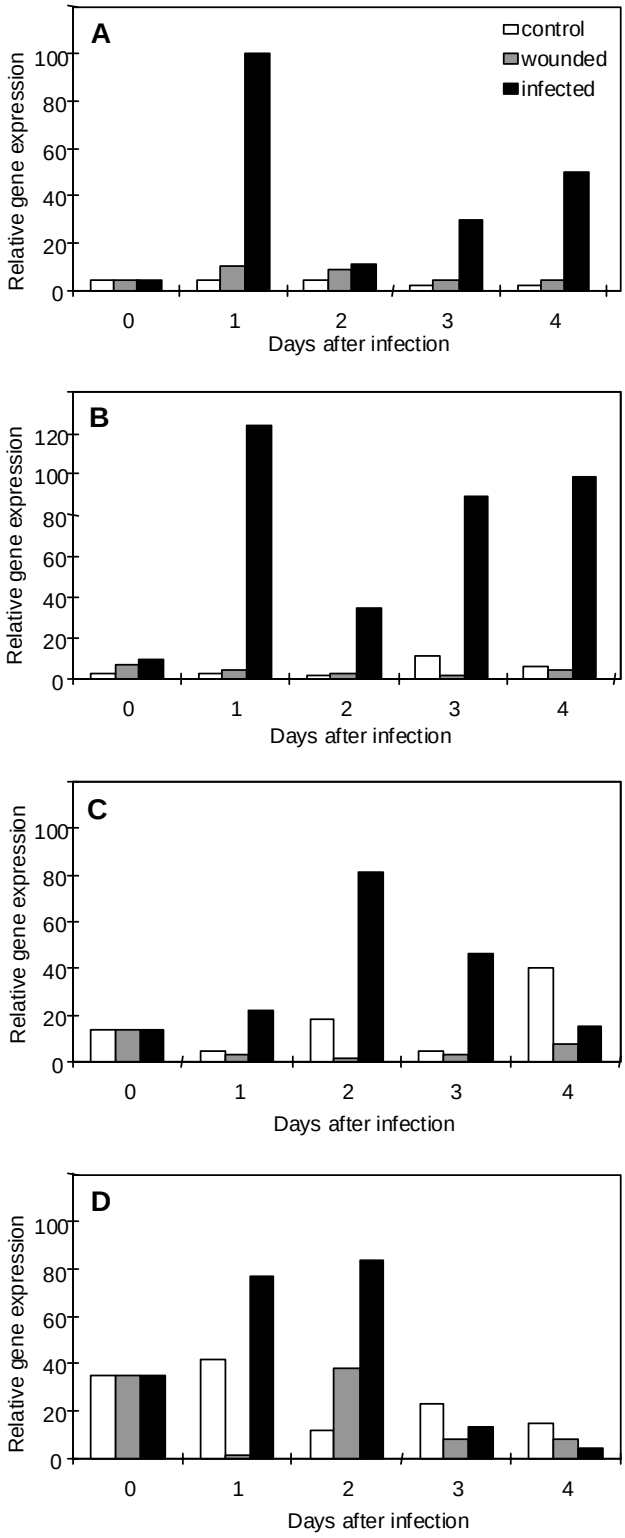


Figure 5. Temporal expression of the *FaPGIP* gene monitored by real-time RT-PCR in control, wounded and *B. cinerea* infected green **(A)**, white **(B)**, turning **(C)** and red **(D)** berries of *F.* x *ananassa* cv. Korona. The assays were performed on cDNA, synthesised from 2 μg RNA isolated from fruits collected after an incubation period of 0, 1, 2, 3 and 4 days. *FaPGIP* gene expression level of infected white berries was used as reference (= 100%). One sample was analysed for each treatment, and all PCR reactions were performed in triplicate.

observed in the wounded, non-inoculated berries during the four days monitored (Fig. 5). Melting point analysis of the *FaPGIP* RT-PCR products resulted in a single peak in all samples, indicating the presence of a single PCR product. The melting temperature ranged from 77.3ºC to 78.6ºC, with no clear differences between the treatments, the four berry types or the time-points. No PCR products were obtained from genomic DNA of *B. cinerea* included as a negative control.

Expression of the *FaPGIP* gene in the *F.* x *ananassa* cultivars Korona, Elsanta, Tenira, Polka and Senga sengana

The five strawberry cultivars examined in this study display differential susceptibility towards *B. cinerea* (Daugaard, 1999); Elsanta, Tenira and Polka are regarded as the least susceptible, while Korona is an intermediate and S. sengana the most susceptible. To investigate the response of these cultivars to infection by *B. cinerea*, white berries harvested one day after infection were examined for expression of *FaPGIP*. The *FaPGIP* gene expression level of infected Polka fruits was used as a reference and referred to as 100%. All cultivars showed a significant induction of *FaPGIP* after infection (Fig. 6), but the differences in induction level of *FaPGIP* between the cultivars were not statistically significant. As in the first experiment with Korona, all the cultivars showed relatively low expression levels of *FaPGIP* in control berries.

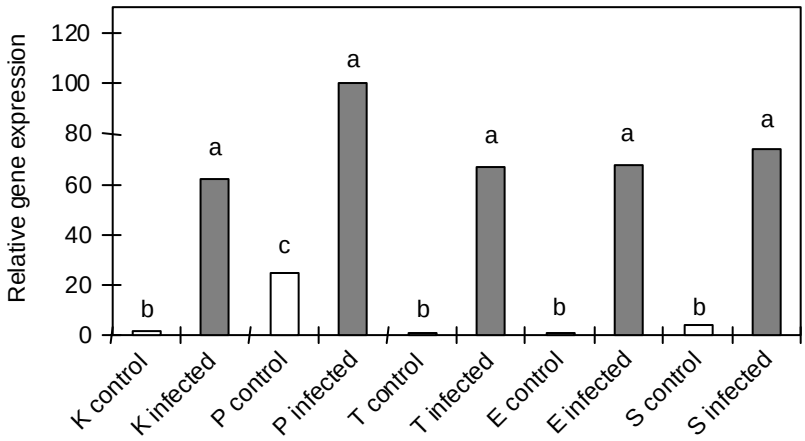


Figure 6. Relative expression of the *FaPGIP* gene monitored by real-time RT-PCR in white berries of five *F.* x *ananassa* cultivars infected with *B. cinerea*. K, Korona; P, Polka; T, tenira; E, Elsanta; S, Senga sengana. The analysis was performed on cDNA obtained one day after infection, and berries inoculated with sterile water served as the control. The *FaPGIP* gene expression level of inoculated Polka fruits was used as the reference (= 100%).Three samples were analysed for each treatment, and all PCR reactions were performed in triplicate. Treatments marked with a different letter differ significantly from each other ($P < 0.05$).

Among the cultivars, Polka showed a significantly higher constitutive expression level of *FaPGIP* in the control berries. Melting point analysis of the *FaPGIP* RT-PCR products resulted in a single peak in all the cultivars, indicating the presence of a single PCR product. The melting temperature ranged from 77.0ºC to 78.6ºC, with no clear differences between the cultivars or the treatments included (data not shown).

Discussion

A cDNA clone encoding a *FaPGIP* was isolated from strawberry using degenerate primers designed based on conserved regions of apricot and apple *PGIP*. Its identity was confirmed by structural data and by its close homology with other characterised *PGIP*s. Strawberry *PGIP* showed a high degree of similarity to *PGIP*s previously isolated from related fruit species, such as apple (Yao et al., 1999), pear (Stotz et al., 1993) and especially raspberry (Ramanathan et al., 1997), where the similarity between the deduced protein sequences was 86%.

PGIPs belong to a group of proteins with repetitive LRR sequences and that are involved in protein-protein interactions (Kobe & Deisenhofer, 1995). The consensus in the strawberry LRR region is similar to plant specific-LRR defined by Kajava (1998), which is also found in most of the characterised plant resistance genes (Mattei et al., 2001). The three-dimensional structure of a PGIP from *Phaseolus vulgaris* has very recently been determined (Di Matteo et al., 2003), and its structure reveals a negatively charged surface on the LRR that is likely involved in binding PGs. Leckie et al. (1999) and Stotz et al. (2000) used site-directed mutagenesis and statistical analysis, respectively, and identified, both within and outside the solvent-exposed region of PGIPs, putative target amino acids involved in PG-PGIP interaction. The alignment of fruit PGIP (Fig. 2A) shows that the ten solvent exposed β-strand regions of these PGIPs are partly conserved between the identified plant species. The residues identified as important for the protein interaction by Leckie et al. (1999) and by Stotz et al. (2000) also show high variation between the identified plants within the Rosaceae. Stotz et al. (2000) propose that these sites are adaptively evolved in response to natural selection by the PGs present in the pathogen population. In accordance with this hypothesis, the strawberry PGIP shows unique residues at many of the sites, that are suggested to be positively selected. Whether these residues confer specificities to *B. cinerea* remains to be examined with techniques as site-directed mutagenesis.

In DNA gel-blot analysis, as many as ten bands were detected with the *FaPGIP* probe in the *F.* x *ananassa* cultivars, whereas five hybridisation bands were detected in the diploid *F. vesca* and

in the octoploid *F. chiloensis*. This pattern agrees with the nature of *F.* x *ananassa,* an interspecific hybrid between the octoploids *F. chiloensis* and *F. virginiana*, and suggests the presence of a small *FaPGIP* gene family in the genus *Fragaria*. In raspberry (Ramanathan et al., 1997) and apple (Yao et al., 1999), both diploid species related to strawberry, three to five hybridisation bands were detected in the DNA blot analysis, suggesting the presence of *PGIP* gene families of a size similar to strawberry. The ancestry of the cultivars is reflected in the banding patterns: the closely related cultivars Korona, Senga sengana and Polka show a different basic pattern than the related cultivars Elsanta and Tenira. The relatively low number of hybridisation bands in the latter two cultivars could suggest more limited allelic variation for the *FaPGIP* genes. The octoploid species *F. chiloensis* was included in the analysis as a reference for complex banding patterns. Surprisingly, the *Eco*RI banding pattern of *F. chiloensis* differs considerably from that of *F.* x *ananassa*.

During fruit maturation the strawberry *PGIP* gene was up-regulated in ripe red fruit only. In contrast, constant transcript levels of *PGIP* were detected throughout fruit maturation in raspberry (Ramanathan et al., 1997), tomato (Stotz et al., 1994) and apple (Yao et al., 1999), whereas harvested apples showed elevated *PGIP* expression levels. This up-regulation of *FaPGIP* in strawberry could be related to factors such as oxidative stress during fruit ripening or changes in the sugar content. Aharoni et al. (2002) showed that pathogenesis-related genes are upregulated in strawberry fruit due to ripening-related stress, whereas Salzman et al. (1998) reported an accumulation of antifungal proteins during fruit ripening in grape, possibly induced by elevated hexose levels.

Strawberry *PGIP* was clearly induced by infection with *B. cinerea* in the present study, whereas wounding seemed to have no impact on the transcript level. The inoculated green, white and red berry stages showed a high induction of the *FaPGIP* gene 24 h after treatment, whereas in turning berries the induction was delayed and occurred 48 h after treatment. *PGIP* response 24 h after *B. cinerea* inoculation has also been shown in other species, for example, in *Arabidopsis* (Ferrari et al., 2003), bean (Bergmann et al., 1994) grape (Bézier et al., 2002) and apple (Yao et al., 1999). Fungal PG is active in the infection process at all fruit maturity stages (van der Cruyssen et al., 1994), and the oligogalacturonides (OG) derived from pectin degradation from early germination of the conidia may be the source of elicitation of the *PGIP* induction (Albersheim et al., 1992). In agreement, Ferrari et al. (2003) showed that the *PGIP* gene *AtPGIP1* of *A. thaliana* was induced by elicitor-active OG as early as 90 min after treatment and sustained for at least six hours; by 24 h the transcripts returned to basal levels. In contrast, the *AtPGIP1* gene was induced by *B. cinerea* 24 h after inoculation, but the peak occurred 48

hours after the treatment. Another *PGIP* gene of *A. thaliana*, *AtPGIP2*, was not induced by OG, but was similarly induced by *B. cinerea* and additionally by jasmonate, the induction peak occurring 48 h after treatment (Ferrari et al., 2003). In the inoculated green and white berries examined in the present study, the decline in *FaPGIP* gene expression level at day 2 was followed by a second induction at days 3 and 4 after treatment. It is likely that the separate induction peaks are derived from different signal transduction pathways, triggering the same or different homologs. If the separate induction peaks observed represent expression of different *PGIP* sequences, the homologs should show high sequence similarity in the target area due to the similar melting points observed.

The maximum induction of *FaPGIP* following *B. cinerea* infection was delayed in turning and red berries compared to green and white berries. Recently Isshiki et al. (2003) showed that the promoter activity of an endo-PG from *Alternaria citri* was induced by pectin but repressed because of catabolic repression by sugars. If this is also the case for *B. cinerea* PG genes, it could explain the delayed induction of *FaPGIP* in red and turning berries, as the sugar content increases drastically during fruit ripening (Seymour et al., 1993). Therefore, it would be useful to monitor the expression levels of fungal PGs simultaneously with the host *FaPGIP*s as the infection progresses to elucidate the timing of this signal.

The induction of *FaPGIP* by *B. cinerea* infection was observed in all the cultivars included in this study. The five cultivars show differential susceptibility towards *B. cinerea.* Elsanta, Tenira and Polka are in general considered less susceptible than Korona, while S. sengana is regarded as the most susceptible (Daugaard, 1999). The high constitutive expression of *FaPGIP* observed in the relatively resistant cultivar Polka is in agreement with this pattern. No significant differences between cultivars were observed in the induction level of *FaPGIP* following inoculation. Apparently, the genetic variation observed between the cultivars in the DNA blot analysis is not reflected in the level of *FaPGIP* gene expression. Time-points earlier than the 24 hours post inoculation employed here will be needed to see if the cultivars show variation in the timing and level of *FaPGIP* induction during the early stages of infection. It should be also kept in mind that PGIP is not the only factor determining host resistance and that the success of the host plant in warding off the pathogen depends on the coordination of different defence strategies and the rapidity of the overall response.

Based on the present study, it is evident that *FaPGIP* expression is downstream induced by *B. cinerea* in the studied *F.* x *ananassa* cultivars. Nevertheless, the data presented here provide only indirect evidence about the impact of PGIP on *B. cinerea* infection in strawberry, and the significance of PGIP in this pathosystem needs to be verified in further studies based on activity

of the proteins using multiple *B. cinerea* isolates, as the strains may differ in the PG isoforms they produce. In transgenic tomato fruits, over-expression of pear *PGIP* resulted in an increased resistance to *B. cinerea* (Powell et al., 2000), but did not provide complete protection against this pathogen, reflecting the specificity of the PGIPs and the pathogen's ability to produce several isoforms of PG. In the present study, the strawberry *PGIP* was cloned from the cultivar Elsanta. Currently, the isolation of different *FaPGIP* isoforms from several strawberry cultivars is in progress, with the aim of also investigating their promoter regions for cis-acting elements. Furthermore, the activity of strawberry *PGIP* against *B. cinerea* PGs will be studied by using purified proteins from transgenic strawberry plants that over-express *FaPGIP* sequences and by challenging the transformed plants with the pathogen.

Acknowledgements

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Chapter 3

Quantification of allele-specific expression of a gene encoding strawberry polygalacturonase-inhibiting protein (PGIP) using Pyrosequencing™

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Abstract

Recent studies indicate that allele-specific differences in gene expression are likely to be a common phenomenon. The extent to which differential allelic expression exists might however still be underestimated, due to the limited accuracy of the methods used so far. We applied a highly quantitative sequencing method called Pyrosequencing using which, for the gene under study, allele frequency differences as small as 4.0% reliably could be determined. To demonstrate allele-specific expression, we investigated the transcript abundance of six individual, highly homologous alleles of a polygalacturonase-inhibiting protein gene (*FaPGIP*) from octoploid strawberry (*Fragaria x ananassa*). By Pyrosequencing of RT-PCR products, it was shown that one *FaPGIP* allele was preferentially expressed in leaf tissue, while two other alleles showed a fruit-specific expression pattern. For fruits that were inoculated with *Botrytis cinerea* a strong increase in overall *FaPGIP* gene expression was observed. This upregulation was accompanied by a significant change in *FaPGIP* allele frequencies as compared to non-treated fruits. Remarkably, in the five cultivars tested, the allele frequencies in cDNA from the inoculated fruits appeared to be similar to that in genomic DNA, suggesting uniform upregulation of all *FaPGIP* alleles present as a result of pathogenesis-related stress.

Introduction

During the last decade a wealth of sequence information has been generated by genome sequencing projects. Also, an immense amount of gene expression data has become available, mainly through the application of DNA microarrays. Recently, attention has been extended to the allelic diversity of genes, which would allow understanding of the relationships between genetic and phenotypic diversity. Despite the fact that, to date, several million single nucleotide polymorphisms (SNPs) have been identified and are available in public databases, only for a limited number of these has the effect on gene function or expression has been investigated.

Only recently, methods have been described that allow expression analysis at the allele level, but these methods are of relatively low accuracy, difficult to perform and are often time-consuming and expensive. Initial reports describing the expression of alleles are mostly based on PCR-techniques, making use of specific SNPs that lead to restriction site polymorphisms or significant insertions/deletions (Indels) that make separation and subsequent quantification of allelic sequences possible (see e.g. Witte et al., 2001). However, transcripts often lack SNPs in restriction sites or large Indels. Furthermore, methods involving quantification of gel

electrophoresis-separated PCR fragments are of low accuracy. Yan et al. (2002) and Cowels et al. (2002) employed single base extension analysis using fluorescent labelled dideoxynucleotides and detection by a DNA sequencer. Cowels et al. (2002) concluded that this assay was of sufficient precision to detect differences in allele expression levels and used an average ratio of 1.5 as the threshold for detection. In doing so they identified 4 out of 69 genes showing allelic differences in expression level of 1.5-fold or greater among two individuals. Other methods described for quantitative discrimination of allelic sequences rely on differential hybridisation of short oligo probes, such as the 5'-nuclease (TaqMan) assay. However, for quantitative analysis of allele frequencies in pooled DNA samples the 5'-nuclease assay seemed unsuitable (von Samson-Himmelstjerna et al., 2003). Lo et al. (2003) used a modified Affymetrix microarray to screen for human allele-specific differences in transcript abundance. Such a microarray approach allows high throughput expression analysis, but due to high production costs, application will be limited to organisms for which dedicated chips are already available. Next to this, it was experienced that only alleles that show a greater than two-fold difference in expression could reliably be distinguished. Interestingly however, in the study by Lo et al. (2003) it was observed that by screening transcripts originating from different tissues of 7 individuals, 54% of 602 genes analysed showed preferential expression of one allele, indicating that allele-specific gene expression must be a common phenomenon. In consequence of the high threshold level used, the already high percentage of genes that showed differences in expression between their alleles might still be an underestimate!

Pyrosequencing is a robust and quantitative sequencing method, based on real-time detection of pyrophosphate, which is released as a result of nucleotide incorporation in a sequencing-by-synthesis reaction (Ronaghi et al., 1996). Assessing allele frequencies in large genomic DNA pools by Pyrosequencing has demonstrated the high level of accuracy of this method. Wasson et al. (2002) reported the reliable detection of allele frequency of 4% differences between DNA pools from human populations. This result was confirmed by Neve et al. (2002) who estimated that, for large DNA pools, allele frequencies that differ by $\geq$ 5.2% would be significant. Because of its high accuracy, Pyrosequencing has been considered as the method of choice for genotyping SNPs in polyploid species, as demonstrated by Rickert et al. (2002) and Oefner (2002). They showed that the different heterozygous states of a binary SNP in tetraploid potato could reliably be distinguished.

We used Pyrosequencing for analysis of allele-specific expression in octoploid strawberry (*Fragaria x ananassa*). Recently, we described the cloning of the *FaPGIP* gene encoding a polygalacturonase inhibiting protein from strawberry (Mehli et al., 2004). This pathogenesis-

related gene was shown to be induced as a result of inoculation of strawberries with the *Botrytis cinerea*, a fungus causing fruit rot in this crop. Several alleles of *FaPGIP* from different strawberry cultivars have been identified now and Pyrosequencing was applied for quantification of allele frequencies in *FaPGIP* transcripts from healthy berries and from berries inoculated with *B. cinerea*. In doing so, we demonstrated that quantitative RT-PCR for expression analysis at the gene level, and Pyrosequencing for expression analysis at the allele level is a powerful combination for quantitative analysis of allele-specific gene expression.

Materials and methods

Cloning and sequencing of *FaPGIP* alleles

Primers were designed for the identification of alleles of the strawberry *FaPGIP* gene based on the genomic DNA sequence described by Mehli et al. (2004). PCR fragments encompassing the main part of the *FaPGIP* gene sequence were obtained from strawberry genomic DNA, and ligated into the pGEMT-easy vector (Promega, Madison, WI, USA) prior to cloning. To minimise PCR errors, a minor fraction (0.25 U / 50 µl) of proofreading DNA polymerase (PfuTurbo Hotstart DNA polymerase, Stratagene, La Jolla, CA, USA) was added to the PCR reaction mixture (Jumpstart Taq DNA Polymerase, Sigma, St. Louis, MO, USA). To minimise the risk of missing polymorphisms due to preferential amplification, two sets of PCR primers (PGIP-FW1, 5'- GGACTACCGACTTGTATATGCAGC and PGIP-REV1, 5'-TATATCTAG-CATCCCTCCGT ; PGIP-FW2: 5'-TGACTTACAGACGCATGCCA and PGIP-REV2, 5'-CAAAATTGTAGGTAACATCTCG respectively) were used, amplifying an overlapping sequence of 1280 bp of *FaPGIP* from the strawberry cultivars Elsanta, Korona, Polka, Senga sengana and Tenira. From this fragment an internal sequence of 600 bp, containing the single intron that was present in strawberry *PGIP*, was sequenced with the forward primer PGIP-seq1 (5'-CCTCCATCGCCAAGCTCAAG) and the reverse primer PGIP-seq2 (5'-CAAGTCCAG-TGAGATCAAGC). In order to identify all the polymorphisms present, 48 cloned *FaPGIP* fragments were sequenced for each cultivar.

Quantitative RT-PCR

Quantitative RT-PCR experiments, for quantification of *FaPGIP* expression at the gene level, have been described elsewhere (Mehli et al., 2004).

Pyrosequencing of *FaPGIP* transcripts

Pyrosequencing was used as a quick and accurate method for genotyping different strawberry cultivars and to quantify allele frequencies in RT-PCR products. Sequencing primers were developed using SNP Primer Design software (version 1.01) from Biotage AB (Uppsala, Sweden). Pyrosequencing was carried out according to manufacturer's standard protocols (Biotage, Uppsala, Sweden).

For expression analysis of *FaPGIP* at the allele level, cDNA was obtained from two experiments conducted by Mehli et al. (2004). In the first case, cDNA samples from leaf and different developmental stages of strawberry fruits of the cultivar Elsanta were used. In the second case, cDNA from control and *B. cinerea* inoculated white-stage fruits of the cultivars Elsanta, Korona, Polka, Senga sengana and Tenira were analysed. All tissues and treatments were sampled in triplicates. For comparison, allele frequencies of *FaPGIP* were also determined from genomic DNA of the five cultivars. Pyrosequencing of RT-PCR products was performed using two different PCR products as sequencing template, one on which SNPs for discrimination of the alleles *FaPGIP*1a, *FaPGIP*1 (a+b+c) and *FaPGIP*3 were present, while the second PCR product was used for discrimination of the *FaPGIP* alleles *FaPGIP*1a, *FaPGIP*1c, *FaPGIP*2c and *FaPGIP*3 (see Fig. 1A). Frequencies for alleles *FaPGIP*1b and *FaPGIP*2a and/or *FaPGIP*2b, could be derived from the other frequencies obtained. Because SNPs that discriminate *FaPGIP*2a and *FaPGIP*2b were only present in the intron sequences, the frequencies of these alleles could not be determined at transcript level. Therefore, for the cultivars Polka and Senga sengana, in which both allele *FaPGIP*2a and *FaPGIP*2b were present, the joint frequency for both alleles is presented. The Pyrosequencing patterns of the separate cloned *FaPGIP* alleles were used as reference patterns for accurate estimation of the allele frequencies in RT-PCR and genomic DNA samples.

Results

Identification of allelic variation

From the five strawberry cultivars, seven different variants of the *FaPGIP* sequence could be identified within the sequenced 600 bp fragment (Fig. 1A). These variants differed in one or more SNPs or Indels. Based on the level of similarity, the *FaPGIP* sequences could be divided into three major groups, *FaPGIP*1, -2 and –3. *FaPGIP*1 and *FaPGIP*2 could be subdivided in three different subforms (a,b,c). The level of homology was very high, ranging from 96.3%

A

```
FaPGIP1a           : CCTCAAGATGCTCAGGCTCAGCTGGAACGGCCTCTCTGGCTCAGTCCCTGACTTCCTCAGCCAGCTTAAGAACCTCACCTTCCTTGAGCTTAACTATAAC : 100
FaPGIP1b           : .................................................................................................... : 100
FaPGIP1c           : .................................................................................................... : 100
FaPGIP2a           : ....................................................................................C.....C......... : 100
FaPGIP2b           : ....................................................................................C.....C......... : 100
FaPGIP2c           : ....................................................................................C.....C......... : 100
FaPGIP3            : .................................T..A...............................................C.....C......... : 100
AF196890 F.iinumae : .................................T..A...............................................C.....C......... : 100
AF196891 F.vesca   : ..................................................................G.................C.....C......... : 100

                                                                    B
FaPGIP1a           : AACTTCACAGGCTCCGTTCCCAACTCGCTTTCAAAGCTACCGAACTTGCTAGCTCTTCATCTAGACCGCAACCAGCTCACAGgtatacatcaaattaact : 200
FaPGIP1b           : .................................................................................................... : 200
FaPGIP1c           : .................................................................................................... : 200
FaPGIP2a           : ......................G.............................................................t............... : 200
FaPGIP2b           : ......................G............................................................................. : 200
FaPGIP2c           : ......................G............................................................................. : 200
FaPGIP3            : ......................G...............T...........................................................t. : 200
AF196890 F.iinumae : .....................GG......C........T...........................................................t. : 200
AF196891 F.vesca   : ......................G............................................................................. : 200

FaPGIP1a           : cta--cagttttttcctcatttagttacagcttatactctaccggtcagaaaatgagtaggtaaatgacgttctagggatacattta-gactaataagtta : 297
FaPGIP1b           : ...--...--............................................................................-............. : 295
FaPGIP1c           : ...--.................................................................................-............. : 297
FaPGIP2a           : .........a............................................................................-............. : 299
FaPGIP2b           : .........a............................................................................-............. : 299
FaPGIP2c           : .........a............................................................................-............. : 299
FaPGIP3            : .........a.............................................------------..........g.......tg............. : 288
AF196890 F.iinumae : .........a.............................................------------..........g.......tg............. : 288
AF196891 F.vesca   : .........a............................................................................-............. : 299

FaPGIP1a           : actgtgttagtaacagagttttgacgttgatatataataatgtgactgcagGTAATATTCCTAGCGCGTACGGGAAATTCGTTGGCACCGTTCCAGATCT : 397
FaPGIP1b           : ...................................................................A................................ : 395
FaPGIP1c           : ...................................................................A................................ : 397
FaPGIP2a           : ..a.............................................................TT.A................................ : 399
FaPGIP2b           : ..a.............................................................TT.A................................ : 399
FaPGIP2c           : ..a.............................................................TT.A................................ : 399
FaPGIP3            : ..a--...........................................................TT.C.T.............................. : 386
AF196890 F.iinumae : ..a--...................t.......................................TT.A.T.............................. : 386
AF196891 F.vesca   : ..a.............................................................TT.A................................ : 399

FaPGIP1a           : CTTCCTCTCCCACAACAAGCTCACAGGCAAAATCCCAACCTCATTTGCTAATATGAACTTTGATCGGATAGACTTGTCACGCAACATGCTGGAAGGAGAC : 497
FaPGIP1b           : ...................................................C................................................ : 495
FaPGIP1c           : ...................................................C...............C................................ : 497
FaPGIP2a           : ...................................................C................................................ : 499
FaPGIP2b           : ...................................................C................................................ : 499
FaPGIP2c           : ...................................................C.....................G.......................... : 499
FaPGIP3            : ...................................................C.............A.............A..........C......... : 486
AF196890 F.iinumae : ...................................................C......................................C......... : 486
AF196891 F.vesca   : ...................................................C................................................ : 499

                                         B
FaPGIP1a           : GCGTCAATGATATTCGGGATGAACAAGACGACCCAGATTGTGGATTTGTCAAGGAACATGCTAGAATTCGATCTGTCCAAGGTGGTGTTTTCGACAAG : 595
FaPGIP1b           : .................................................................................................. : 593
FaPGIP1c           : .................................................................................................. : 595
FaPGIP2a           : .................................................................................................. : 597
FaPGIP2b           : .................................................................................................. : 597
FaPGIP2c           : .................................................................................................. : 597
FaPGIP3            : .................................................................................................. : 584
AF196890 F.iinumae : .................................................................................................. : 584
AF196891 F.vesca   : .................................................................................................. : 597
```

B

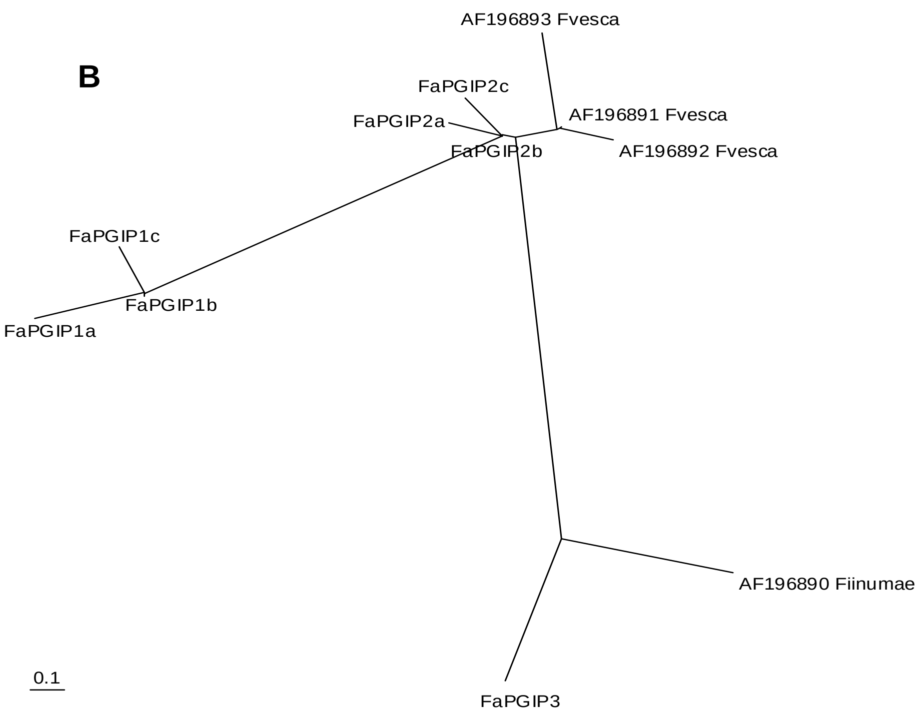

Figure 1. (previous page) Comparison of a 600 bp fragment of genomic *FaPGIP* sequences obtained from the five studied *F*. x *ananassa* cultivars. *F. vesca* and *F. iinumae* are included as representatives for diploid *Fragaria* species. (**A**) DNA sequence alignment of *FaPGIP*. Sequencing primers are located directly outside the presented *FaPGIP* sequence. Solid arrows indicate primers for amplification of fragments for Pyrosequencing; arrows marked with 'B' are biotinylated primers. Interrupted arrows indicate Pyrosequencing primers. Intron sequences are in lower case. The boxed sequences were analysed by Pyrosequencing. hyphen = deletion; dot = identical nucleotide. (**B**) Phylogenetic tree showing the close relationship of the different *FaPGIP* sequences and *PGIP* sequences from diploid *Fragaria* species.

Table 1. An overview of the presence of different alleles of the strawberry *FaPGIP* gene in different strawberry cultivars used in this study. X= present; - = absent.

	FaPGIP alleles						
Strawberry cultivar	1a	1b	1c	2a	2b	2c	3
Elsanta	X	X	-	X	-	-	-
Korona	-	X	X	-	X	-	X
Polka	-	X	X	X	X	-	X
Senga sengana	-	X	X	X	X	X	X
Tenira	-	X	-	X	-	-	-

(*FaPGIP*1b compared with *FaPGIP*3) to 99.8% identity (*FaPGIP*1b compared to *FaPGIP*1c). Published *PGIP* sequences from the wild diploid *Fragaria* species *F. vesca,* showed a high level of similarity to *F. x ananassa* PGIP sequences belonging to group 2 (*FaPGIP*2a,b,c), whereas the sequenced *PGIP* from *F. iinumae* showed high homology to *FaPGIP*3 (Fig. 1B). Sequencing revealed that *FaPGIP*1b was common to all cultivars and that in all the cultivars tested at least two different alleles of *FaPGIP* were present. A maximum of six different *FaPGIP* alleles was found in Senga sengana (Table 1). The variation in the number of *FaPGIP* alleles between the different cultivars corresponds to the variation in number of hybridisation bands found by Southern blot analysis of *Eco*RI digested DNA in our previous study (Mehli et al., 2004). For Tenira, for which two *FaPGIP* alleles were found, the smallest number of hybridisation bands (five) was obtained in this Southern blot, whereas for Senga sengana the highest number of bands (nine) was found. This result suggests that the number of sequenced clones was sufficient to discover most of the allelic variation present in the strawberry cultivars tested. This was also confirmed by Pyrosequencing of genomic DNA of the different cultivars (Fig. 3).

Expression of alleles

Analysis of RT-PCR products of leaf tissue, and from the developmental fruit stages of Elsanta, showed that the three *FaPGIP* alleles found in Elsanta were present in variable frequencies, suggesting differential expression (Fig. 2B). In leaf cDNA, the main allele that was present was *FaPGIP2*a, whereas this allele was underrepresented in fruit cDNA samples. *FaPGIP*1a and 1b showed fruit specific expression. In cDNA from small green fruit tissue, *FaPGIP*1a was the main allele present, but during fruit development the proportion of the alleles *FaPGIP*1b and *FaPGIP*2a increased at the cost of *FaPGIP*1a. Quantitative RT-PCR analysis (Fig. 2A) has shown that the overall *FaPGIP* gene expression was relatively low in all tissues tested, except

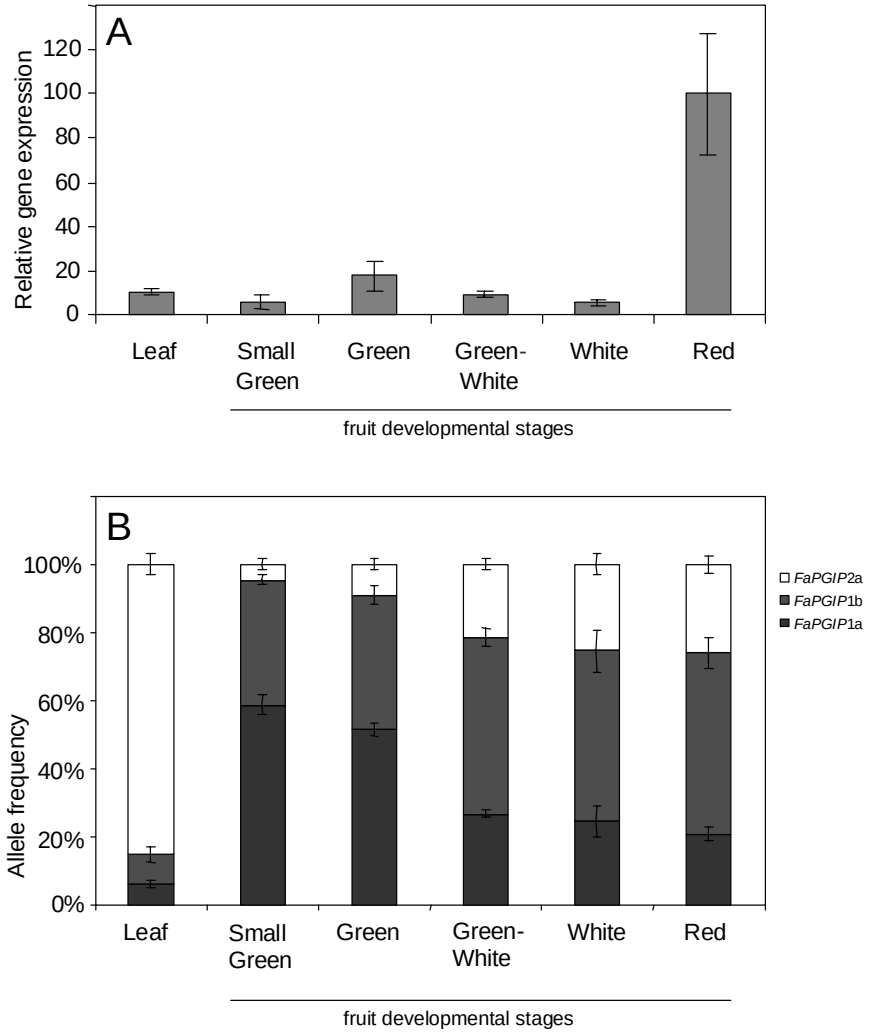

Figure 2. *FaPGIP* gene expression and allele frequency analysis in leaf and strawberry fruit tissues from the strawberry cultivar Elsanta at different stages of development. Three samples were analysed for each tissue, values are ± SE. (**A**) Overall *FaPGIP* gene expression as assessed by quantitative RT-PCR in strawberry leaf and unripe fruit stages of the cultivar Elsanta. (**B**) Distribution of *FaPGIP* alleles estimated by Pyrosequencing using RT-PCR products as sequencing template.

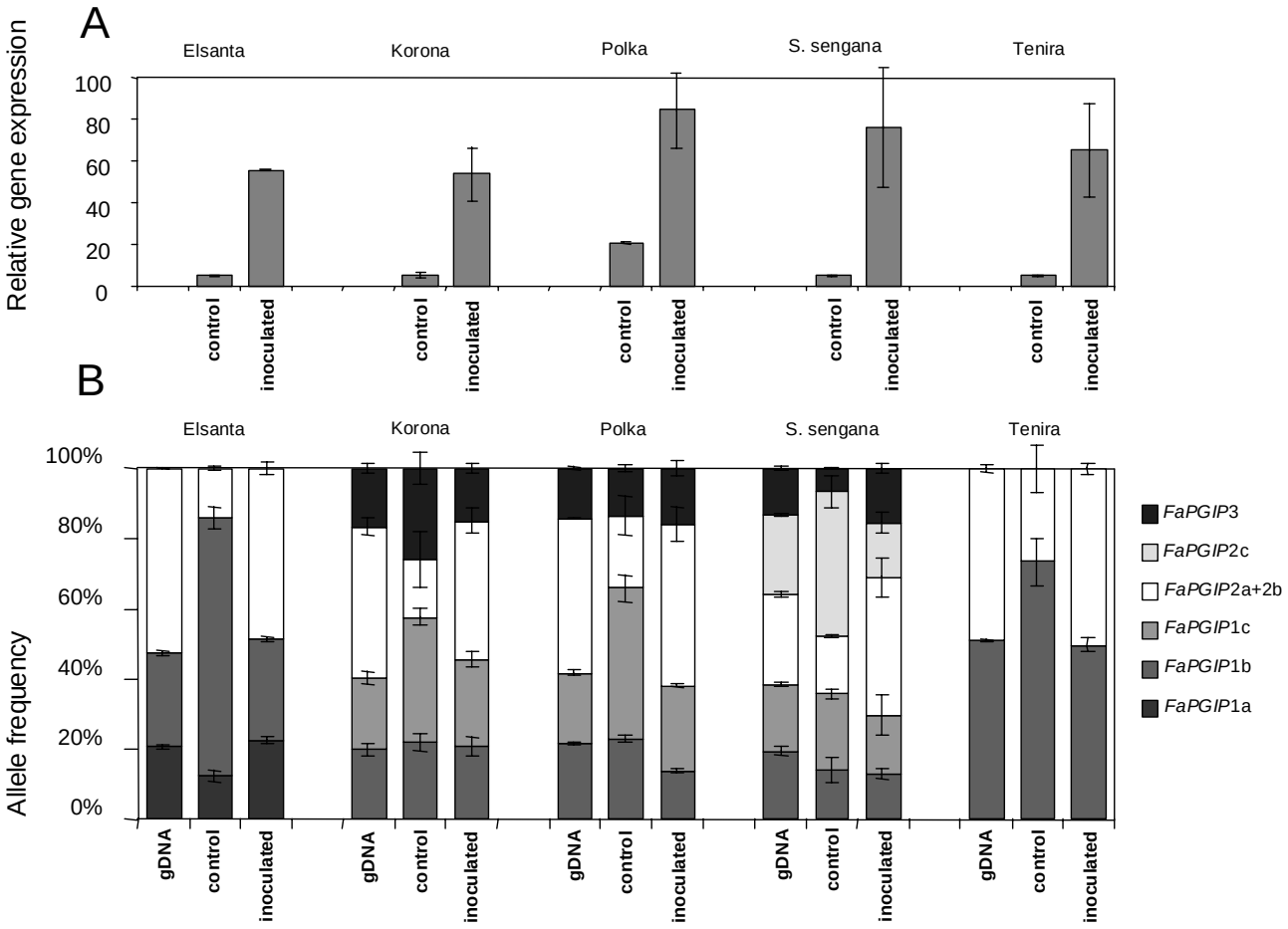

Figure 3. *FaPGIP* gene expression and allele frequency analysis in control white fruits and white fruits inoculated with *B. cinerea*. Three samples were analysed for each treatment, values are ± SE. (**A**) Quantitative RT-PCR of overall *FaPGIP* gene expression, 1 day after inoculation of white strawberry fruits of the strawberry cultivars Elsanta, Korona, Polka, Senga sengana and Tenira. The *FaPGIP* gene expression level of one inoculated Polka fruit sample was used as reference (100%). (**B**) Distribution of *FaPGIP* alleles estimated by Pyrosequencing using RT-PCR products as sequencing template. The distribution of *FaPGIP* alleles in genomic DNA is added as reference.

for red fruit tissue where *FaPGIP* was significantly upregulated. However, this *FaPGIP* upregulation did not result in significant differences in relative allele frequencies between white and red fruits (Fig. 2B).When white fruits of the five strawberry cultivars were challenged with *B. cinerea*, *FaPGIP* gene expression was upregulated significantly one day after infection (Fig. 3A) (Mehli et al., 2004). Compared to untreated control fruits, a clear increase in the relative frequency of allele *FaPGIP*2a and/or *FaPGIP*2b was observed in the inoculated fruit cDNAs (Fig. 3B). This increase coincided with a decrease in the frequency of *FaPGIP*1(a,b,c) alleles. Only for Senga sengana the frequency of *FaPGIP*1b and *FaPGIP*1c did not alter, but the

frequency of *FaPGIP*2c, which is specific for this cultivar, decreased strongly. Remarkably, for all the five cultivars the frequency distributions of the different *FaPGIP* alleles in inoculated fruit cDNAs corresponded to their representation in genomic DNA (Fig. 3B).
The allele frequencies determined for genomic DNA samples, which were analysed in triplicate, showed an average variation of 4.0 ± 2.8% (n=18). For cDNA samples the avarage variation between the repeats in the experiment with leaf and developmental fruit stages (Fig. 2B) was 7.8 ± 1.3% (n=18), while for the experiment in which the effect of inoculation with *B. cinerea* was studied (Fig. 3B), the average variation was 10.0 ± 2.1% (n=36).

Discussion

We investigated the suitability of Pyrosequencing, a novel quantitative sequencing method, for quantitative analysis of allele-specific gene expression. Pyrosequencing promises to be a valuable tool for this purpose, as Wasson et al. (2002) and Neve et al. (2002) reported that this technique was highly quantitative for the detection of allele frequencies in large genomic DNA pools. Furthermore, Rickert et al. (2002) showed that 82% of the polymorphic sites tested were amenable to allelic discrimination by Pyrosequencing, which compared favourably to other SNP genotyping studies. Another advantage of Pyrosequencing is the possibility to determine multiple SNP frequencies in a single measurement, allowing analysis of more than two alleles simultaneously. This may be desirable for genetic studies involving polyploid genotypes.

Identification of *FaPGIP* alleles

To validate Pyrosequencing as a useful tool for genetic analysis at the transcript level, we made use of strawberry, an octoploid and hybrid species, in which genetic analyses are complicated. As an example, the expression of different alleles of the pathogenesis-related strawberry *FaPGIP* gene was studied during strawberry fruit development and after induction by fungal inoculation of fruit tissue. This *FaPGIP* gene is considered to be a member of a small gene family in *F. x ananassa* (Mehli et al., 2004), so if two or three *FaPGIP* gene copies are present, in principle up to 16 or 24 different alleles, respectively, might be found in the octoploid strawberry genome. Sequencing of genomic *FaPGIP* clones however revealed that variation for this gene was limited. For a fragment of 600 bp only 7 different *FaPGIP* variants were found in 5 different strawberry cultivars. For some cultivars only 2 or 3 of these *FaPGIP* alleles could be identified. The present study may therefore focus on just one *FaPGIP* gene copy only. However, it cannot be excluded that more than one *FaPGIP* gene copy was involved. Anyhow,

for convenience we refer in this publication to the different *FaPGIP* sequences as alleles. Based on SNPs in the cDNA sequence, 6 different *FaPGIP* alleles could be discriminated at transcript level.

FaPGIP allele-specific expression

The frequencies of the three different *FaPGIP* alleles found for the cultivar Elsanta were estimated in cDNA samples from leaf tissue and from fruits at different stages of development. It was shown that expression of the *FaPGIP*1a and 1b alleles was mainly restricted to fruit tissue. Allele *FaPGIP*2a was preferentially expressed in leaf tissue, but this allele was also present at increasing frequencies during development of fruits. The different alleles therefore appear to be controlled by different regulatory mechanisms in different tissues. The frequency distribution of the three *FaPGIP* alleles was not affected by ripening induced *FaPGIP* expression in red fruit. Only the total level of expression of all *FaPGIP* alleles was enhanced, which implies that during ripening the *FaPGIP* alleles were co-ordinately upregulated.
Allele frequencies were also estimated from cDNA from control and *B. cinerea* inoculated white-stage fruits of the five different strawberry cultivars. This upregulation overruled tissue-specific *FaPGIP* expression and was accompanied by significant changes in *FaPGIP* allele frequencies as compared to non-treated fruits. Interestingly, for all five cultivars studied, *FaPGIP* allele frequencies in cDNA from inoculated fruits appeared to be similar to allele frequencies as determined for genomic DNA. This suggests that the timing and the high induction level of expression of all the *FaPGIP* alleles present in the strawberry genome is similar due to the pathogenesis-related stress. We can, therefore, conclude that the upregulation of *FaPGIP* following pathogen infection is highly context specific, and involves different regulatory mechanisms than the ripening related upregulation of gene expression. Apparently, the synchronous strong initiation of expression of all the *FaPGIP* alleles present is important to achieve maximum potential inhibitory action towards polygalacturonase activities of invasive pathogens.

Regulatory variation

Analysis of allele-specific expression has the potential to enhance the ability to identify regulatory genetic variation that results in differential allelic expression (Knight, 2004). The observed differential allelic expression of the *FaPGIP* gene suggests the presence of allele-specific variation in *cis*-regulatory elements. We have examined 5'-upstream DNA sequences from the three *FaPGIP* alleles present in the cultivar Elsanta and identified several

polymorphisms (results not shown) which may allow differential binding of regulatory factors. Database-assisted screening of the promoter sequences revealed that several *cis*-acting elements found in other plant species are also present in *FaPGIP* promoter sequences, and that some are unique for one or two of the alleles. However, without experimental data it cannot be determined which of the specific *cis*-acting regulatory variants are functionally important. Deletion mapping analysis, site-specific mutagenesis and gel mobility shift experiments are required to get insight into the location of the functional important regulatory regions.

At present, in general, it is not known what proportion of regulatory variants has functional consequences. For PGIPs it is known that individual PGIPs from a single plant can exhibit differential inhibitory activities against polygalacturonases from various fungi or even towards different polygalacturonases from the same fungus as demonstrated by Desidiro et al. (1997). Recent studies have also demonstrated that PGIPs may also play a role in developmental processes (Jang et al., 2003). The combination of such variation in gene function with allelic differences in gene expression, will contribute significantly to phenotypic diversity.

Pyrosequencing is an accurate method for allele-specific expression analysis

The experiments described in this paper demonstrate the suitability of Pyrosequencing for gene expression analysis at the allele level, enabling discrimination of only subtle differences in transcript abundance of the different alleles. With the Pyrosequencing assay designed for SNP frequency analysis in *FaPGIP* alleles, frequency differences as small as 4.0 ± 2.8% could reliably be identified in replicated genomic DNA samples. This is in agreement with that reported by Wasson et al. (2002) and Neve et al. (2002). This means that application of Pyrosequencing for analysis of allele frequencies in RT-PCR products, provides an important improvement to earlier described methods. The relatively low level of variation in allele frequencies observed within replicated cDNA samples demonstrates that the allele frequency distributions for each tissue or treatment are highly context-specific. The somewhat larger variation observed in the experiment with *B. cinerea* inoculated fruits might be explained by the more dynamic regulation of *FaPGIP* expression as a result of inoculation, which was shown to be transient and was downregulated again after 2 days (Mehli et al., 2004).

Yan et al (2002b) indicated the need for highly quantitative methods for analysing gene expression at the allelic level. In their brief communication they showed that in humans, a 50% decrease in expression level of one allele of a tumor suppressor gene was associated with a pronounced predisposition to hereditary colorectal tumors. Based on this and other observations, they predict that similar and smaller changes in allelic gene expression will be

found to cause other hereditary diseases. Recent studies (Cowels et al., 2002; Yan et al., 2002; Lo et al., 2003) give evidence that allele-specific expression is relatively common among non-imprinted autosomal genes in human and mouse. Lo et al. (2003) demonstrated this by showing that for more than 50% of the 602 genes examined, preferential expression of one allele was observed. Since their method could only discriminate expression levels with a greater than a two-fold difference, this percentage is certainly an underestimate and implies that differential allelic expression is likely to be even more common than expected. Interestingly, in our study, in which we focussed on just one gene, we found indication of different regulatory mechanisms, depending on tissue type, developmental stage and environmental conditions. The observed diversity in regulation of *FaPGIP* alleles confirms the supposed generality of allele-specific expression.

Conclusion

If SNPs in coding regions are known for the alleles of a gene of interest, quantitative RT-PCR combined with Pyrosequencing of the RT-PCR products offers a highly quantitative method for analysis of allele-specific expression. The method described here is generally applicable for determination of frequencies of any polymorphism that discriminates allelic sequences. Since there are strong indications that differential allelic expression is a relatively common phenomenon, and because it is suggested that small allele-specific differences might be physiologically important (Yan et al., 2002; Knight, 2003), Pyrosequencing may be a valuable technology for uncovering important small variations in allele-specific gene expression.

Acknowledgements

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Chapter 4

Tissue-specific expression of the ß-glucuronidase reporter gene in transgenic strawberry (*Fragaria x ananassa*) plants

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Abstract

The strawberry (*Fragaria* spp) is regarded as a false fruit because it originates from the receptacle, which is a non-ovarian tissue. For this reason fruit-specific promoters, isolated from plant species in which the fruit is derived from the ovary wall, might not be suited to control gene expression in a fruit-specific way in strawberry. In order to achieve (false) fruit-specific expression in strawberry the petunia *FBP7* (floral binding protein7) promoter, which proved to be active in the receptacles of petunia flowers, was tested in transgenic strawberry fruits. In strawberry plants containing the *FBP7* promoter fused to the *ß*-glucuronidase (GUS) reporter gene (*gus*), GUS activity was found in floral and fruit tissues of all developmental stages tested, but not in leaf, petiole and root tissue. Surprisingly, Northern blot analysis showed the presence of *gus* derived mRNA's in root (strong) and in petiole (weak) tissue of *FBP7-gus* plants in addition to the floral and fruit tissues. Therefore, it is concluded that histological GUS phenotype does not necessarily correspond with expression at the mRNA level. mRNA quantification using the TaqMan PCR technology confirmed the Northern results and showed that in red strawberry tissue the cauliflower mosaic virus (*CaMV*) *35S* promoter is at least sixfold stronger than the *FBP7* promoter.

Introduction

For functional gene analysis, generally transgenic plants are produced in which the isolated genes are either overexpressed or silenced. For this, usually a constitutive promoter like the cauliflower mosaic virus (*CaMV*) *35S* promoter is used to drive expression of the transgene, but this will affects its function in all plant tissues. To allow a more focussed control of transgene expression, like in strawberry fruits, the use of tissue-specific promoters is desired. For strawberry no fruit-specific promoters have been isolated yet and for this reason the applicability of heterologous promoters is presently being tested.

Mathews et al. (1995) used the tomato fruit-specific *E4* promoter in strawberry transformation experiments. However, fruit-specific expression in strawberry was not demonstrated by these experiments. In tomato, the *E4* promoter is activated by an increase in ethylene concentration associated with the onset of ripening (Cordes et al. 1989). The non-climacteric strawberry fruit, however, shows a decline in ethylene production during ripening (Perkins-Veazie et al. 1995) and the *E4* promoter, therefore, might not be active in strawberry fruits. Manning (1997)

suggested the use of other tomato fruit-specific promoters, which are not regulated by ethylene. A problem in that approach might be the different origin of both fruit tissues, being the pericarp for tomato and the receptacle for strawberry. Due to its non-ovarian origin, the strawberry fruit is really a false fruit and the achenes, which contain the pericarp tissue, are the true strawberry fruits. Therefore, the achenes are the sites in which expression of the proposed tomato promoters can be expected. In the following text for convenience we shall refer to the "false" strawberry fruit as the fruit.

Colombo et al. (1997) reported the cloning of a promoter from petunia which regulates the expression of the MADS box floral binding protein7 (*FBP7*) gene. Expression studies using RNA in situ hybridisation techniques with developing petunia floral buds (Angenent et al. 1995) and analysis of transgenic petunia plants in which *FBP7* promoter-reporter gene fusions were introduced (Colombo et al. 1997) showed that the *FBP7* promoter was active in the seed coat of developing seeds. In addition, it was found that the *FBP7*-promoter showed a high activity in the receptacles of petunia flowers (Colombo and Angenent, unpublished results). Because the strawberry fruit is in fact an outgrown receptacle we decided to determine the suitability of the petunia *FBP7* promoter for giving fruit-specific expression in strawberry, by analysing the expression pattern of a chimeric *FBP7* promoter-*gus* reporter gene in transgenic strawberry plants.

Materials and methods

Plant material and transformation

Young folded leaves of the strawberry (*Fragaria x ananassa*) cultivars Gariguette and Polka and of the Plant Research International breeding line number 88312 were collected and surface sterilized using a 2% (w/v) sodium hypochlorite solution as described by Nehra et al. (1990a). Segmented (4-6 mm) leaflets were transfected according to Puite and Schaart (1996), using the supervirulent *A. tumefaciens* strain AGL0 (Lazo et al. 1991) containing either the binary plasmid pMOG410 (Hood et al. 1993) harbouring the *gus* (Vancanneyt et al. 1990) reporter gene under the control of a *CaMV 35S* promoter or pFBP202 harbouring the *gus* gene controlled by the *FBP7* promoter (Colombo et al. 1997) (Fig. 1A). After cocultivation for 4 days in the dark at 25°C, the leaf explants were transferred to selection medium which consists of modified shoot regeneration medium (Nehra et al. 1990a), in which benzyladenine was replaced by 1 mM thidiazuron and Bacto agar by Phytagel (0.4%, w/v), supplemented with 250

mg/l cefotaxime for elimination of *Agrobacterium* and 50-75 mg/l kanamycin for selection of transgenic shoots. Regenerated shoots were multiplied on proliferation medium containing 25 mg/l kanamycin (Nehra et al. 1990b) and subsequently transferred to the greenhouse.

Southern analysis

DNA was isolated from young folded leaves from greenhouse plants according to the method described by Doyle and Doyle (1987) but including 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. Five µg of DNA of control plants and of plants transformed with the pMOG410 binary vector was digested with *Hin*dIII and DNA of plants transformed with the pFBP202 binary vector was digested with *Hin*dIII together with *Eco*RI. After electrophoresis on a 0.8% agarose gel, blotting of the DNA onto Hybond-N membrane (Amersham) and hybridization with the ^{32}P-labelled coding region of *nptII* was done as described earlier (Puite and Schaart, 1996).

Histochemical GUS assay

Histochemical GUS staining of leaf discs, petioles, root ends and manually-cut sections of different developmental stages of flower buds, flowers and fruits was carried out as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40.

Northern analysis and TaqMan quantitative PCR assay

Total RNA of the strawberry receptacle at different ripening stages and of roots, leaves and petioles was isolated as described by Schultz et al. (1994). For each transgenic line and control plants, RNA was isolated from pooled tissue of fruits at the same stage of development.

For Northern analysis 10 µg total RNA of each sample was denaturated, electrophoresed, blotted onto Hybond N+ membrane (Amersham) and hybridised with ^{32}P- labelled *gus*-coding sequence as described by Aharoni et al. (2000).

The TaqMan quantitative PCR technology makes use of a fluorogenic TaqMan probe, labelled with a reporter dye (FAM, 6-carboxyfluorescein) and a quencher dye (TAMRA, 6-carboxytetramethylrhodamine), that hybridises to sequences located between the forward and the reverse primer. During PCR the TaqMan probe is cleaved due to the 5' nuclease activity of

Taq polymerase leading to uncoupling of the reporter dye and the quencher dye, which results in an increase in reporter fluorescence. The fluorescence intensity is directly related to the amount of input target cDNA. For the Real Time TaqMan quantitative PCR assay, first strand cDNA was synthesised from DNaseI (Gibco BRL, Life Technologies) treated total RNA using the TaqMan Reverse Transcription Reagents (Perkin-Elmer, Applied Biosystems). For monitoring *gus* cDNA levels the TaqMan quantitative PCR was set up in triplets of 20 µl volumes containing first strand cDNA from 10 or 50 ng total RNA, 300 nM forward *gus*-primer (5'-CGGAAGCAACGCGTAAACTC-3'), 300 nM reverse *gus*-primer (5'-TGAGCGTCGCA-GAACATTACAT-3'), 100 nM fluorogenic TaqMan *gus*-probe (FAM-5'-CGCGTCCGATCA-CCTGCGTC-3'-TAMRA), 10x TaqMan buffer A, including a passive internal reference dye, 5.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 0.01 U/µl AmpErase Uracil-n-glycosylase and 0.025 U/µl AmpliTaq Gold DNA Polymerase (all reagents were supplied with the TaqMan PCR Core Reagent Kit (Perkin-Elmer, Applied Biosystems)). Cycling parameters for the PCR reaction were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. As endogenous control a cDNA from strawberry (kindly provided by A. Aharoni) was selected after testing its expression pattern in different strawberry tissues by Northern blot hybridisation. This cDNA showed strong homology to a putative DNA-binding protein gene from *Arabidopsis thaliana* and will be indicated in this report as *dbp*. For monitoring *dbp* cDNA levels, a forward *dbp* primer (5'-TTGGCAGCGGGACTTTACC-3'), a reverse *dbp*-primer (5'-CGGTTGTGTGACG-CTGTCAT-3') and a fluorogenic TaqMan *dbp*-probe (FAM-5'- TCCTTCTTCAGCTGCATC-AATATGTTTTCCA-3'-TAMRA) were used in the TaqMan quantitative PCR reaction. All PCR-reactions were incubated in the ABI Prism 7700 Sequence Detection System (Perkin Elmer, Applied Biosystems). For the real time analysis, PCR products were detected directly by monitoring the increase in fluorescence from the dye labelled *gus*- or *dbp*-specific DNA probe. The amplification was plotted as the normalised reporter signal, ΔR_n (the reporter dye was normalised to the internal reference dye and corrected for the baseline value), towards the number of PCR cycles. For each reaction the threshold cycle, C_T, which is defined as the PCR cycle at which a statistically significant increase of ΔR_n is first detected, was determined. The final relative quantitation was done using the comparative C_T-method (User bulletin #2, ABI PRISM 7700 Sequence Detection System, December 1997, Perkin-Elmer, Applied Biosystems) in which the differences in the C_T for the *gus*-amplicon and the C_T for the endogenous control *dbp*, called ΔC_T, were calculated to normalise for the differences in the total amount of cDNA

present in each reaction and the efficiency of the RT step. For comparison of two samples the ΔC_T values were subtracted from each other, giving a $\Delta\Delta C_T$ value and finally the relative amount of *gus* mRNA copies was calculated by $2^{-\Delta\Delta CT}$.

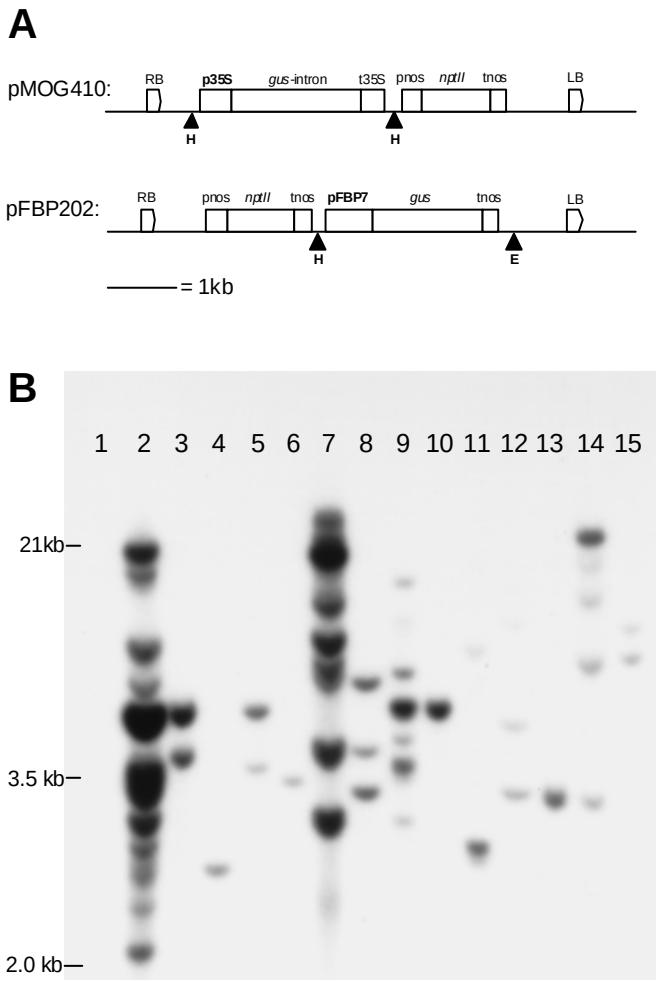

Figure 1. Southern blot analysis of genomic DNA isolated from several independent transgenic strawberry plants. (A) Schematic diagram of the T-DNA of the binary vectors used to study fruit-specific *gus* expression in strawberry. pMOG410, harboring a chimeric *CaMV 35S-gus*-intron gene construct; pFBP202, harbouring a chimeric *FBP7-gus* gene construct. Restriction sites relevant for Southern blot analysis are indicated. RB, right border sequence; LB, left border sequence; p35S, *CaMV 35S* promoter; t35S, *CaMV 35S* terminator; pnos, nopaline synthase promoter; tnos, nopaline synthase terminator; *gus,* ß-glucuronidase gene; *npt*II, neomycin phosphotransferase gene; E, *Eco*RI; H, *Hin*dIII. (B) Total genomic DNA was digested with both *Hin*dIII and *Eco*RI for the pFBP202 plants and with *Hin*dIII for the pMOG410 and the control plants. Probing with the coding region of the *npt*II gene gives an indication of the number of integrated T-DNA copies. Lane 1, DNA of an untransformed control plant; lanes 2-10, DNA of plants transformed with pFBP202; lanes 11-15, DNA of plants transformed with pMOG410. Lanes 8, 9 and 15 represent hybridisation patterns of the transgenic strawberry lines cv. Gariguette/*FBP7-gus-B*, cv. Gariguette/*FBP7-gus-A* and cv. Gariguette/*CaMV 35S-gus* respectively.

Results

Transformation of strawberry

To study the expression pattern of the *FBP7* promoter in strawberry, different strawberry genotypes were transformed with the *Agrobacterium tumefaciens* strain AGL0 containing respectively pFBP202, harbouring a *FBP7* promoter-*gus* fusion and pMOG410 harbouring a *gus* fusion with the constitutively expressed promoter *CaMV 35S* as a control (Fig. 1A). Southern blot analysis was performed on 14 kanamycin resistant clones together with a non-transformed plant as control. The hybridisation patterns that were obtained using the *nptII*-coding region as probe, indicated that in most transgenic plants one or two copies of the transgenes had been integrated into the genome (Fig. 1B).

Activity of the FBP7 promoter in strawberry plants

To investigate the spatial and temporal distribution of *FBP7* promoter activity in transgenic strawberry plants, tissue samples of leaves, petioles, roots, flowers and fruits at different stages of development were analysed by means of histological GUS staining. Plants of the transgenic clone cv. Gariguette/*FBP7-gus-A* were initially selected for this expression study. Clone *FBP7-gus-A* was supposed to be a high GUS expresser as indicated by the intense blue staining of red fruit tissue in histochemical GUS assays. For comparison the corresponding tissues of a cv. Gariguette/*35S-gus* clone were also analysed for GUS expression. Later the same GUS expression patterns were also observed in the other cultivars when transformed with the same binary vector. In summary, in 15 out of 19 *FBP7-gus* and in 9 out of 13 *CaMV 35S-gus* transgenic strawberry plants, a similar GUS expression pattern was observed as found in cv. Gariguette/*FBP7-gus-A* or cv. Gariguette/*35S-gus* respectively. In the remaining plants no or a very low GUS expression level was observed. Representative cv. Gariguette plant material stained with X-gluc is shown in Fig. 2 (page 57).

When plant material of *FBP7-gus* strawberry plants was stained with X-gluc, GUS activity was not found in leaf, petiole or root tissue (Fig. 2A-C), whereas *CaMV 35S-gus* strawberry plants showed strong GUS activity in all these tissues after staining (Fig. 2L-N). Staining of flowers and fruits of *FBP7-gus* (Fig. 2D-K) and of *CaMV 35S-gus* (Fig. 2O-R) plants showed GUS expression at all developmental stages of both transgenic types.

A more detailed study showed some remarkable differences in the GUS expression patterns. In longitudinal sections of flowers and fruits of *FBP7-gus* plants, the proportion of receptacle

tissue demonstrating GUS activity increased during flower and fruit development, starting at the outside of the receptacle of young flowers (Fig. 2D, E) and proceeding towards the pith in senescing flowers (Fig. 2G) and small green fruits (Fig. 2H, I). In fully expanded white and in red fruits the GUS activity was present throughout the entire receptacle (Fig. 2J, K) (for a schematic reproduction of a typical strawberry fruit see Fig. 3.) At all developmental stages, GUS activity was routinely observed in cross-sections of achenes. In flowers a strong GUS activity was also found in the stigmatic region of pistils (Fig. 2F). GUS activity of the *FBP7* promoter in calyx or pedicel tissues was not observed.

In contrast with *FBP7-gus* strawberry plants, the GUS activity observed in floral buds of *CaMV 35S-gus* plants was predominantly located in the pith whereas the greater part of the cortex showed no GUS activity (Fig. 2O). In later stages of floral development GUS activity was found both in the pith as well as in the cortex (Fig. 2P). In all stages of fruits of *CaMV 35S-gus* plants high GUS activity was found throughout the entire receptacle and in the achenes (Fig. 2Q, R). In addition, strong GUS activity was present in calyx and pedicel tissues of *CaMV 35S-gus* transgenic flowers and fruits.

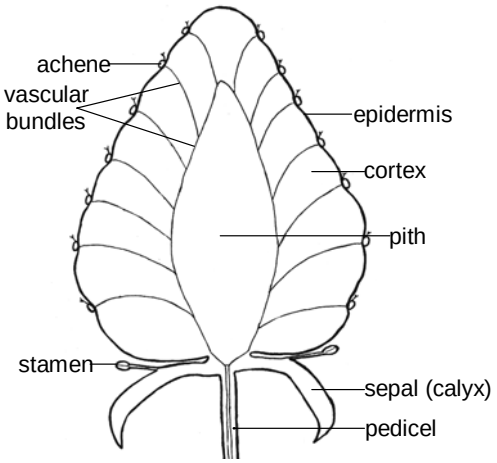

Figure 3. Schematic reproduction of a typical strawberry. The strawberry receptacle consists of a fleshy pith at the center surrounded by a cortex and an epidermis. The strawberry "seeds", the achenes, which are in fact the true strawberry fruits, are located in pits in the surface of the strawberry and are connected to the pedicel via a ring of vascular bundles that extend from the pedicel through the pith and cortex and then branch out towards the achenes (Perkins-Vaezie et al. 1995).

RNA analysis

Northern analysis of *gus* expression was performed on tissues of cv. Gariguette/*FBP7-gus*-A and cv. Gariguette/*35S-gus* both showing strong histochemical GUS staining in red fruits, as well as on cv. Gariguette/*FBP7-gus*-B, which showed weak GUS staining of red fruit tissue (Fig. 4). As a control non-transgenic cv. Gariguette plants were used (results not shown). For clone *FBP7-gus*-A *gus* expression was found at all developmental fruit stages tested (Fig. 4A). Furthermore, a remarkably high *gus* mRNA level was present in roots. In leaf tissue of *FBP7-*

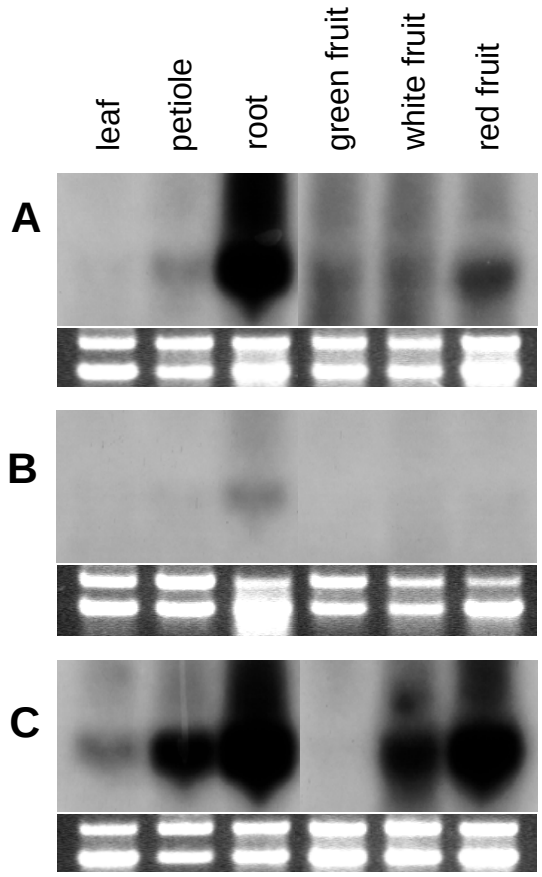

Figure 4. Comparison of *gus* gene expression in different tissues of the transgenic strawberry clones cv. Gariguette/*FBP7-gus-A* (**A**), cv. Gariguette/*FBP7-gus-B* (**B**) and cv. Gariguette/*CaMV 35S-gus* (**C**) by Northern blot analysis. Each lane contains 10 μg of total RNA isolated from different tissues. Ethidium bromide staining of total RNA (lower panels) was used to demonstrate equivalent loading.

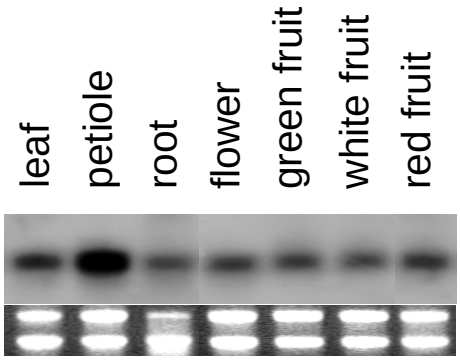

Figure 5. Comparison of *dbp* gene expression in different tissues of non-transgenic strawberry plants by Northern blot analysis. Each lane contains 10 μg of total RNA isolated from different tissues. Ethidium bromide staining of total RNA (lower panels) was used to demonstrate equivalent loading.

gus-A plants *gus* expression was absent, while in the petioles a minor *gus* mRNA signal could be detected. In *FBP7-gus*-B plants, a clear signal was found in the root sample only (Fig. 4B). No other tissue of this clone showed hybridisation with the *gus* probe. In *35S-gus* plants, a high expression of *gus* was found in white and red fruit tissues but on the Northern blot no *gus* expression could be detected in samples of small green fruits (Fig 4C). In petiole and root tissue a high *gus* expression was found while in leaf tissue of *35S-gus* plants a relatively low level of *gus* expression was found. As expected, hybridisation of the *gus*-probe with RNA of the non-transgenic samples gave no signal on the Northern blot (results not shown).

The relative *gus* mRNA levels in red fruit and root tissues of several *FBP7-gus* and *CaMV 35S-gus* transgenic strawberry plants were quantified by TaqMan PCR. For standardisation of the amount of sample mRNA added to the reaction, amplification of an endogenous control has been performed. As endogenous control we selected a cDNA from strawberry which showed a similar level of expression in a Northern blot for several strawberry tissues (Fig. 5). Only for petiole tissue was a higher *dbp* expression level observed. To relate the *gus* mRNA levels of all red fruit samples, we took the level of *gus* mRNA in red fruits of cv. Gariguette/*35S-gus* plants as a calibrator, so that the level of *gus* mRNA in red fruit samples of the different transgenic clones is expressed as an *n*-fold difference to *gus* mRNA in red fruits of cv. Gariguette/*35S-gus*. For the root samples of cv. Gariguette/*35S-gus* and cv. Gariguette/*FBP7-gus*-A and B, the level of *gus* mRNA in red fruit of the same transgenic plant was taken as a calibrator. The results of a TaqMan PCR on samples of red fruit and root mRNA are presented in Tables 1 and 2, respectively. It is shown that the levels of *gus* mRNA measured in red fruits of the *35S-gus* transgenic cv. Polka and 88312 clones are respectively a factor 2.2 and 4.5 higher than the corresponding mRNA level in cv. Gariguette/*35S-gus*, showing the variation present in individual transgenic plants. Whether this variation is due to the genetic background of the cultivars or is inherent to the different individuals is unclear. The *gus* mRNA level in red fruit tissue of cv. Gariguette/*FBP7-gus*-A is a factor 5.9 lower than the *gus* mRNA level in cv. Gariguette/*35S-gus* red fruits (Table 1). In cv. Gariguette/*FBP7-gus*-B, in which the GUS staining in the red fruit was restricted to the vascular bundles and to the outside of the cortex (data not shown), the level of mRNA in the red fruit tissue was only 1/115 times the *gus* mRNA level in red fruits of cv. Gariguette/*35S-gus*. In the roots of all three transgenic clones tested, the level of *gus* mRNA was higher than in red fruit tissue of the same clone (Table 2).

Table 1. Relative quantitation of *gus* mRNA in transgenic strawberry plants using the comparative C_T method. Expression levels of *gus* in red fruit tissue of the different transgenic clones are related to *gus* expression levels in red fruit tissue of cv. Gariguette/*35S-gus.*

Red fruit tissue	*gus*	*dbp*[b]	*gus*
Transgenic clone	average C_T [a] (n=3)	average C_T (n=3)	Relative to cv.Gariguette/*35S-gus*[c]
cv. Gariguette/*35S-gus*	21.90±0.17	20.79±0.04	1.0
			(0.9-1.1)
cv. Polka/*35S-gus*	21.30±0.15	21.39±0.23	2.2
			(1.9-2.8)
#88312/*35S-gus*	21.01±0.15	22.07±0.06	4.5
			(4.2-4.8)
cv. Gariguette/*FBP7-gus*-A	26.22±0.16	22.59±0.14	0.17
			(0.15-0.20)
cv. Gariguette/*FBP7-gus*-B	28.40±0.16	20.44±0.30	0.0087
			(0.0068-0.0110)

[a] The C_T value was determined in three separate RT-PCR reactions

[b] *dbp* is the endogenous control

[c] The range given for *gus* mRNA relative to the mRNA level of cv. Gariguette/*35S-gus* is determined by evaluating $2^{-\Delta\Delta CT}$, with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$ (see also material and methods)

Table 2. Relative quantitation of *gus* mRNA in transgenic strawberry plants using the comparative C_T method. Expression levels of *gus* in root tissue of the different transgenic lines are related to *gus* expression levels in red fruit tissue (Table 1) of the same transgenic plant.

Root tissue	*gus*	*dbp* [b]	*gus*
Transgenic line	average C_T [a] (n=3)	average C_T (n=3)	Relative to Red fruit[c]
cv. Gariguette/*35S-gus*	19.23±0.07	20.62±0.30	8.28
			(6.68-10.3)
cv. Gariguette/*FBP7-gus*-A	19.69±0.05	20.18±0.04	14.4
			(13.8-15.0)
cv. Gariguette/*FBP7-gus*-B	23.21±0.10	19.90±0.19	25.1
			(21.7-29.0)

[a] The C_T value was determined in three separate RT-PCR reactions

[b] *dbp* is the endogenous control

[c] The range given for root-*gus* mRNA relative to *gus* mRNA levels of the corresponding red fruit samples is determined by evaluating $2^{-\Delta\Delta CT}$, with $\Delta\Delta C_T + s$ a nd $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$

Discussion

This study describes the successful identification of promoters that drive gene expression in the (false) fruit of strawberry, thus making modification of strawberry fruit characteristics feasible.
Using a transformation protocol, based on the procedure described by Nehra et al. (1990a), several transgenic strawberry plants have been produced in which the *gus* gene driven either by the petunia flower-specific *FBP7* or the constitutive *CaMV 35S* promoter was introduced into the strawberry genome at low copy number.
For representative *FBP7-gus* and *35S-gus* plants striking differences in *gus* expression patterns were seen during the development of the receptacle of the flower. The observed differences illustrated that both the *CaMV 35S* and *FBP7* promoter regulated gene expression in a unique way in receptacle tissue of strawberry flowers. Whereas in the *35S-gus* plants *gus* expression was observed in all tissues analyzed, in *FBP7-gus* plants the activity of the petunia *FBP7* promoter seemed to be restricted to the flower and fruit tissue. In more detail, in red fruits of both *FBP7-gus* and *35S-gus* plants, GUS staining was seen throughout the entire receptacle, this means in the epidermis, cortex and pith and also in the vascular bundles of the fruit, but staining of the green parts, i.e. pedicel and calyx, was only observed in *35S-gus* transgenic strawberries. These GUS staining results indicated that in strawberry the *FBP7* promoter regulated gene expression in a receptacle specific way, and because of the absence of any detectable GUS staining in other tissues this promoter was considered as a good candidate to direct gene expression specifically in the strawberry fruits.
In order to confirm the GUS activity results and to compare quantitatively the expression levels of *gus* driven both by the *CaMV 35S* and the *FBP7* promoter, RNA analysis was performed using Northern blotting and TaqMan quantitative PCR techniques. The constitutive presence of *gus* mRNA in *35S-gus* strawberry plants was confirmed by Northern blot analysis. Only in small green fruits *gus* mRNA was present at low levels. In *FBP7-gus* strawberry fruits *gus* mRNA was detected in all fruit stages but not in leaf and at low level in petiole tissue. A surprisingly high level of *gus* mRNA was detected in roots of *FBP7-gus* plants. This does not correspond to the absence of GUS enzyme activity in roots of *FBP7-gus* plants as observed in histochemical GUS-stainings. In transgenic *FBP7-gus* petunia plants (Colombo et al. 1997) analysis of root tissue is not described. It would seem that in roots of *FBP7-gus* plants either *gus* mRNA is not translated or GUS enzyme activity is inhibited. This phenomenon appears to be restricted to the roots and related to the use of the *FBP7* promoter. To clarify the disparity

between *gus* transcript levels and the absence of GUS activity in roots of *FBP7-gus* plants, further research is needed. Recently, Gygi et al. (1999) studied the relationship between mRNA and protein expression levels for several yeast genes and concluded that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. This underlines the importance of checking both mRNA and enzyme activity levels when characterising gene expression.

To quantify the level of mRNA in red fruit and root tissue, TaqMan quantitative PCR analysis was performed. This is a very sensitive and accurate technique that can be used to detect relative levels of specific DNA and RNA sequences between samples. So far, TaqMan PCR has been used successfully in numerous medical studies, but it can also be of great value in plant research as demonstrated in this paper. Comparing the maximum *gus* mRNA levels measured in red fruit tissue of *FBP7-gus* and *35S-gus* plants, the *gus* mRNA level seemed to be at least 6 times higher in red fruits of *35S-gus* than in *FBP7-gus* red fruits. This is in agreement with the fact that the *CaMV 35S*-promoter is generally seen as a strong promoter. The *gus* mRNA level in roots of three different transgenic clones was compared to the *gus* mRNA level in red fruit tissue of the same plant. The strong signals observed by the Northern blot analysis are reflected in the TaqMan data.

The results described in this study show the possibilities for the use of heterologous promoters in directing gene expression in strawberry fruits. For modification of strawberry receptacle specific traits like firmness, color and sweetness the *FBP7* promoter is preferable to the *CaMV 35S* promoter, because the *FBP7* promoter directs gene expression in a more receptacle specific way. One should be aware, however, that when using the *FBP7* promoter, transcription also occurs in roots and expression levels are relatively low. For more specific and higher levels of gene expression, other promoters that are highly receptacle specific have to be identified and isolated from strawberry itself. Currently, we are testing the activity and specificity of different putative promoter sequences identified in the 5' upstream sequences of different receptacle specific strawberry cDNA's.

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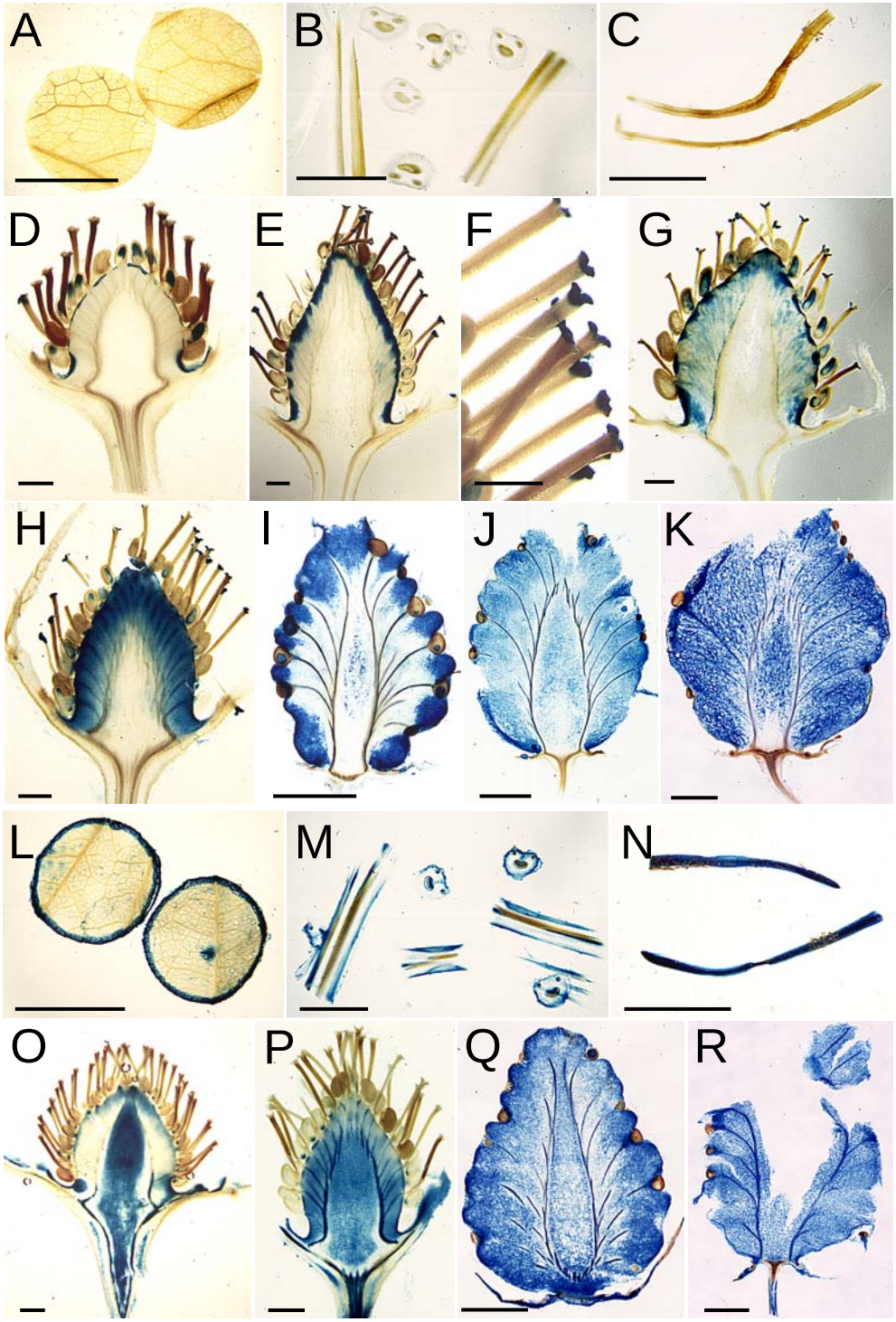

Figure 2. Histochemical localisation of GUS activity in different tissues of transgenic strawberry clones cv. Gariguette/*FBP7-gus-A* (A-K) and cv. Gariguette/*CaMV 35S-gus* (L-R). Leaf disks (A, L), cross and longitudinal sections of petioles (B, M), root ends (C, N), longitudinal sections of flowers and fruits at different stages of development: flower buds (D, O), young open flowers (E, P), pistils of young open flowers (F), mature flower (G), senescing flower (H), small green fruit (I), big white fruits (J, Q) and ripe-red fruits (K, R) were stained with the chromogenic substrate X-gluc. Size bars represents 5.0 mm in A-C, I-N, Q and R, and 1.0 mm in D-H, O and P.

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Chapter 5

Isolation and characterisation of a strawberry fruit-specific promoter

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Abstract

In order to achieve specific expression of transgenes in strawberry fruits, the availability of tissue- (receptacle) specific promoter sequences is desired. For this reason, 5'-upstream sequences of the strawberry expansin gene *FaExp2*, which is expressed in a fruit-specific manner, have been isolated. To characterise the promoter activity of the isolated sequences, fragments of 0.7 kb (0.7p*FaExp2*) and 1.6 kb (1.6p*FaExp2*) have been fused to the β-glucuronidase reporter gene (*gus*). In transgenic strawberry plants transformed with either 0.7p*FaExp2-gus* or 1.6p*FaExp2-gus,* the expected fruit-specific expression pattern was generally observed for both promoter constructs. However, quantitative RT-PCR revealed that *gus* expression levels driven by the 1.6p*FaExp2* promoter fragment were much more higher. In addition to the expression in fruits, both promoter fragments also seemed to direct gene expression in the achenes (strawberry seeds) and to some extent in epidermal and subepidermal tissues of petioles and stems of flowers and fruits. For one transgenic plant transformed with the 1.6p*FaExp2-gus* construct, a deviating expression pattern was observed, possibly caused by effects related to the site of integration. It is concluded that both promoter sequences are suitable for directing transgene expression in strawberry fruits.

Introduction

Genetic modification of crop plants is gaining importance. A key feature of genetic modification is that a cultivar of particular interest can undergo improvement of one or a few traits, while the cultivar's own characteristic properties are in principle not disturbed. Most currently cultivated genetically modified crops have been modified by introducing genes that provide the plant with resistances to herbicides, bacterial or fungal pathogens or insects. In these crops generally strong heterologous constitutive promoters like the cauliflower mosaic virus 35S (*CaMV 35S*) promoter have been employed, but the advantages of regulating transgene expression more finely and specifically are increasingly being recognised (Potenza et al., 2004).

Breeding of improved strawberry cultivars is difficult and time-consuming. Amongst others, breeding of strawberries is hampered because of their octoploid, hybrid and highly heterozygous genome. In addition, the availability of genetic resources is limited for many important traits such as disease resistance. Genetic modification of strawberry looks promising

for a relatively quick improvement of existing important strawberry cultivars. Depending on the cultivar of interest, the production of transgenic strawberry plants is rather easy to realise. However, the number of suitable genes and specific regulatory sequences that will result in the desired improvements is still rather limited.

Initially, we described the possibility of two heterologous promoter sequences to direct gene expression in strawberry fruits (Schaart et al., 2002). However, for more specific and higher levels of gene expression, other promoters, for example promoters that are highly strawberry receptacle-specific, have to be identified and isolated from strawberry itself. Only recently the isolation and characterisation of some strawberry promoters have been described. Spolaore et al. (2003) demonstrated differences in timing and levels of expression of two endo-ß-1,4-glucanase genes and studied promoter activities of the corresponding regulatory sequences using *gus*-reporter gene fusions. It was concluded that both promoter sequences could be candidates for genetic modification approaches involving modification of fruit-specific characteristics. Unfortunately, no data showing spatial expression patterns of both promoters were shown. The strawberry *agamous* homolog *STAG1* showed a low level of fruit-specific expression as determined by Northern blot analysis (Rosin et al., 2003). A detailed expression study was performed using transgenic strawberry plants containing *STAG1*-promoter-*gus* fusions. Histochemical GUS staining revealed that *STAG1*-promoter activity was mainly localised in the achenes and in vascular strands leading to the achenes. In mature fruits a faint GUS staining was observed throughout the cortex and the pith of the strawberry fruit. This promoter may, therefore, be suitable for certain applications for which low expression levels are required.

We aimed at the identification of a strawberry promoter sequence, that is able to direct transgene expression to a high level in a receptacle-specific way. For this, we selected the strawberry expansin 2 gene (*FaExp2*) which showed a high expression level in strawberry fruit during ripening (Civello et al., 1999; Aharoni et al., 2002; Salentijn et al., 2003) and cloned and characterised 5'-upstream genomic DNA fragments of this gene.

Materials and methods

Isolation of nucleic acids

Genomic DNA was isolated from young folded leaves from greenhouse plants according to the method described by Doyle and Doyle (1987), including 1% (w/v) polyvinylpyrrolidone-10 in

the DNA extraction buffer. Total RNA was isolated from young leaves, root and immature and ripe fruits as described by Asif et al. (2000).

Isolation of promoter fragments

Isolation of 5'-upstream sequences was performed as described by Rosin et al. (2003). In short, for the isolation of expansin promoter fragments, genomic DNA libraries of the strawberry cultivar Elsanta were used which had been constructed using the Universal Genome Walker™ kit (Clontech, Palo Alto, CA, USA). For the primary and nested PCR the *FaExp2* gene specific primers GSP1 (5'-CCAGAAGCATCACCACCTCCATAGA-3') and GSP2 (5'-GATACCAG-AAGAGTAATAGCCAAGC-3') were used, together with the corresponding adapter primers (AP1 and AP2, respectively). Three cloned PCR fragments of respectively 400 bp, 700 bp and 1600 bp were obtained from a *Sca*I-, *Stu*I- and *Dra*I-digested genomic DNA library, and were completely sequenced.

Sequence-specific PCR

Because in strawberry different expansin gene sequences have been identified, sequence-specific PCR was applied to check correlation of the obtained promoter fragments with the different expansin gene sequences. For this PCR, forward primers unique for each of the 700 bp and 1600 bp promoter fragments (5'-TTCTGCTTCTTACAATCCACCAC-3' and 5'-TTCTG-CTTCGAGTTCTCATTATCC-3', respectively; Fig. 2) were combined with reverse primers, which have been described by Harrison et al. (2001) and which are specific for six different expansin genes (FaExp 2 –7).

Construction of transformation vector

To study promoter activities of the 700 bp and 1600 bp *FaExp2* promoter fragments, both promoter fragments were cloned upstream of the ß-glucuronidase (GUS) reporter gene (*gus*) that was equipped with a nopalin synthase terminator sequence. The promoter-*gus* fusions, which are indicated as 0.7p*FaEXP2-gus* and 1.6p*FaEXP2-gus,* were cloned into the binary vector pBinplus (van Engelen et al., 1995) (Fig 1), and the ultimate construct was subsequently transferred to the supervirulent *Agrobacterium tumefaciens* strain Agl0 (Lazo et al., 1991).

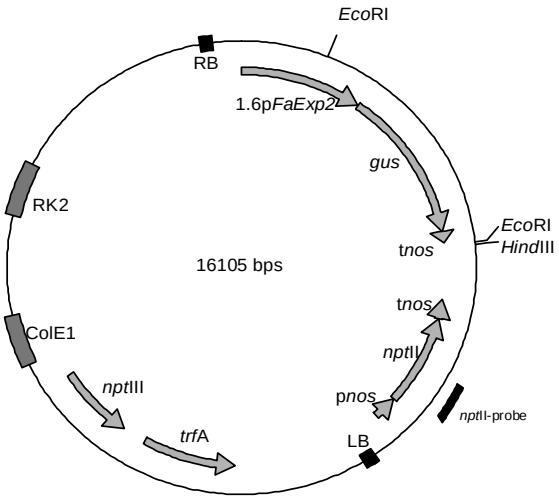

Figure 1. Schematic representation of the binary vector used for transformation of strawberry for expression analysis of the *FaExp2* promoter fragments. For analysis of the shorter promoter fragment, the 1.6p*FaExp2* in the binary vector promoter sequence is replaced by 0.7p*FaExp2*. The *CaMV 35S* promoter was used for control plants. The restriction enzyme sites *Eco*RI and *Hin*dIII and the indicated *npt*II probe are used in DNA-gel blot analysis.

Strawberry transformation

For expression analysis of the cloned promoter fragments, transgenic strawberry plants of the cultivar Calypso harbouring T-DNA with the described constructs were produced according to Schaart et al. (2002). As a control, transgenic plants with the *gus* gene under the control of the constitutive *CaMV 35S* promoter were produced. From the obtained transgenic strawberry plants containing 0.7p*FaExp2-gus* or 1.6p*FaExp2-gus,* respectively three and four independent transgenic lines, showing intense GUS staining of red fruit tissue were selected for further analysis. One *35S CaMV-gus* plant showing intense blue staining of red fruit tissue was included as control.

DNA gel-blot analysis

Ten microgram of genomic DNA of the different transgenic lines and a non-transgenic strawberry plant was digested with the restriction enzymes *Eco*RI or *Hin*dIII. After electrophoretic separation of the fragmented DNA on a 0.9% agarose gel, the DNA was transferred onto Hybond-N+ membranes (Amersham Biosciences). Hybridisation with an alkaline phosphatase-labelled *npt*II probe and chemiluminescent detection was performed according to the 'gene images AlkPhos direct labelling and detection system' (Amersham Biosciences).

Histochemical GUS-assay

Histochemical GUS staining of leaf discs, root ends, cross-sections of petioles and longitudinal sections of petioles, flowers and fruits at various developmental stages was performed as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl ß-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40.

Quantitative RT-PCR

For quantification of *gus* expression levels quantitative RT-PCR was performed. In order to limit the number of RNA isolations, for each tissue type RNA was isolated from pooled tissue of the different independent transformants produced with the same construct. cDNA was synthesised using the SuperScript first-strand cDNA synthesis system for RT-PCR (Invitrogen) according to the instruction manual. Quantitative RT-PCR was performed using the ABI Prism7700 Sequence Detection System (Perkin Elmer, Applied Biosystems) as described by Schaart *et al*. (2002), but instead of the fluorogenic TaqMan probes, SYBR Green was used for detection of PCR products. For amplification of *gus*, the forward and reverse *gus*-primers (5'-CGGAAGCAACGCGTAAACTC-3' and 5'-TGAGCGTCGCAGAACATTACAT-3') were used (product size: 80 bp). As endogenous control, a strawberry gene encoding a DNA binding protein with high homology to a gene coding for a putative DNA binding protein from *Arabidopsis thaliana*, was selected as a reference gene. This gene, indicated as *dbp* (forward primer 5'-TTGGCAGCGGGACTTTACC-3', reverse primer 5'-CGGTTGTGTGACGCTGTC-AT-3', product size: 72 bp), has shown a similar level of expression in multiple strawberry tissues (Schaart *et al*., 2002). All PCR reactions were performed in triplicate. For each reaction the threshold cycle, C_T, which is defined as the PCR cycle at which a statistically significant increase of ΔR_n is first detected, was determined. The relative quantification was done using the comparative C_T-method (User bulletin #2, ABI PRISM 7700 Sequence Detection System, December 1997, Perkin-Elmer, Applied Biosystems) in which the differences in the C_T for the *gus*-amplicon and the C_T for the endogenous control *dbp*, called ΔC_T, were calculated to normalise for the differences in the total amount of cDNA present in each reaction and the efficiency of the RT step. For comparison of two samples the ΔC_T values were subtracted from each other, giving a $\Delta\Delta C_T$ value and finally the relative amount of *gus* mRNA copies was calculated by $2^{-\Delta\Delta CT}$.

Results

Isolation of *FaExp2* promoter fragments

In order to isolate a strawberry receptacle specific promoter, the strawberry expansin 2 gene (*FaExp2*) was selected. The expression of this gene was described to be relatively strong and highly strawberry fruit-specific (Civello et al., 1999; Harisson et al., 2002; Aharoni et al 2002; Salentijn et al., 2003). Furthermore, its expression was demonstrated not to be affected by either auxin (Civello et al., 1999; Aharoni et al., 2002) or ethylene treatment (Aharoni et al., 2002) which made this gene a good candidate for isolation of a strawberry receptacle-specific promoter sequence. Following a genome walking approach using gene specific primers for *FaExp2*, three different 5'-upstream genomic DNA sequences of 400 bp, 700 bp and 1600 bp have been amplified from *Sca*I, *Stu*I, and *Dra*I-digested Elsanta genomic libraries, respectively.

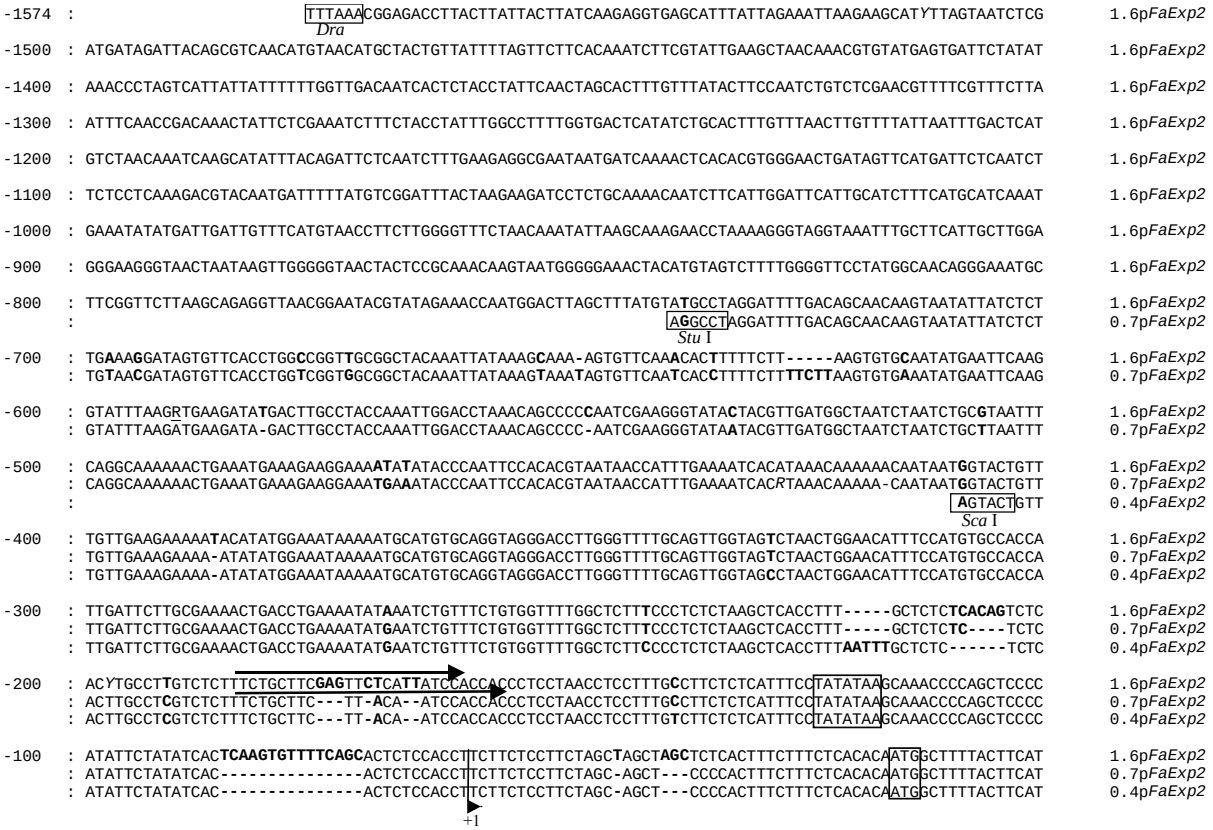

Figure 2. Sequence alignment of the 0.4, 0.7 and 1.6 kb *FaExp2* promoter fragments. Nucleotides in bold indicate polymorphic sites, hyphen indicates deletion. The restriction enzyme sites, *Dra*I, *Stu*I and *Sca*I, which have been used in the construction of strawberry genome-walking libraries, are boxed as well as a TATATAA-box and the first ATG of the *FaExp2*-gene. The putative start of transcription is indicated by an arrowhead. Forward primers used for the sequence-specific PCR are indicated by arrows above the 1.6p*FaExp2* and 0.7p*FaExp2* sequences.

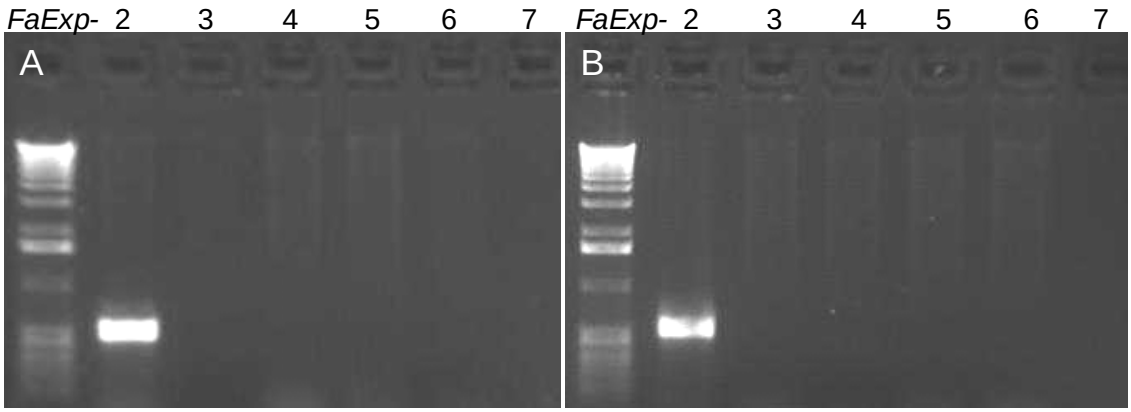

Figure 3. Sequence-specific PCR using forward primers specific for 0.7p*FaExp2* (**A**) or 1.6p*FaExp2* (**B**) and reverse primers specific for the strawberry expansin genes *FaExp2-7*.

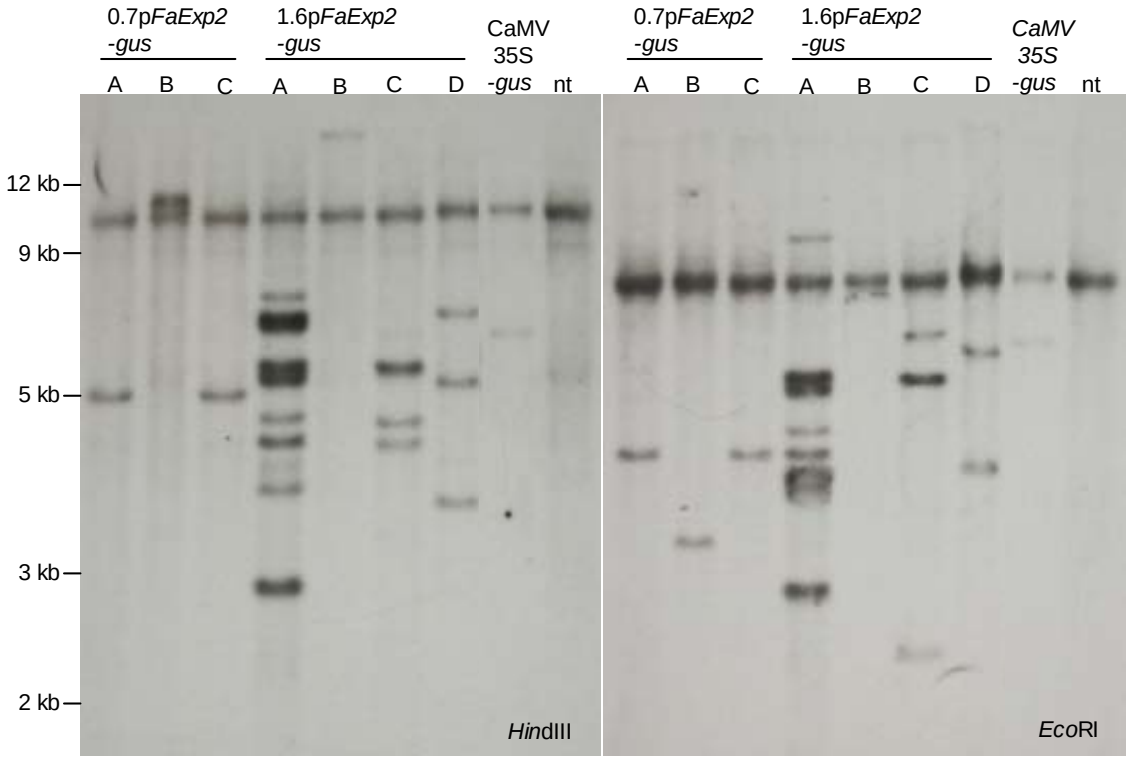

Figure 4. DNA-gel blot analysis of genomic DNA of independent transgenic strawberry lines harbouring the 0.7p*FaExp2-gus*, the 1.6p*FaExp2-gus* or the *CaMV 35S-gus* constructs. DNA of a non-transgenic plant (nt) is included as control. Total DNA was digested with *Hin*dIII (left blot) or *Eco*RI (right blot) and the blotted DNA was hybridised with a *npt*II probe. The number of hybridisation bands indicate the integrated T-DNA copy number. Note the a-specific hybridisation band which is present in all lanes, including the non-transgenic control lane.

DNA sequence alignment of the three different fragments showed a high degree of similarity. The sequences differed in several SNPs and small Indels (inserts/deletions) and a larger (15 bp) Indel (Fig. 2). SNPs in the *Sca*I and *Stu*I-restriction sites, that were responsible for promoter fragment length polymorphisms, could be traced back in the larger sequences (Fig. 2).

For strawberry several homologous expansin genes have been identified and especially *FaExp2* and *FaExp7* show a high degree of similarity (Harrison et al., 2001). In order to check the origin of the obtained promoter sequences, sequence-specific PCR was performed using specific forward primers for the two longest promoter fragments in combination with reverse primers that are specific for six different strawberry expansin genes (see Harrison et al., 2001). Only for

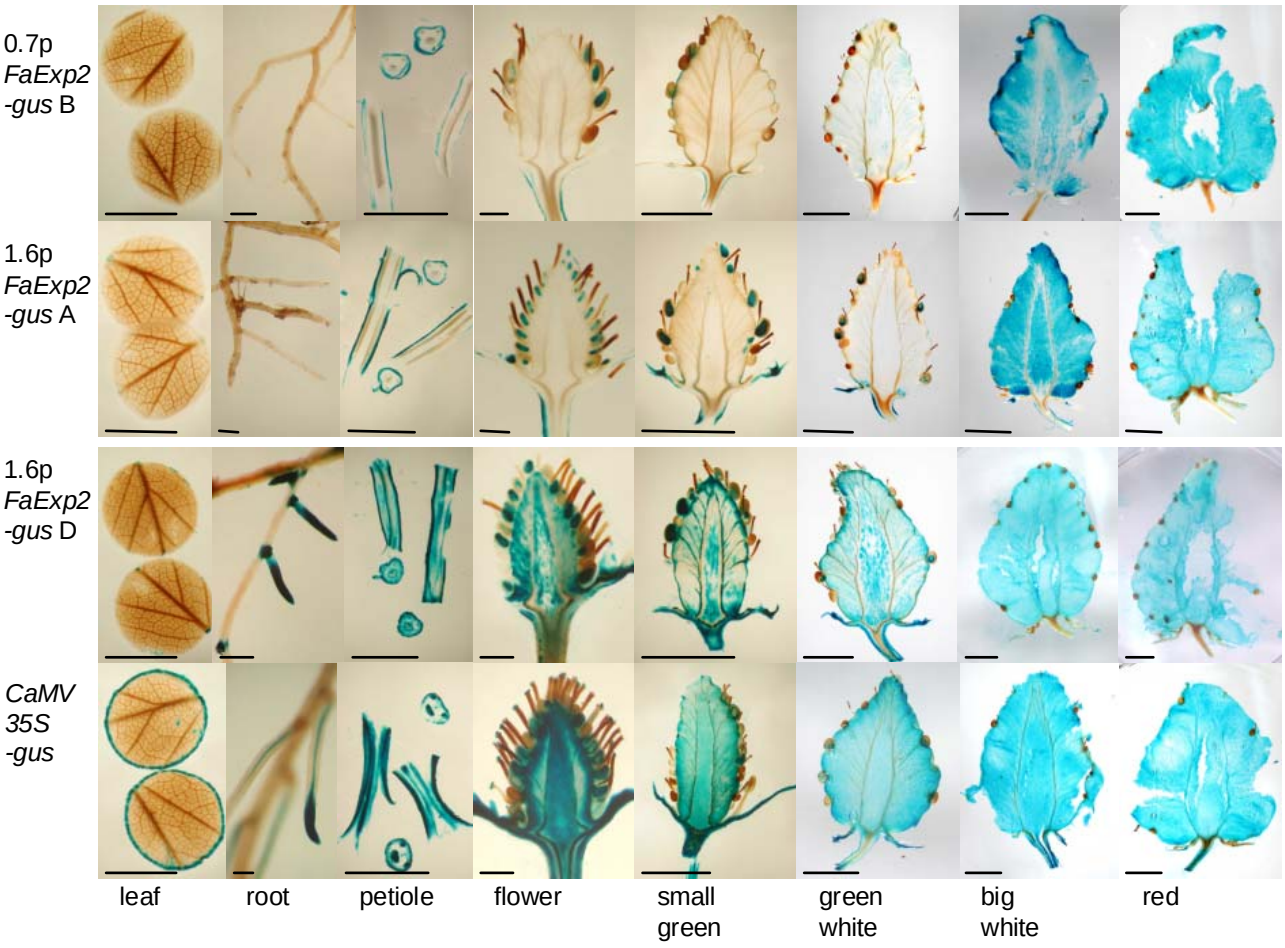

Figure 5. Histochemical GUS staining of leaf, root, petiole and flower tissue and of fruits at different developmental stages. Typical GUS staining patterns for the 0.7p*FaExp2* and the 1.6p*FaExp2* promoters are represented by tissues of plant 0.7p*FaExp2-gus* B and plant 1.6p*FaExp2-gus* A, respectively. For plant 1.6p*FaExp2-gus* D the deviating GUS staining pattern is shown. Tissue of a *CaMV 35S-gus* transgenic strawberry plant is included as reference. Size bars: 1.0 mm for roots and flowers; 5.0 mm for all other tissues.

the combination of both forward promoter primers together with the reverse primer specific for *FaExp2,* amplification of a fragment of expected size was obtained (Fig. 3), indicating that both different 5'-upstream sequences belong to the *FaExp2* gene and represent most likely allelic sequences.

Construction of the reporter vector and introduction into strawberry plants

For the promoter activity study the 700 bp and 1600 bp fragments were fused to the *gus* reporter gene and transferred to the strawberry cultivar Calypso using *A. tumefaciens* mediated transformation. For comparison, a construct in which the heterologous *CaMV 35S*-promoter was combined with the *gus* reporter gene was introduced in the same strawberry cultivar. For all constructs several transgenic lines expressing *gus* in fruit tissue have been produced, and three 0.7p*FaExp2-gus* (A-C) and four p1.6*FaExp2-gus* (A-D) lines, showing a relatively high intensity of histochemical GUS staining in ripe fruits, were selected for further analysis. DNA gel-blot analysis using genomic DNA of all transgenic lines was performed in order to determine the number of integrated T-DNA copies (Fig. 4). Both *Eco*RI and *Hin*dIII cut the T-DNA at one side of the *nptII* gene (Fig. 1), while the other relevant restriction site is located in the host DNA that flanks the *nptII* gene at the other side after T-DNA integration. Depending on the position of the restriction site in the host DNA, hybridisation bands of different sizes may be obtained after hybridisation with a *npt*II probe, representing different T-DNA integration events. For both blots an a-specific hybridisation band is visible at a similar position in all lanes (Fig. 4; approximately 10 kb for the *Hin*dIII-blot and 8 kb for the *Eco*RI-blot). Since this band is also present in the non-transgenic DNA lane, it should be left out in consideration when determining the T-DNA copy number. From the blots could be deduced that all three selected 0.7p*FaExp2-gus* plants, one of the 1.6p*FaExp2-gus* plants (B) and the *CaMV 35S-gus* plant seem to contain a single T-DNA insertion. In two 1.6p*FaExp2-gus* lines (C and D) at least three T-DNA copies are present, while for the 1.6p*FaExp2-gus* A plant a multiple hybridisation banding pattern was obtained, suggesting the presence of at least nine T-DNA copies.

Histochemical GUS staining

For all three selected 0.7p*FaExp2-gus* plants and for three out of the four selected 1.6p*FaExp2-gus* transgenic plants, a an identical, fruit-specific GUS staining pattern was observed (Fig. 5, 0.7*pExp2* B, *1.6pExp2* A). In flowers and young fruits of these plants, no visible GUS staining was found in receptacle tissue, but in big white and red fruits a clear blue staining was

observed. It was remarkable however, that the development of GUS staining in big white and red fruits of 1.6p*FaExp2-gus* transgenic plants was extremely fast as compared to similar fruits from 0.7p*FaExp2-gus* or *35S CaMV-gus* transgenic plants, indicating a high level of GUS activity in these fruits (results not shown). In addition to the GUS-staining found in receptacle tissue, blue staining was also found in achenes (i.e. strawberry seeds) and in epidermal and sub-epidermal layers of petioles and stems of flowers and fruits. No GUS staining was observed in roots, and for leaf-discs only plants transformed with the 1.6p*FaExp2-gus* construct showed some GUS staining near the vascular tissue.

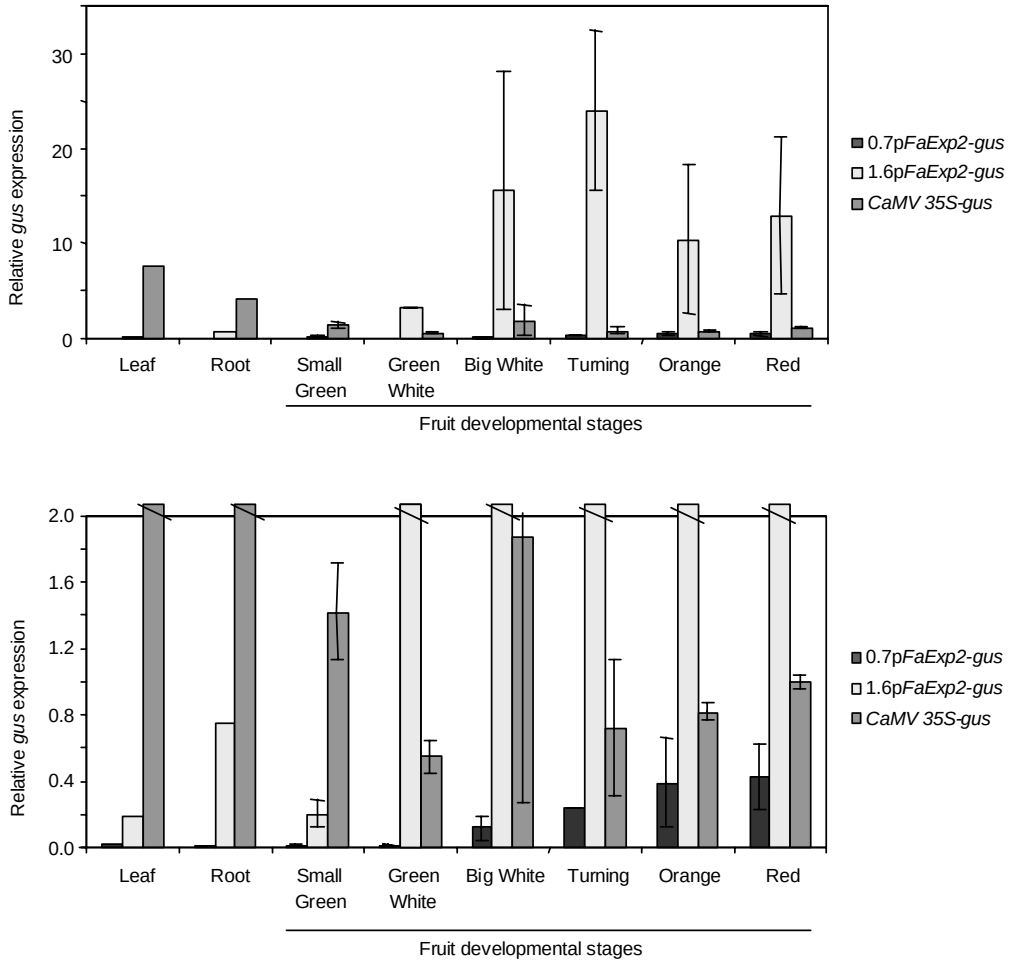

Figure 6. *Gus* gene expression analysis by quantitative RT-PCR. Due to large differences in *gus* transcript levels, the data are plotted against two different scales. Expression levels of all samples are related to the *gus* expression level in red fruits of the *CaMV 35S-gus* control plant. Error bars represent ±SE values. All fruit sample values are replicates in two-fold. Leaf and root samples have been analysed once. RT-PCRs for 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus* have been performed on RNA samples obtained from pooled tissues of respectively three and four different independent transgenic plants.

The 1.6p*FaExp2-gus* D plant showed a GUS staining pattern that deviated from all other plants. In this particular transgenic plant strong GUS activity was observed in flowers and in fruits of all developmental stages, as well as in petiole and root tissue (Fig. 5, *1.6pExp2* D), while in leaves only some staining was observed along the wound edge, near the vascular tissue. The GUS staining pattern of this line resembled that of *CaMV 35S-gus* transgenic strawberry plants (Fig. 5, *CaMV 35S*).

Gus expression analysis

For the different transgenic lines made with p*FaExp2-gus* and *CaMV 35S-gus* constructs the level of *gus* expression was determined by quantitative RT-PCR. In order to limit the number of RNA isolations, for each tissue type RNA was isolated from pooled tissue samples from independent transgenic lines harbouring the same construct. Therefore, this will give average expression levels for the different constructs, rather than specific expression results for each transgenic line. Figure 6 shows that for 0.7p*FaExp2-gus* plants (Fig. 6, lower panel) as well as for 1.6p*FaExp2-gus* plants (Fig. 6, upper panel) expression levels are upregulated in ripening fruits, starting with big white fruits. For 1.6p*FaExp2-gus* transformants there was also considerable expression in the green-white fruit stage. For *CaMV 35S-gus* plants *gus* expression was not correlated with any fruit stage and highest expression levels were observed for leaf and root tissue. Also in 1.6p*FaExp2-gus* plants a reasonable level of *gus* expression was found for roots, but this may originate from the single 1.6p*FaExp2-gus* transgenic line that showed, also for root tissue, a deviating GUS-staining pattern.

Discussion

For tissue-specific expression of genes in strawberry, the availability of specific regulatory sequences is desirable. Because *FaExp2* was known to be tightly regulated during ripening (Civil et al., 1999; Aharoni et al., 2002; Salentijn et al., 2003), we selected this gene for the isolation of a strawberry receptacle and ripening-specific promoter sequence. A genome walking approach resulted in three different *FaExp2* 5'-upstream PCR fragments of 0.4 kb, 0.7 kb and 1.6 kb, obtained from Elsanta genomic DNA, and sequencing revealed that the sequences were highly homologous. The overlapping corresponding parts of the sequences differed by several SNPs and smaller or larger Indels. This sequence variation indicated that the amplified fragments could be corresponding to different expansin gene sequences. In

strawberry, up to seven homologous expansin genes have been identified showing variability in expression (Harrison et al., 2001). PCR-analysis using a specific forward primer for the 0.7 kb and 1.6 kb *FaExp2* promoter fragments in combination with sequence specific reverse primers for six of the seven known strawberry expansin genes (*FaExp2-7*), revealed that both promoter sequences belong to *FaExp2* gene. However, whether the promoter fragments belong to different alleles or different gene copies of *FaExp2*, could not be determined from this experiment.

In general, promoter sequences do not have strictly defined borders. For some genes regulatory elements as far as 20 kb upstream from the transcription start have been identified (Potenza et al., 2004). However, most promoter sequences used for regulating transgene expression range between 0.5-2.0 kb in length (Potenza et al., 2004). In order to investigate the promoter activity of the 0.7 kb and 1.6 kb *FaExp2* promoter sequences, both fragments were fused to the *gus* reporter gene and the resulting gene fusion was transferred to the strawberry genome by means of *Agrobacterium*-mediated transformation.

Histochemical GUS staining showed that both 5'-flanking fragments of *FaExp2* were sufficient to confer receptacle-specific and ripening-regulated expression of *gus*. For white and red fruits of transgenic plants containing the 1.6 kb promoter fragment fused to the *gus* reporter gene, a much faster staining was obtained than for the 0.7 kb fragment, indicating a higher GUS activity in these fruits. However, in the end the tissue specific GUS staining pattern seemed to correspond quite well between fruits of all three selected 0.7p*FaExp2-gus* plants and three out of the four selected 1.6p*FaExp2-gus* transgenic strawberry plants. Although the three 1.6p*FaExp2-gus* plants A-C showed quite some variation in T-DNA copy number as determined by DNA-gel blot analysis, these differences do not seem to have much influence on the *gus* expression pattern. GUS staining of petiole tissue and achenes indicated that the expression of *FaExp2* is not completely restricted to ripening strawberry fruits. Aharoni and O 'Connell (2002) determined, using DNA microarrays, that the expression level of *FaExp2* in receptacle of red fruits was 13-fold higher than in achenes. For a number of other expansin genes from strawberry, pear and tomato also an overlapping expression has been reported (Harrison et al. 2001; Hiwasa et al, 2003; Brummel et al. 1999). Possibly, it is a common feature of certain expansin genes to be expressed in different tissues, suggesting variable functions for these genes.

Quantitative *gus* expression analysis showed a large difference in *gus* transcript level in ripening fruits of 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus* plants. This suggests that in the 1.6p*FaExp2* promoter sequence one or more additional positive regulatory *cis*-acting elements

were present as compared to the 700 bp fragment, that gave rise to an overall higher level of gene expression. The temporal and spatial control of gene transcription is generally mediated by the interaction of negative and positive regulatory elements. For example, for a fruit-specific promoter of the tomato polygalacuronase gene (*PG*), deletion analysis indicated the presence of different positive and negative regulatory regions which modulated tissue-specific gene expression (Montgomery et al., 1993). For the *FaExp2* promoter it is most likely that *cis*-acting elements, which promote the level of gene expression, are located in the proximal 900 bp region of the *FaExp2* promoter. However, the observed differences in expression could also be due to polymorphisms that discriminate the two promoter sequences in the first 700 bp (Fig. 2). In order to clear up the location of relevant regulatory elements, more detailed experimental data are required.

In the 1.6p*Exp2-gus* line which showed a deviating GUS staining pattern, staining was also observed in immature fruits and vegetative tissues in addition to receptacle tissue of ripening fruits. With regard to this expression pattern, this transgenic line resembled more the expression pattern as found for the *CaMV 35S-gus* transgenic strawberry line with exception of the leaves. PCR analysis, using specific primers for the 1.6p*Exp2* promoter fragment in combination with *gus* primers revealed that this transgenic plant was a true 1.6p*Exp2-gus* line (results not shown), suggesting that the deviating expression pattern might be related to the T-DNA integration site.

In order to achieve strawberry fruit-specific expression of transgenes, both *FaExp2* promoter sequences may be good candidates, depending on the desired level of expression. Currently, we have applied the 1.6p*FaExp2* promoter to direct expression of an antifungal gene with the aim to enhance the level of resistance to fruit rot caused by *Botrytis cinerea*.

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Chapter 6

Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene

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Abstract

Public concerns on the issue of the environmental safety of genetically modified plants have led to a demand for technologies allowing the production of transgenic plants without selectable (antibiotic resistance) markers. We describe the development of an effective transformation system for generating such marker-free transgenic plants, without the need for repeated transformation or sexual crossing. This system combines an inducible site-specific recombinase for precise elimination of undesired, introduced DNA sequences, with a bifunctional selectable marker gene used for the initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants. The described system can be generally applied to existing transformation protocols and was tested in strawberry, using a model vector in which site-specific recombination leads to a functional combination of a CaMV 35S promoter and a GUS encoding sequence, thereby enabling histochemical monitoring of recombination events. Fully marker-free transgenic strawberry plants have been obtained following two different selection/regeneration strategies.

Introduction

Up to date, two different approaches for the removal of selectable marker genes from transgenic plants have been described (for recent review see Puchta, 2003). In the first approach the selectable marker genes and the gene of interest are introduced at different loci of the plant genome by co-transformation, after which the selectable marker gene is segregated out by crossing sexually. The second method entails the elimination of selectable markers either by transposition or by intrachromosomal site-specific recombination. The second system would be the one of choice for crops that are vegetatively propagated or have a long reproductive cycle, like most fruit crops. To eliminate the selectable marker using site specific-recombination, it has to be flanked by a pair of two directly repeated specific recombination sites allowing exact excision of the enclosed DNA sequence in the presence of the corresponding site-specific recombinase activity. The first reported example of selectable marker elimination in plants employed the bacteriophage P1 Cre/*lox* system comprising Cre catalysed recombination between *lox* sites (Dale and Ow, 1991). In this study, a lox-flanked *hpt*-gene was removed from transgenic plants upon re-transformation with a construct expressing the Cre-recombinase gene.

An important improvement of this early technique was reported by Zuo et al. (2001) using a chemical-inducible artificial transcription factor for indirect, transcriptional regulation of Cre-recombinase gene expression. Thus, the recombinase gene and the *lox* recombination sites could co-exist without leading to premature recombination. Following selection of transgenic tissue, chemical induction of the recombinase gene produced the desired excision events. A major drawback of this method was the formation of genetic chimeras due to incomplete DNA excision.

Sugita et al. (2000) described removal of a positive selectable marker using the site specific recombination system R/*Rs* from *Zygosaccharomyces rouxii*, in which expression of the recombinase was directly regulated by a chemical-inducible promoter. The isopentenyl transferase (*ipt*) gene from *Agrobacterium tumefaciens*, which they used as selectable marker, leads to cytokinine overproduction and results in transgenic shoots with abnormal shooty morphology. Following induced excision of the *ipt*-gene, the appearance of normal looking plants emerging from abnormal tissues facilitated simple recognition of non-chimeric marker-free plants. Although the successful use and removal of *ipt* was demonstrated in four different plant species (Ebinuma and Komamine, 2001), the system was not very efficient and the use of the *ipt* selectable marker might require optimisation of transformation protocols due to changes in tissue culture conditions.

One way to reduce the appearance of chimeras might be the use of a negative selectable marker such as the *E. coli* cytosine deaminase gene (*cod*A), which in plant and mammalian cells was shown to confer sensitivity to 5-fluorocytosine (5-FC) (Mullen et al., 1992; Stougaard, 1993). *Cod*A is a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-FC to cytotoxic 5-fluorouracil (5-FU). For example, Gleave et al. (1999) employed the negative selection capabilities of this gene for the production of marker-free plants. They selected kanamycin-resistant transgenic tobacco plants in which lox sequences flanked the *npt*II gene together with the *cod*A gene. Transient expression of the Cre recombinase gene allowed selection of completely marker-free transgenic plants on 5-FC containing medium, albeit at low frequency.

We describe the construction and application of a system consisting of a plant-adapted version of the *Z. rouxii* R recombinase and a *cod*A-*npt*II bifunctional selectable marker gene. The recombinase is ligand-regulated (post-translationally) as first described by Logie and Stewart (1995). The *npt*II component of the bifunctional marker gene is used for selection of transgenic tissue, whereupon the *cod*A gene can be used for selection against cells that have held on to the marker despite the action of the recombinase. The application of this inducible site-specific

recombination system in conjunction with a bifunctional selectable marker provides an effective and versatile procedure for the production of non-chimerical marker-free transgenic plants.

Materials and methods

Construction of binary plasmid

The binary plasmid pRCNG (Fig. 1) is based on pMOG22 (Goddijn et al., 1993). The R recombinase coding sequence was resynthesised increasing its G+C content from 41% to 49% and its frequency of favourable XXG/C codons from 41 to 63%, without altering the amino acid composition. For this, 24 sense and antisense oligonucleotides with an average size of 81 nucleotides were used in an overlap extension PCR method as described by Ho et al. (1989) and Rouwendal et al. (1997), and this yielded the full-length product. The ligand-binding domain (LBD) of the rat glucocorticoid receptor was translationally fused to the C-terminus of the R-recombinase gene. The LBD sequence was obtained from the rat cDNA clone 6RGR (kindly provided by KR Yamamoto) by overlap extension mutagenesis that eliminated an internal *Eco*RI site (Miesfield et al., 1986). The coding sequence of the hybrid selectable marker gene consists of the cytosine deaminase (*cod*A) gene, isolated from *E. coli* strain JM109 by PCR using primers 5'-GTGAACCATGGCTAATAACGCTTTACAAACAA-3' and 5'-GCAGTGG-ATCCACGTTTGTAATCGATGG-3', translationally fused to the *npt*II gene isolated from pBIN19 using primers 5'-TCGCAGATCTGAACAAGATGGATTGCACG-3' and 5'-GCTCA-GGATCCCGCTCAGAAGAACTCGTC-3'. Both hybrid recombinase R-LBD and *cod*A-*npt*II coding sequences are flanked by a downstream nopaline synthase gene terminator (t*nos*) and an upstream translational enhancer consisting of the alfalfa mosaic virus (AMV) 5' untranslated region followed by intron 5 from the potato *gbbs* gene (IVS) (Jobling and Gehrke, 1987; van der Leij et al., 1991). The *cod*A-*npt*II hybrid gene was regulated by the cauliflower mosaic virus (CaMV) 35S promoter. The combination of the promoterless recombinase R-LBD gene and the *cod*A-*npt*II hybrid gene is flanked by 58 bp directly repeated recombination sites (*Rs*) which had been isolated from *Z. rouxii* total DNA by PCR using primers 5'-AGGCGAGATCT-TATCACTGT-3' and 5'-GTCACGGATCCACGATTTGATGAAAGAAT-3'. This Rs-flanked segment separates an enhanced CaMV 35S promoter and a ß-glucuronidase (*gus*) reporter gene in such a way that its recombinase-mediated elimination will lead to GUS activity. Initially, this CaMV 35S promoter controls the recombinase R-LBD hybrid gene. The plasmid pRCNG contains also an *hpt*-gene available for control experiments.

Transformation of strawberry and elimination of selectable marker genes

For plant transformation the binary construct pRCNG was transferred to *A tumefaciens* strain AGL0 (Lazo et al., 1991). Transformation of the strawberry cultivar Calypso was essentially performed as described by Schaart et al. (2002). Leaf explants were infected with AGL0(pRCNG) and co-cultivated for three days on shoot regeneration medium (SRM) consisting of MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) glucose, 5 µM thidiazuron and 1 µM NAA and 0.4% (w/v) gelrite, after which the leaf explants were transferred to selective SRM containing 250 mg/l cefotaxime for elimination of *A. tumefaciens*, and 150 mg/l kanamycin for the selection of transgenic tissue. After one month on this medium, leaf explants were subcultured in two different ways. In the first case the leaf explants were incubated overnight in liquid MS-medium supplemented with 10 µM dexamethasone (DEX) for the activation of the R recombinase protein, after which they were transferred to fresh SRM containing 250 mg/l cefotaxime, 1 µM DEX and 150 mg/l 5-FC each month. In this medium 5-FC is present for negative selection of marker (*cod*A)-free regenerating shoots. Alternatively, after the first month on selective SRM, the leaf explants were transferred to fresh SRM containing 250 mg/l cefotaxim and 100 mg/l kanamycin in order to regenerate kanamycin-resistant shoots first. These shoots were isolated from the leaf explants and subcultured on shoot propagation medium (MS-medium with 3% (w/v) sucrose and 0.9% (w/v) Daishin agar) supplemented with kanamycin at a concentration of 25 mg/l. Shoots that rooted on this medium were regarded as kanamycin resistant transgenic shoots. Leaf explants from these shoots were incubated overnight in liquid MS-medium supplemented with 10 µM DEX and then transferred to SRM containing 1 µM DEX and 150 mg/l 5-FC to obtain regenerants again. Putative marker-free plants obtained following both approaches were rooted and propagated on shoot propagation medium containing 150 mg/l 5-FC and were moved to the greenhouse for further analysis.

Histochemical GUS-assay

Histochemical GUS staining of leaves was carried out as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40.

PCR and DNA gel- blot hybridisation analysis

DNA was isolated from young folded leaves from greenhouse grown plants according to the method described by Doyle and Doyle (1987), but including 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. In order to obtain representative sampling for DNA isolation, leaf material was pooled from several individuals from the same transgenic line. For PCR analysis of the putative marker-free plants the following primer sets were used (see also Fig. 1). B4-primers (5'-CTCTTCCTCTCTGTAACACC-3' and 5'-CTGCTGCATCTCTTCCTCTACCAT-3'), corresponding to a strawberry polygalacturonase-like gene (GenBank accession no. AY280662), were used as a quality check of the template DNA. Vir-primers (5'-GCCGGGGC-GAGACCATAGG-3' and 5'-CGCACGCGCAAGGCAACC-3'), which will amplify the *A. tumefaciens vir*G gene, served to exclude the presence of *A. tumefaciens* bacteria. Gus-primers (5'-CTGTAGAAACCCCAACCCGTG-3' and 5'-CATTACGCTGCGATGGATCCC-3') were used for detection of the T-DNA. Primers CodNpt-up and –dw (5'-AAGGTGATTGCCAGCA-CACA-3' and 5'-TACGTGCTCGCTCGATGCGA-3', respectively) allowed detection of selectable marker sequences. 35S- and gus primers (5'-CCACTATCCTTCGCAGACC-3' and 5'-TATCTGCATCGGCGAACTGA-3', respectively) were used for detection of recombination events. As control templates, DNA from non-transgenic cv. Calypso plants and *A. tumefaciens* AGL0(pRCNG) bacteria were included in each PCR-test. For DNA-gel blot hybridisation analysis, 5 µg of DNA of control and transgenic plants was digested with *Eco*RI and separated by electrophoresis on a 0.8% (w/v) agarose gel. The DNA was then transferred to a Hybond-N membrane (Amersham) and hybridised with the ^{32}P-labelled coding region of *npt*II, *gus* and a PCR fragment of the CaMV 35S promoter sequence, respectively, as described earlier by Puite and Schaart (1996).

Results

Construction of binary vector

A binary vector, pRCNG, was constructed that contains a unique combination of features aimed at efficient production, selection and detection of marker-free transgenic plants (Fig. 1). First of all, the R recombinase gene was completely re-synthesised to create a plant-adapted version of the gene. Next, the C-terminus of the R recombinase gene was fused to the LBD of the rat glucocorticoid receptor to achieve stringent post-translational regulation of recombinase activity, as originally described by Logie and Stewart (1995).

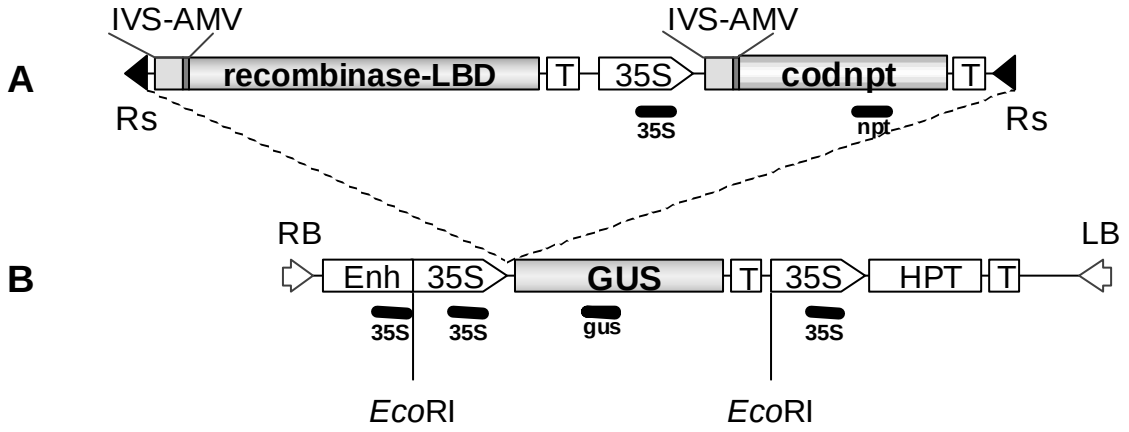

Figure 1. Diagram depicting the T-DNA of pRCNG consisting of segment A inserted between the enhanced CaMV 35S promoter and the *gus* gene in segment B. Segment A minus one of the Rs sequences is removed upon recombinase mediated deletion. 35S-, gus- and npt-marked bars represent probe regions used for Southern blot analysis. *Eco*RI is the restriction enzymes used for Southern blot analysis. RB, right border; LB, left border; Rs, recombination site; 35S, CaMV 35 promoter; Enh, enhancer; IVS, intron 5 of potato *gbss* gene; AMV, 5' untranslated region of alfalfa mosaic virus; T, terminater of *A. tumefaciens nos* gene; HPT, hygromycin resistance gene; codnpt, hybrid gene for positive (*npt*II) and negative selection (*cod*A); GUS, ß-glucuronidase reporter gene. Drawing is not on scale; internal *Eco*RI fragment is 8.7 Kb and 2.9 Kb before and after recombination, respectively.

By combining the open reading frames of *cod*A and *npt*II into a hybrid enzyme, a new bifunctional selectable marker was created. The *cod*A-*npt*II hybrid gene was tested in transformation experiments with the potato variety Bintje, from which 10 independent transgenic lines were selected on kanamycin containing shoot regeneration medium. Axillary buds of single-node cuttings taken from shoots of all 10 transgenic lines failed to grow on 5-FC containing propagation medium, whereas shoot growth from buds of untransformed cv Bintje cuttings was not affected on the same medium (results not shown).

The R recombinase-LBD fusion (R-LBD) and the hybrid marker genes were located in adjacent positions and flanked by directly repeated Rs sequences. This Rs-flanked fragment was inserted between an enhanced CaMV 35S promoter (E35S) and the *gus* gene with the R-LBD gene immediately downstream of and under the control of the E35S promoter (Fig. 1). DEX-induced recombinase activity would lead to excision of the fragment, leaving behind a short Rs sequence between the E35S promoter and the *gus* gene. Thus, with the *gus* gene now being positioned under the control of the E35S promoter, recombinase-mediated deletion could be easily visualised.

Strawberry transformation and selection of putative marker-free regenerants

Cultivar Calypso was used to test the pRCNG vector in strawberry because of its high shoot regeneration efficiency (Passey et al., 2003). After co-cultivation with AGL0(pRCNG), the leaf explants were cultured on kanamycin containing selective shoot regeneration medium (SRM) for one month. Then, two strategies were followed for the selection of marker-free transgenic strawberry plants. In the early negative selection approach, half of the leaf explants were incubated overnight in liquid medium supplemented with 10 µM DEX to induce recombinase activity, and the leaf explants were subsequently transferred to kanamycin-free SRM containing 1 µM DEX for sustained R recombinase induction and 150 mg/l 5-FC for selection against cells retaining the marker.

Following the early approach, 126 regenerating shoots and shoot-like structures were isolated from the leaf explants and fragments from all regenerants were subjected to a GUS- staining assay. Partially or completely blue shoot fragments resulting from recombinase-mediated removal of the sequences between 35S promoter and the *gus* gene were observed for 28 of these shoots. Most of the regenerants were in the form of small shooty clusters and were often vitrified. Attempts to generate shoots from those on shoot propagation medium without selection eventually gave rise to 4 normal growing shoots, 20 weeks after the start of the experiment. These putative marker-free shoots were denoted as ‘early’ shoots.

In the alternative, delayed (negative) selection strategy, the remaining half of the leaf explants were transferred to fresh kanamycin-containing SRM from which eventually 56 individual kanamycin resistant shoots were regenerated and propagated on shoot propagation medium containing 50 mg/l kanamycin. With 51 from these lines the marker elimination procedure was started by treating leaf explants with 10 µM DEX as described above to induce recombinase activity. The leaf explants were subsequently subjected to a second round of shoot regeneration in the presence of 1 µM DEX and 150 mg/l 5-FC and secondary regenerants were obtained from all lines, 35 weeks after the start of the experiment. These putative marker-free regenerants (‘late’ shoots) were propagated and subjected to further analysis.

A remarkable observation was made when leaf explants of the kanamycin resistant plants (primary regenerants), destined for the delayed selection approach, were subjected to a histochemical GUS assay, immediately following a DEX treatment. As a control, untreated leaf explants from the same plants were also assayed for GUS activity. In 32, out of 51 independent transgenic plants treated with DEX and tested for GUS activity, staining was observed, the

remainder being GUS-negative. This suggests DEX-induced recombination in leaf explants of at least 62% of the primary regenerants. Surprisingly, for all lines tested, a similar GUS-staining intensity and pattern was observed for untreated leaf explants of corresponding plants, indicating that (partial) recombination had already occurred before the start of the DEX-treatment. The primary regenerants, which may have undergone premature recombination, were not subjected to further molecular analysis.

Analysis of putative marker-free plants

The four putative marker-free plants from the early selection and 13 randomly selected putative marker-free plants obtained via the delayed selection protocol were tested for GUS activity. Two out of four of the early selected putative marker-free plants (E2 and E4) showed strong GUS-staining, whereas in a third plant (E1) a very faint staining was observed and in the fourth (E3) no GUS-staining was observed (data not shown). Of the 13 supposedly marker-free plants obtained by delayed selection, 10 plants showed strong GUS-staining and in three plants reduced staining was found. All four putative marker-free plants from the early selection and four of the strongly GUS-positive putative marker-free plants from the late selection (L1-4) were subjected to further molecular analysis.

PCR and Southern blot analysis of genomic DNA showed that only line E1 still contained a copy of the marker gene (Fig. 2A,B, *npt*II panels). Hybridisation of DNA from line E1 with the *gus*-probe revealed three *Eco*RI fragments (Fig. 2B, *gus* panel); one weakly and two strongly hybridising fragments, one of which also hybridising to the *npt*II-probe. Although, the size of this common hybrid fragment corresponds to the size of the full-length *Eco*RI fragment that was part of the original T-DNA, the lack of expected hybridisation with the 35S-probe precludes such a straightforward explanation (Fig. 2B, 35S panel). PCR analysis demonstrated that one of the 4 plants obtained from the early selection (E3) was likely to be a non-transgenic escape, as it lacked not only the marker and the 35S promoter, but also the *gus* gene as was confirmed by Southern blot analysis using a *gus*-probe.

Using a primer pair consisting of a forward primer specific for the CaMV 35S promoter and a reverse primer specific for the *gus* gene, PCR analysis revealed the small fragment of expected size in all GUS-positive plants including the plant that still contained the marker gene. The appearance of this fragment indicates that a 6.1 kb deletion took place that positioned the *gus* gene immediately downstream of the enhanced CaMV 35S promoter. This indicates that

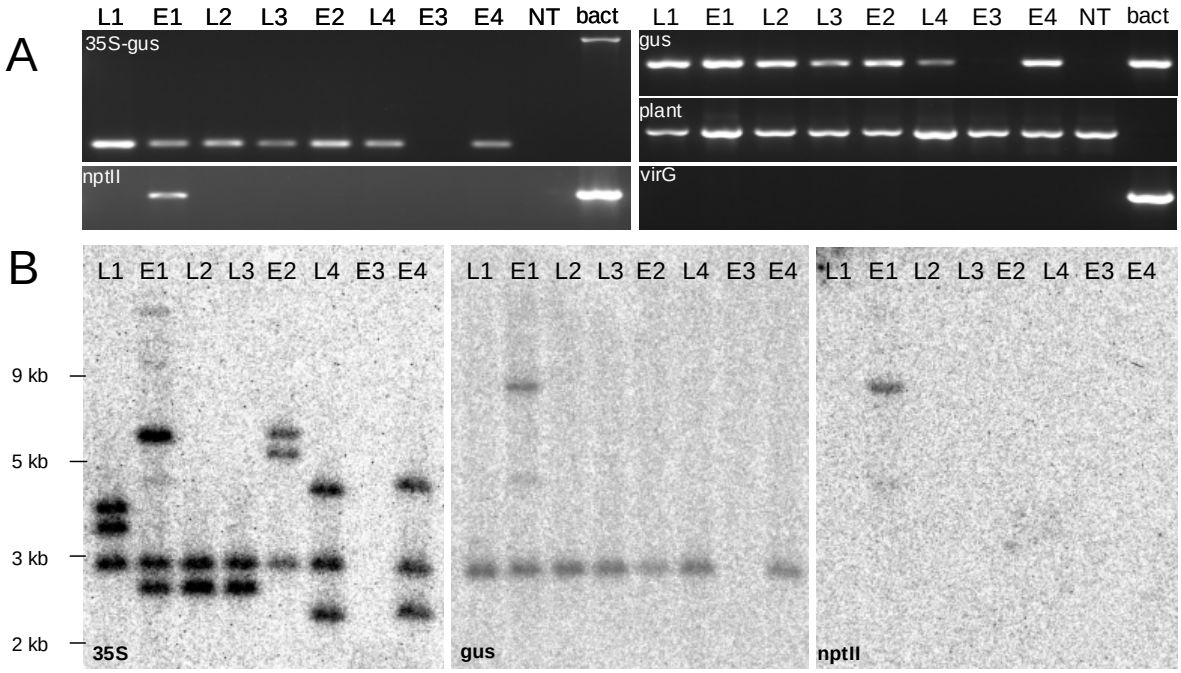

Figure 2. Molecular analysis of putative marker-free plants. E1-E4, putative marker-free transgenic plants obtained through early negative selection; L1-L4, putative marker-free transgenic plants from late negative selection; NT, non-transgenic control; bact, *A. tumefaciens* AGL0(pRCNG) as non-recombined positive control. (**A**) PCR analysis using primers specific for 35S-promoter and *gus* gene, *nptII*-gene, *gus*-gene, endogenous strawberry polygalacturonase gene (plant) and *A. tumefaciens vir*G-gene. (**B**) Southern blot analysis of *Eco*RI-digested DNA, hybridized with 35S-, *gus*-, and *npt*II-probe.

complete recombinase-mediated removal of the recombinase and the selectable marker did occur in all GUS-positive plants. In line E1 however, amplification with these primers might also have yielded a much longer PCR fragment, amplified from non-recombined T-DNA as shown for the pRCNG template (Fig 2A, 35S-gus-primers, bacterial control), if the 9.3 kb fragment observed in the Southern blot hybridising with the 35S-probe, would have represented a complete T-DNA. The absence of this larger fragment encompassing part of the 35S promoter, the recombinase-LBD, the complete selectable marker and part of the *gus* gene might be due to PCR conditions being biased towards amplification of small fragments. However, it is also likely that the 9.3kb fragment of E1 is not derived from a full-length T-DNA, but instead corresponds to a truncated T-DNA copy.

Southern blot analysis using the 35S-probe yields information on the number of T-DNA copies integrated into the strawberry genome. Four out of six marker-free transgenic plants displayed two *Eco*RI fragments in addition to the internal one present in all GUS-positive lines, which

would seem to indicate the integration of one T-DNA. Apparently, the other two plants (L2 and L3) only showed one additional *Eco*RI fragment, but the relatively high intensity of this additional fragment, as compared to the internal one, indicates the presence of different fragments of similar length.

Discussion

We developed an effective method for the production of transgenic plants from which selectable marker genes have been removed. The unique combination of a chemical-inducible recombinase activity and a bifunctional selection system allowed the straightforward production of completely marker-free transgenic plants, without the need for repeated transformation or sexual crossing.

The described method was tested by applying two different selection procedures. The efficiencies of the two negative selection procedures were found to differ considerably. The delayed selection procedure starting with leaf explants from fully-grown kanamycin resistant transformants produced more than thirty putative marker-free plants. Molecular analysis revealed that all four selected plants were completely free of selectable markers. On the contrary, early selection of marker-free plants from leaf explants that had only just begun to yield shoots (this is after four weeks of positive selection), produced only very few marker-free transgenic plants. Here, one of the putative marker-free plants appeared to be non-transgenic, which indicates that positive selection was insufficient. In another plant the selectable marker was still present. Southern blot analysis suggests that it is likely that in this case more than one T-DNA was integrated into the genome, and that one truncated, incomplete T-DNA-copy had resulted in loss of recombination capability and *cod*A expression. For the two remaining putative marker-free plants from the early selection, the selection markers were proven to be eliminated completely. Considering the fact that the number of leaf explants originally infected with pRCNG was roughly the same for the two selection procedures, implies that delayed selection was much more effective. For early negative selection, however, increased efficiencies may be obtained by optimising the timing of selection procedures.

One of the key features of the system presented here is the hybrid combination of a positive and a negative selectable marker gene. For positive selection of transgenic tissue the *npt*II gene conferring kanamycin resistance was used because of its effectiveness in strawberry transformation experiments (Schaart et al., 2002). The *E. coli cod*A gene had been successfully

used as a negative selectable marker in plant transformations, especially at the stage of shoot regeneration (Schlaman and Hooykaas, 1997) and has also been used as a selection marker in another marker removal scheme (Gleave et al., 1999). Concentrations ranging from 50-500 mg/l were described to be effective for negative selection at the regeneration stage (Schlaman and Hooykaas, 1997). In our hands 5-FC at a concentration of 150 mg/l was sufficient for the regeneration of transgenic plants, which are completely devoid from marker genes.

The use of a chemical-inducible recombinase activity allowed the simultaneously introduction of the R recombinase gene and the Rs recombination sites, so there was no need for retransformations or sexual crossing in order to combine the separated recombinase and its recombination sites after transgenic plants had been selected. Although marker-free plants could be obtained in an effective way using the inducible R recombinase activity, it was demonstrated by GUS-staining that significant recombination had already occurred in non-induced leaves of kanamycin resistant transgenic shoots from the delayed selection. Since these shoots showed sustained growth on kanamycin containing medium, and since they could only be GUS-positive due to deletion of the DNA fragment carrying the selectable marker, this would imply that the plants were chimeric for the selectable marker. Apparently, early partial elimination of the selectable marker did not affect the initial positive selection ability, necessary for production of the transgenic plants.

The observation that significant recombination had already taken place before treatment with DEX, suggests that the control exerted by the LBD was incomplete. Up to now, the principle of the LBD-mediated regulation of recombinase activity is not really clear and might be based on preventing nuclear localisation of the recombinase protein or on the inability of complexed recombinase to engage in recombination, or both (Picard and Yamamoto, 1987). In contrast to the direct enzyme activity control system (post-translational control) employed in this study, the steroid receptor LBD has also been used for indirect control of enzyme activity (transcriptional control) by fusing it to the transcription factor that regulates expression of the target gene. For example, Aoyama and Chua (1997) described the use of a hybrid transcription factor fused with the LBD of the rat glucocorticoid receptor and demonstrated that this allowed stringent control of luciferase reporter gene. The same system used for *gus* reporter gene regulation also allowed tight regulation in most transgenic lines studied (Ouwerkerk et al., 2001). Kunkel et al. (1999) however, showed leakiness of this LBD-mediated inducible system, when they used it for regulation of *ipt* expression. Possibly, non-induced gene expression levels are too low to be detected by the reporter genes used by Aoyama and Chua (1997) and Ouwerkerk et al. (2001). It is only when even low expression levels of a reporter lead to phenotype differences, like with

the *ipt* gene, that background activity of the system becomes evident. Our system is likely to be sensitive to low expression levels too, since it is essentially an irreversible system, resulting in accumulation of recombination events in time and a concomitant increase in GUS activity levels. Although such a low basal expression level is generally seen as undesirable, in our system it can be beneficial, as elimination of undesired sequences continues as long as the recombinase gene is present, even under non-inductive situations. On the other hand, premature recombination might reduce the frequency of transgenic plants with single copy T-DNA insertions, as untimely removal of the single marker would render them kanamycin sensitive at an early stage. However, this does not seem to be the case, in view of the relatively high number of single copy transformants obtained in this study.

So far, other methods describing production of marker-free plants without the requirement of sexual crossing or repeated transformations are not very efficient or application is limited to specific species and varieties. For example, recently, de Vetten et al. (2003) reported on a transformation system for the production of marker-free potato plants in which the use of a selectable marker was omitted. The practicality of their approach of using PCR to separate transgenic from untransformed shoots relies on high transformation efficiencies. Zuo et al. (2002) reflected on the use of regeneration promoting genes, like the *ipt* gene, for the production of marker-free plants, but application to new systems generally needs time-consuming re-optimisation of existing transformation protocols. The strategy for production of marker-free plants described by us is effective and is in principle widely applicable to any plant species for which transformation protocols have been developed. Our system differs from other marker-free transformation approaches in the possibility of selection for completely marker-free transgenic plants, so that the formation of genetic chimeras, a major drawback of other systems, is prevented. In order to substantiate the wide applicability of the system described here, we applied it to the apple cultivar Elstar, which can only be transformed at low frequencies (Puite and Schaart, 1996), and we were successful in obtaining completely marker-free transgenic apple shoots (results not shown). In addition, in tobacco, the use of chemical-induced recombination activity combined with the secondary regeneration under negative selective conditions, was quite effective in obtaining completely marker-free transgenic plants (results not shown).

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Chapter 7

General discussion: production of cisgenic strawberry plants

In this thesis different molecular biological aspects are described which are related to the production of consumer-friendly genetically modified strawberry plants. Part of the described work has been performed within the frame-work of a multidisciplinary EU-project entitled '*Sustainable production of transgenic strawberry plants. Ethical consequences and potential effect on producers, environment and consumers*'. One of the aims of this project 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 a strawberry *PGIP* gene. To ensure consumer and producer acceptance of such genetically modified strawberry plants, it was considered desirable that the ultimate genetically modified strawberry plants were free of any foreign regulatory and coding DNA sequences. Because solely species-own DNA was applied for modification of the trait of choice, it was proposed to call these plants cisgenic rather than transgenic.

Consumer acceptance of genetically modified crops

In the same EU-project the attitude of consumers was monitored toward genetic modification in general and particularly towards genetically modified strawberries. 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 was increased in case beneficial traits to consumers could be recognised (Fig. 1b). Furthermore, it was shown that modifications involving the use of strawberry-own DNA exclusively, the so-called cisgenic approach, also enhanced acceptance (Fig. 1c). This latter finding confirmed a recent consumers survey in the USA, which showed that the majority of the respondents would eat vegetables with an extra gene from the same species (81%) or from another vegetable species (61%), while this was only 14% in case viral genes had been used. (Lusk and Sullivan, 2002).

For assessing the novelty of specific genetic modifications, Nielsen (2003) proposed to categorise genetically modified organisms based on genetic relatedness of donor and recipient

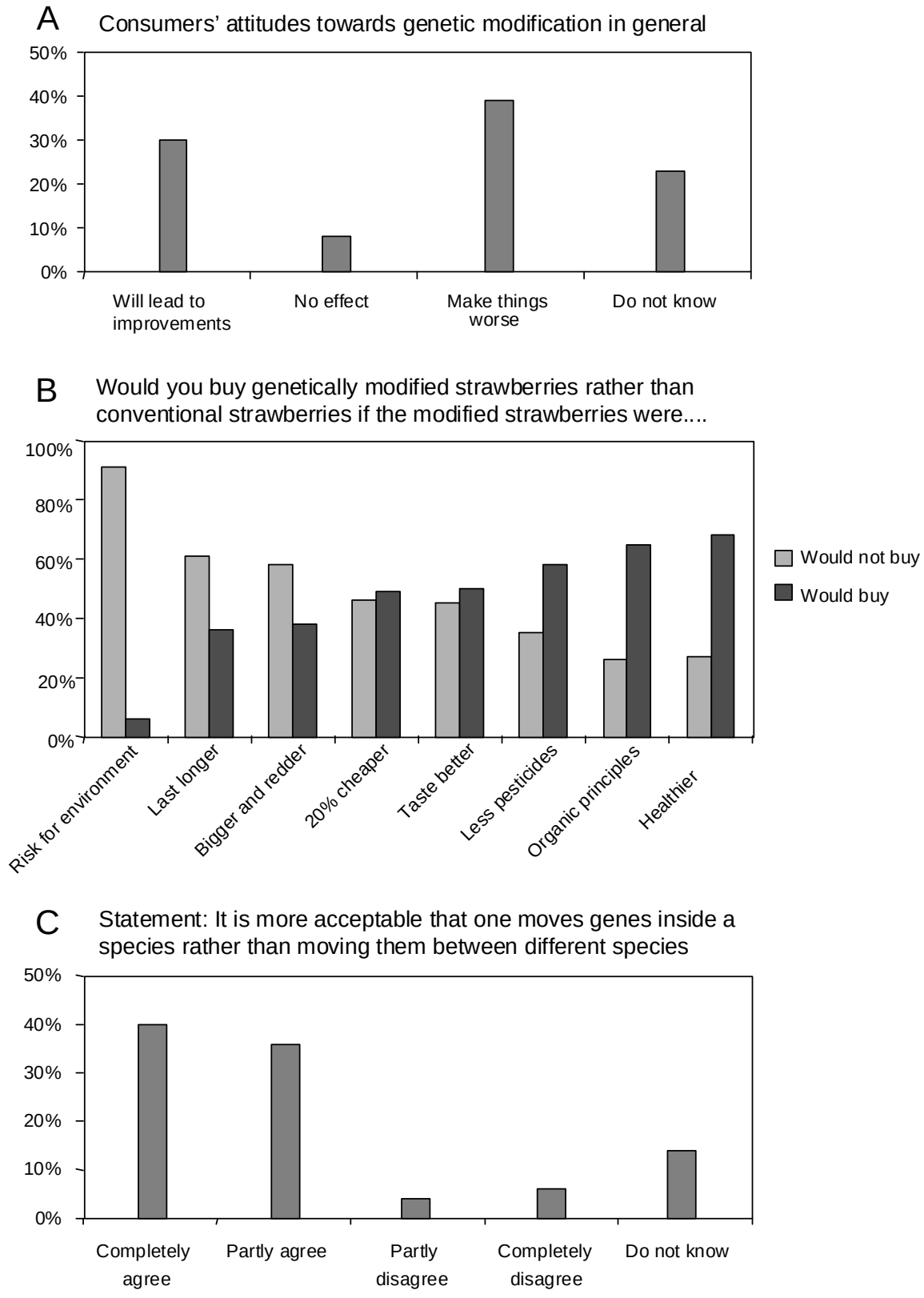

Figure 1. Sociological inquiry among 720 consumers in Norway, Denmark and the UK. Data are kindly provided by Reidun Heggem, Centre for Rural Research, Norwegian University of Science and Technology (NTNU).

organisms. The genetic distance between donor and recipient organisms should then indicate the potential for the modified trait to evolve spontaneously. According to Nielsen (2003), this could clarify the limited novelty of such genetic modifications in which donor sequences from the same species have been used. From a biological point of view however, there is less basis for using genetic relationships as functional criteria for assessing the novelty of the genetic modification. This can be illustrated by the fact that sufficient examples are known in which, within individuals, minimal genetic variation, like single nucleotide polymorphisms, can cause dramatic changes in function or expression of genes (also discussed in Chapter 3). On the other hand, there are examples known that the use of gene sequences from distant related species in transgenics does not necessarily lead to catastrophes. Therefore, for biologists, the ultimate impact of the genetic modification rather than the type of modification, or origin of donor sequences, would be important for acceptance.

The referred sociological studies showed, however, that general public perception of genetic modification is driven by emotions, invoked by the way the genetic modification has been applied, rather than by understanding the possibilities and limitations of the molecular biological art. In order to bridge the gap in the way that scientists and consumers approach the acceptance of genetic modification, it should be stressed that ethicists and sociologists have to be involved in discussing the concept of consumer-friendly genetically modified organisms. For example, in order to fully utilise possibilities of genetic modifications for crop improvement, the interesting question what minimal genetic distance is required to ensure sufficient public acceptance has to be resolved by ethical and sociological studies. The application of interspecific crosses as an existing practice in conventional breeding can be used as basis for such a study. Also, it remains to be clarified to what extent it is acceptable to make (intraspecific) modifications to genes and regulatory sequences in order to adapt gene expression and gene functions in transgenic crops.

Isolation and characterisation of strawberry *PGIP*

The above mentioned sociological studies have suggested the relatively high level of public acceptance of cisgenic crop plants. For the ultimate production of cisgenic crops with improved new characteristics, 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 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. After isolation of gene and regulatory sequences from the species to be engineered, accurate functional characterisation of the sequences to be used for the ultimate modification 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, since studies have shown that PGIP from a variety of origins is able to inhibit *B. cinerea* PG activity in vitro (Sharrock and Labavitch, 1994; Yao et al., 1995). Moreover, the introduction of a *PGIP* from pear into transgenic tomato plants resulted in a enhanced level of resistance towards *B. cinerea* (Powell et al., 2000). To this end, we isolated a *PGIP* gene from strawberry and characterised its expression pattern (Chapters 2 and 3). To isolate the *PGIP* gene from strawberry, degenerated primers were designed based on known *PGIP* sequences from related rosaceous species. Subsequently, *FaPGIP* expression analysis was performed which showed that *FaPGIP* was expressed at relatively low level in leaves and immature fruit tissue, but was upregulated during strawberry fruit ripening. Inoculation, but not wounding, led to a rapid upregulation of *FaPGIP*, but this upregulation seemed to be transient and *FaPGIP* was downregulated again two days after inoculation. Furthermore, it was demonstrated that the different *FaPGIP* alleles that were identified, showed variation in expression pattern in different tissues, at different developmental fruit stages and after inoculation of fruits with *B. cinerea*. These observations prompted us to aim at modifying *FaPGIP* gene expression in such a way that sufficient *FaPGIP* activity would be and stay present in *B. cinerea* susceptible tissues.

In order to investigate functional activity of *FaPGIP*, transgenic strawberry plants have been produced in which either of the two *FaPGIP* allelic sequences *FaPGIP1a* or *FaPGIP2a* have been introduced. In these transgenic plants both alleles are regulated by the strong and constitutive *CaMV 35S* promoter, which allows early screening of *B. cinerea* resistance, using leaf tissue. Although these plants still have to be thoroughly characterised, preliminary results (Fig. 2) show that for a certain number of these transgenic plants, inoculation of leaves with *B. cinerea* does not result in significantly different reaction as compared to control (water) inoculations on the same leaf. This suggests that those plants are resistant to *B. cinerea*. For non-transgenic control plants as well as for some of the transgenic plants inoculation with *B. cinerea* resulted in significantly larger lesions. The relation of the level of resistance to *B. cinerea* and expression pattern and levels of *FaPGIP* at both the gene and protein level is

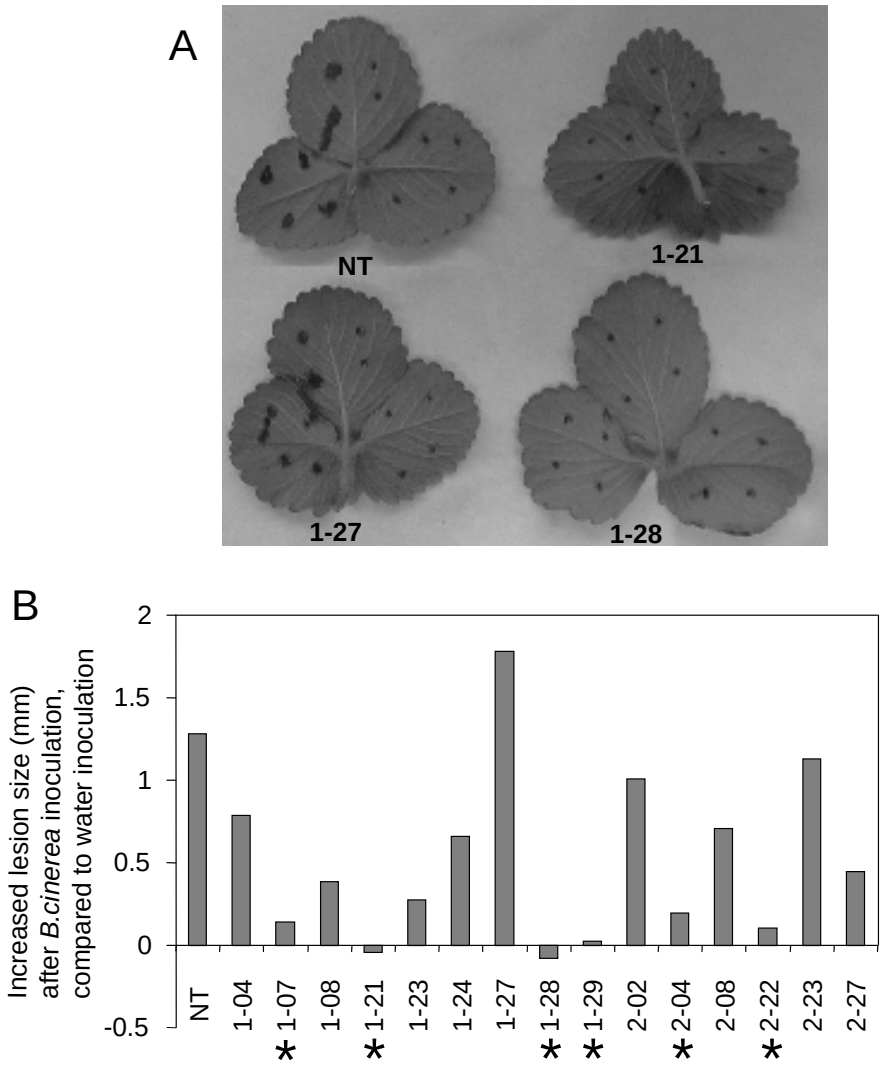

Figure 2. *B. cinerea* colonisation test on detached leaves of non-transgenic control (NT) and genetically modified strawberry plants transformed with a construct containing *FaPGIP* under the regulation of the *CaMV 35S* promoter. Detached leaves were wounded with a needle, giving an approximately 1 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). Transgenic lines marked with an asterisk differ significantly from the non-transgenic control at P-values < 0.05. Data are kindly provided by Trygve Kjellsen, Plantebiosenteret, Department of Biology, Norwegian University of Science and Technology (NTNU).

currently under investigation. Furthermore, the *B. cinerea* resistance tests of the transgenic strawberry plants are currently extended to flowers and to fruits at different developmental stages.

Identification of suitable promoter sequences

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 ripe and unripe fruits. In order to restrict *B. cinerea* in an effective way, *FaPGIP* expression should be upregulated at least in ripe fruits, 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, we focussed on the heterologous *CaMV 35S* and the petunia *fbp7*-promoter sequences that were already available, and we tested these promotor sequences for their expression pattern in transgenic strawberry plants (Chapter 4). 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*. In order to follow the cisgenic 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 for the isolation of its promoter.

By following a genome walking approach three 5'-upstream sequences were isolated and two fragments of 700 bp and 1600 bp respectively were analysed for their promoter activity. For this, transgenic strawberry plants were produced in which the *FaExp2*-promoter-*gus* fusions had been introduced. Analysis of leaf, root, petiole, flower and fruit tissue of these transgenic plants showed that both *FaExp2* promoter fragments regulated *gus* expression in a fruit-specific way, which was in agreement with the earlier described *FaExp2* expression. Interestingly, plants with the 1600bp-*FaExp2*-promoter-*gus* construct showed a 25-130-fold higher *gus* transcript abundance than what was found for transgenic plants with the shorter *FaExp2*-promoter fragment. In order to achieve high levels of *FaPGIP* expression for inhibition of *B. cinerea* in the ultimate cisgenic strawberry plants, the 1.6p*FaEXP2*-fragment was considered to be most suitable and was subsequently chosen for further experimentation.

Production of marker-free plants

For the efficient production of genetically modified plants the use of selectable marker genes is a prerequisite. In many transformation protocols either herbicide or, most often, antibiotic resistance genes have been shown to act as very effective selectable markers for genetically modified tissue and have, therefore, found wide application. However, public debate concerning health and environmental risks focuses 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. In this thesis the development and testing of a recombinase based system for elimination of undesired DNA sequences has been described. Such a marker removal system is especially valuable for vegetatively propagated crops, like strawberry, and for crops with long reproductive cycles. It has been demonstrated that the described system for production of marker-free genetically modified plants can be applied effectively to species and genotypes for which transformation protocols have already been developed. Employing this method for marker removal, even for apple, for which the production of transgenic plants is relatively difficult to achieve, marker-free plants could effectively be produced.

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. An additional advantage of recombinase-based systems for marker removal is that complex T-DNA integration patterns may be resolved due to the activation of the recombinase (Srivastava et al., 1999). An disadvantage, however, is that with the used recombinase system one specific recombination sites remains at the locus of T-DNA integration. In case T-DNAs have been integrated at different loci within a genome, recombinations between recombination sites of different T-DNAs could lead to chromosomal deletions or inversions or to chromosomal translocations. In yeast it has been demonstrated that the recombination system can mediate recombination between two recombination sites that are present about 180 kb apart on one chromosome or between recombination sites that are located on two non-homologous chromosomes (Matsuzaki et al., 1990). The rate at which such

recombination between distantly located recombination sites occurs in plants is not clear yet. Therefore, to prevent chromosomal rearrangements, the application of recombination mediated marker removal should be restricted to transgenic plants with a single locus T-DNA insertion.
Because marker-removal allows retransformation of transgenic plants employing the same selectable marker gene as used for the initial selection of the transgenic plant, this application has often been associated with stacking of transgenes through repeated series of transformations and recombination-mediated marker removal. Because newly introduced T-DNAs will usually integrate at different loci, a definite risk of chromosomal rearrangements exists (Ebinuma et al., 2001), necessitating a thorough screening of the ultimate genetically modified plants.

Integration of T-DNA

One item which has not been described in this thesis, but which is of importance for the production of acceptable genetically modified plants, is the frequent occurrence of integration of vector backbone sequences in the genome of transgenic plants. Depending of the left border sequence and vector sequences flanking this left border, a significant number of transgenic plants may contain vector backbone sequences of variable length (for example see De Buck et al., 2000). These sequences often include antibiotic resistance gene sequences and bacterial replication origins that are located on the vector backbone. Therefore, genetically modified plants have to be screened for the presence/absence of such vector backbone sequences, preferably by the isolation and sequencing of DNA-sequences flanking the left and right border of the introduced T-DNA.
Alternatively, transformation vectors can be constructed which prevent the presence of vector backbone sequences in the genetically modified plant. In such vectors the complete transformation vector is located between the T-DNA border sites (Fig. 3a), resulting in initial integration of the complete binary vector sequence into the host genome. Smart positioning of recombination sites allows subsequent elimination of sequences that originate to the vector backbone, as well as undesired sequences of the original T-DNA sequence, leaving behind the gene of interest, one recombination site sequence and fragments of both T-DNA right and left borders (Fig. 3b). Although both the recombination site and T-DNA border sequences are of foreign origin, these sequences are not expected to have any biological function in plants. If desired, the T-DNA border sequences can be replaced by so-called P-DNA (plant-derived DNA) border sequences, which have been isolated from potato and show a high level of homology to the left and right T-DNA borders of *A. tumefaciens* nopaline strains (Rommens et

al., 2004). These P-DNA borders have been demonstrated to be equally effective for T-DNA transfer as conventional T-DNA borders.

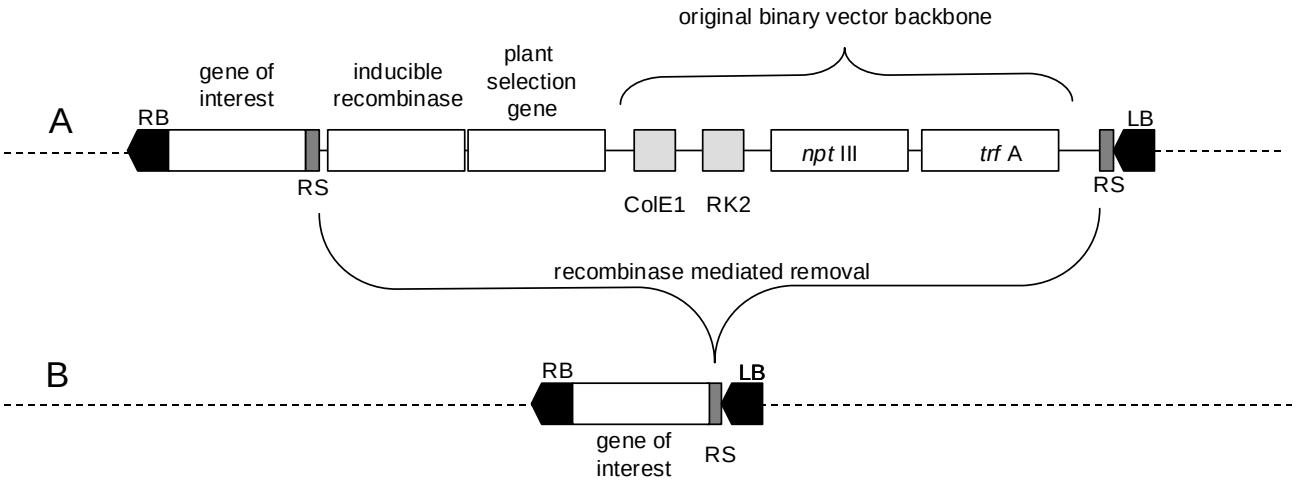

Figure 3. Schematic representation of the T-DNA sequence of a binary vector for production of 'vector backbone-free' transgenic plants. The complete binary vector is located in between the right and left T-DNA border sequences (RB and LB, respectively). Interrupted line represents host genomic DNA. After T-DNA integration (situation **A**) and selection of transgenic plants, induction of recombination will result in removal of the DNA-sequence flanked by recombination sites (RS), leaving behind the gene of interest, T-DNA border sequences and one RS-site (situation **B**). In practice, T-DNA borders that will flank the transferred sequence will be incomplete. The origin of replication sequences ColE1 and RK2, and the gene sequences *npt*III (bacterial selection gene) and *trf* A (bacterial replication initiation gene), are located on the backbone DNA sequence of the binary vector pBINplus. The gene of interest ideally consists of regulatory and coding sequences originating from the species to be genetically modified. The plant selection gene may consist of the bifunctional *cod*A-*npt*II hybrid gene (see Chapter 6). For explanation of recombinase-mediated removal of DNA sequences, see Chapter 6.

Production of cisgenic strawberry plants

In the end, the combined use of all aspects described above enables the production of genetically modified plants which contain only native genes and native promoters and which may therefore be more acceptable to consumers. To demonstrate the possibility of producing such cisgenic plants, the described techniques have been applied to strawberry with the aim to generate strawberry plants that are less susceptible to infection by *B. cinerea*. For this aim the strawberry *FaPGIP1a* gene, which natural expression level is expected to be insufficient for inhibition of *B. cinerea* spread, has been combined with the 1.6p*FaExp2*-promoter sequence, which directs gene expression to a high level in ripening strawberry fruits. This new strawberry

gene combination has been inserted into a binary vector in which the inducible recombinase gene and the bifunctional selectable marker gene are flanked by recombination sites, enabling recombination mediated removal of both genes at the desired point in time. Using this vector for transformation of strawberry and successive removal of the selectable marker and recombinase gene have resulted in 14 putative cisgenic strawberry plants (results not shown). PCR analysis has shown that in 11 of these plants the selectable marker gene has successfully been removed and that the new gene combination is present. These plants have recently been transferred to the greenhouse for production of fruits for further characterisation.

It is envisaged that a significantly higher level of *B. cinerea* resistance in fruits of these plants will result in reduction of fungicide applications, which will be favourable to both producers, consumers and environment. Since these plants contain no regulatory or coding sequences of foreign origin, it is envisaged that this particular genetically modified strawberry will find good acceptance by producers and consumers of strawberries. In the end, the use of such cisgenic strawberry plants, for example in the production according to organic principles, could lead to a new way of sustainable crop production practices (Ronald et al., 2004).

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Summary

Fruit rot, caused by *Botrytis cinerea*, may result in significant losses during strawberry production. Since *Botrytis* is proliferating particularly on ripe fruits, it may lead to pre- and postharvest damage. Therefore, in the commercial strawberry production, fungicides have to be applied at least on a weekly basis and the application has to be continued until a few days before harvest. This may lead to fungicide residues on the ripe fruits.

For a more sustainable production of strawberries, the application of fungicides has to be reduced. For this reason, it is desirable to have strawberry cultivars available, that have a high level of resistance towards *Botrytis*. Since there are no strawberry cultivars or closely related strawberry species known that express a high level of *Botrytis* resistance, it will be very difficult to achieve sufficient resistance through conventional breeding.

Genetic modification is a technique with which new genetic qualities can be introduced into an organism, without further changing it's own qualities. So, genetic modification seems to be a very useful technique for the improvement of existing commercially important strawberry cultivars by introducing resistance towards *Botrytis*. For the realisation of such a genetic modification, certain conditions have to be met. First of all, suitable genes will have to be identified that may equip strawberry with resistance against *Botrytis*. Next to this, for the specific regulation of transgene expression, suitable promoter sequences have to be identified and isolated.

Plant-pathogenic fungi, like *Botrytis*, often produce cell wall degrading enzymes with which they attack the plant. The activity of certain cell wall degrading enzymes, however, may be inhibited by the polygalacturonase inhibiting protein (PGIP), which has been found in many plant species. Published results, concerning transgenic tomato plants in which a *PGIP*-gene from pear has been introduced, show that this modification resulted in an increase in resistance towards *Botrytis.* This information suggests that the *PGIP*-gene is a suitable candidate for introduction into strawberry, in order to achieve an enhanced level of *Botrytis* resistance.

The *PGIP*-gene appears to be present naturally in strawberry. Isolation and characterisation of the strawberry *PGIP*-gene (*FaPGIP*) indicated that several variants of this gene are present in strawberry, and that these variants can vary in expression level. *FaPGIP* showed a relatively low expression level in leaves, petioles, roots, flowers and immature fruits. However, in ripe red fruits the expression of *FaPGIP* is upregulated. Next to this, *FaPGIP* expression is also strongly upregulated after inoculation with *Botrytis.* This occurs both in immature as well as in

red ripe strawberry fruits. Nevertheless, this upregulation of gene expression does not last very long and is probably insufficient to restrict colonisation of the fruit by *Botrytis*. Modification of the expression level of *FaPGIP* may, therefore, be helpful in accomplishing a higher level of inhibition of *Botrytis*.

The expression of a gene can be modified by combining it with a different promoter sequence. Until recently, in strawberry no fruit-specific promoter sequences have been isolated yet. Therefore, initially, the activity of the *fbp7*-promoter of petunia was tested in strawberry. The *fbp7*-promoter was reported to direct strong gene expression in receptacles of petunia flowers. Since the strawberry fruit actually consists of swollen receptacle tissue, it was supposed that the *fbp7*-promoter would also be active in strawberry fruits. In fruits of transgenic strawberry plants, in which the *fbp7*-promoter combined with the *gus*-reporter gene had been introduced, histochemical GUS-staining confirmed the expected tissue-specific activity of the *fbp7*-promoter. In addition to this heterologous promoter, promoter fragments of strawberry itself have subsequently been isolated. For this purpose, the strawberry expansin-2 gene (*FaExp2*) was selected, which plays a role in fruit ripening and shows specific expression in ripening fruits. Characterisation of two promoter fragments of the *FaExp2* gene, which differ in length (700 bp and 1600 bp, respectively), showed that in transgenic plants which contain a combination of either of the two promoters with the *gus*-reporter gene, the fruit-specific activity could be confirmed. Both promoters seemed to be active predominantly in full-grown, but still unripe fruits and they stayed active during further fruit ripening. Apart from that, it was remarkable that, despite the common gene expression pattern observed for both promoter fragments, the 1600 bp promoter fragment induced gene expression to a much higher level. Since the *FaExp2*-promoter is active at those stages of fruit development at which primary *Botrytis* infection takes place, this promoter is regarded as a suitable candidate to direct the fruit-specific overexpression of *FaPGIP*. Ultimately, it is expected that the combination of the *FaExp2*-promoter with the strawberry *PGIP*-gene in transgenic plants will lead to a reduction in susceptibility to *Botrytis*-induced fruit rot.

It is known that, particularly in Europe, a negative attitude exists towards genetic modification. However, a sociological study in Europe, regarding genetic modification of strawberry, has shown that if consumers recognise introduced traits as beneficial for themselves, acceptation of genetic modification increased. Furthermore, in this sociological study it was shown that modifications involving genetic material only from the species itself would support acceptance even more. By using the strawberry's own *FaPGIP*-gene and *FaExp2*-promoter sequences in making genetically modified strawberry plants, this condition will be met.

For the efficient production of genetically modified plants the use of (antibiotic resistance) selection genes is a prerequisite. The selection gene, which is usually of bacterial origin, is introduced simultaneously with the gene-of-interest, thereby allowing easy selection of transgenic plants. After the selection of transgenic plants has been completed, the selection gene has no function anymore, and the presence of such a foreign gene is actually undesired in the ultimate transgenic plants. In this thesis an effective method is described which enables the removal of the selection gene from the transgenic plant by means of a chemically inducible recombination step. By using this method it is possible to produce genetically modified plants in which, in the end, only species-own genes and promoter sequences are present. Because this type of genetically modified plants contains no foreign genes or promoter sequences, it is proposed to call these plants cisgenic rather than transgenic. Currently, cisgenic strawberry plants, in which the *FaPGIP*-gene combined with the *FaExp2*-promoter has been introduced, are cultured in the greenhouse for further investigation. In case these plants are found to exhibit a sufficient level of *Botrytis* resistance the described approach might contribute to a more sustainable production of strawberries.

Samenvatting

In de aardbeiproductie kan vruchtrot, veroorzaakt door de schimmel *Botrytis cinerea,* tot aanzienlijke verliezen leiden. Omdat *Botrytis* met name op rijpe vruchten groeit, kan de economische schade niet alleen vóór, maar ook na de oogst optreden. In de commerciële aardbeiproductie moet daarom vanaf de bloei zeker wekelijks met middelen tegen *Botrytis* gespoten worden en deze middelen moeten tot aan enkele dagen voor de pluk worden toegepast. Dit kan leiden tot fungicidenresiduen op de rijpe vruchten.

Voor een duurzame productie van aardbeien zal het gebruik van bestrijdingsmiddelen moeten worden teruggedrongen en is het dus wenselijk om de beschikking te hebben over aardbeirassen met een hoge mate van vruchtrot-resistentie. Omdat er geen rassen of wilde verwanten van aardbei bekend zijn die voldoende resistentie bezitten tegen vruchtrot, zal het zeer moeilijk zijn om met traditionele veredeling tot aardbeirassen met een hoge mate van *Botrytis* resistentie te komen.

Genetische modificatie is een techniek waarmee in principe nieuwe genetische eigenschappen aan een organisme kunnen worden toegevoegd, zonder verandering aan te brengen in de bestaande eigenschappen van dat organisme. Genetische modificatie lijkt dus zeer geschikt om bijvoorbeeld reeds bestaande commercieel belangrijke aardbeirassen zodanig aan te passen dat ze minder vatbaar voor vruchtrot zijn. Om zo'n modificatie te verwezenlijken moet aan een aantal voorwaarden worden voldaan. Ten eerste zullen geschikte genen moeten worden geïdentificeerd welke resistentie geven tegen vruchtrot. Daarnaast moeten regel-sequenties, zogenaamde promotoren, beschikbaar zijn die het in te brengen gen op een juiste manier aansturen.

Plantpathogene schimmels, zoals *Botrytis*, produceren vaak celwand-afbrekende enzymen, waarmee ze de plant kunnen aantasten en binnendringen. Planten kunnen echter een eiwit aanmaken, het polygalacturonase inhibiting protein (PGIP), dat de activiteit van bepaalde celwand-afbrekende enzymen kan remmen, waardoor de aantasting door *Botrytis* vertraagd wordt. Gepubliceerde resultaten over genetisch gemodificeerde tomatenplanten, die uitgerust waren met een *PGIP*-gen uit peer, laten bijvoorbeeld zien dat deze planten minder schade ondervonden van inoculaties met *Botrytis*. Deze bevindingen geven aan dat het *PGIP*-gen een geschikte kandidaat is om resistentie tegen vruchtrot in aardbei te bewerkstelligen. Het *PGIP*-gen blijkt van nature ook in aardbei voor te komen. Isolatie en karakterisering van het aardbei *PGIP*-gen (*FaPGIP)* laten zien dat er verschillende varianten van dit gen in aardbei aanwezig

zijn, welke deels verschillend tot expressie komen. *FaPGIP* vertoont een relatief laag expressieniveau in blad, bladsteel, wortel, bloemen en onrijpe vruchten. In rode rijpe vruchten echter, treedt er een verhoging van *FaPGIP*-genexpressie op. Daarnaast wordt de expressie van dit gen ook sterk gestimuleerd als reactie op inoculatie van zowel onrijpe als rijpe aardbeivruchten met *Botrytis* sporen. Deze verhoging van *FaPGIP* expressie is echter van korte duur en is waarschijnlijk onvoldoende om uitbreiding van *Botrytis* aantasting tegen te gaan. Verandering van het expressieniveau van *FaPGIP* kan mogelijk tot een betere remming van *Botrytis* leiden.

De expressie van een gen kan worden aangepast door het van een andere promotor te voorzien. Tot voor kort waren nog geen vrucht-specifieke promotoren van aardbeigenen beschikbaar. Daarom werd in eerste instantie de activiteit van de promotor van het *fbp7*-gen uit petunia in aardbei bestudeerd. Van de *fbp7*-promotor was beschreven dat deze sterke expressie vertoont in de bloembodem van petunia-bloemen. Daar de aardbeivrucht in feite uit een opgezwollen bloembodem bestaat, werd verondersteld dat de *fbp7*-promotor ook actief zou zijn in aardbeivruchten. In transgene aardbeiplanten, die de *fbp7*-promoter gecombineerd met het *gus*-reportergen bevatten, kon door middel van histochemische GUS-kleuringen inderdaad het vruchtspecifieke karakter van de *fbp7*-promotor worden aangetoond. Naast deze heterologe promotor zijn vervolgens ook promotorfragmenten uit aardbei zelf geïsoleerd. Hiervoor werd het expansine-2-gen (*FaExp2*) uitgekozen, dat een rol speelt bij de vruchtrijping en specifieke expressie vertoont in rijpende aardbeivruchten. Voor karakterisering van twee geïsoleerde promotorfragmenten van het *FaExp2*-gen, die verschillen in lengte (respectievelijk 700 bp en 1600 bp), werden transgene planten gemaakt waarin combinaties van de afzonderlijke promotorfragmenten met het *gus*-reportergen waren geïntroduceerd. Beide promotoren bleken voornamelijk actief in volgroeide, maar nog onrijpe vruchten en bleven actief tijdens de rijping van de vrucht. Het was overigens opmerkelijk dat, ondanks het overeenkomstige expressiepatroon, met het 1600 bp promotorfragment een veel hoger niveau van genexpressie werd verkregen. Omdat de *FaExp2*-promotor actief is gedurende het stadium van vruchtontwikkeling waarin de primaire *Botrytis* infectie tot stand komt, is deze promotor een geschikte kandidaat om het *FaPGIP* tot overexpressie te brengen. Het is de verwachting dat de combinatie van de *FaExp2*-promoter met het aardbei *PGIP*-gen in transgene planten zal leiden tot een verminderde gevoeligheid voor vruchtrot.

Het is bekend dat, met name in Europa, er een negatieve houding bestaat ten aanzien van genetische modificatie in algemene zin. Een sociologische studie in Europa naar acceptatie van genetische modificatie van aardbei wijst echter uit dat in specifieke gevallen, waarbij de

modificatie tot een voor de consument herkenbare positieve eigenschap leidt, er een betere acceptatie van genetische modificatie van aardbei is. Verder kwam uit de studie naar voren dat de acceptatie nog verder verbeterde indien er alleen erfelijk materiaal van de soort zelf voor de genetische modificatie was gebruikt. Met het gebruik van het aarbei-eigen *FaPGIP* gen en de *FaExp2*-promotor in genetisch gemodificeerde aardbeiplanten wordt aan deze voorwaarde tegemoet gekomen.

Voor een efficiënte productie van genetisch gemodificeerde planten moet gebruik worden gemaakt van selectiegenen (antibioticum-resistentie), die meestal van bacteriële oorsprong zijn. Zulke selectiegenen worden tegelijkertijd met het gen-van-interesse in de transgene plant ingebracht waardoor transgene planten gemakkelijk kunnen worden geselecteerd. Na selectie van transgene planten heeft het selectiegen geen functie meer en is de aanwezigheid in principe ongewenst. In dit proefschrift is een methode beschreven waarmee, door middel van een chemisch geïnduceerde recombinatie-stap, effectief het gebruikte selectiegen uit de plant kon worden verwijderd. Deze methode biedt dus de mogelijkheid om op een efficiënte wijze genetisch gemodificeerde planten te produceren waarin uiteindelijk alleen soorteigen genen en promotorsequenties voor de modificatie gebruikt zijn. Omdat dit type genetisch gemodificeerde planten geen vreemdsoortige genen bevat, wordt voorgesteld om ze cisgeen in plaats van transgeen te noemen. Recentelijk zijn in ons laboratorium cisgene aardbeiplanten gemaakt, welke de combinatie van de *FaExp2*-promoter met het *FaPGIP*-gen bevatten. Deze planten worden momenteel in de kas opgekweekt voor nader onderzoek. Indien deze planten over een voldoende mate van vruchtrotresistentie blijken te beschikken, zal het hier beschreven onderzoek wellicht kunnen bijdragen aan een meer duurzame productie van aardbeien.

Nawoord

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Curriculum vitae

Jan Gerrit Schaart was born on the 21st of December 1963 in Delfzijl, the Netherlands. In 1985 he finished successfully the school for laboratory technicians (HLO), botanical section, after which he started working as research assistant at the University of Groningen (RUG). In 1986 he moved to Wageningen were he obtained a position as technician at research institute Ital. Ital was successively incorporated in CPO, CPRO-DLO and finally in Plant Research International, to which the author is still connected. In 1986 Jan started also a part-time study at the University of Utrecht (RUU), and obtained a Master degree on Biology in 1993. Since April 2000 he has been working as junior scientist on a EU-project which was aimed at the production of consumer-friendly transgenic strawberries. Part of the results obtained in this project are described in the present thesis.

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