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KATI HANHINEVA

Metabolic Engineering of Phenolic Biosynthesis Pathway and Metabolite Profiling of Strawberry *(Fragaria x ananassa)*

Doctoral dissertation

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ABSTRACT

In this Thesis garden strawberry (*Fragaria* × *ananassa*) was genetically modified in order to enhance resistance to the grey mould fungus *Botrytis cinerea*. A gene encoding stilbene synthase from frost grape *Vitis riparia* (*NS-Vitis3*) was introduced in strawberry to create biosynthetic pathway for resveratrol, a phenolic phytoalexin compound. Several genes encoding stilbene synthase have been used widely in genetic modification of plants, resulting in a succesful synthesis of resveratrol derivatives and providing enhanced resistance to pathogens including *B. cinerea*. The coding sequence of the gene *NS-Vitis3* was introduced in strawberry under the control of two different promoters, i.e., a flower-specific (fil1) and general (cauliflower mosaic virus 35S) promoter. In the leaf tissue of the *35S:NS-Vitis3* line the integrated transgene was shown to cause down-regulation of an endogenous gene, chalcone synthase, resulting in the depletion of flavonoids and accumulation of phenylpropanoid compounds, as revealed by metabolite profiling by UPLC-qTOF-MS. An *in vitro* leaf test suggested decreased resistance to grey mould, most likely resulting from the significant alteration of the metabolite composition. A detailed metabolite profiling was crucial for the understanding of the unexpected consequences of the genetic modification. The modification did not have clear impact on the floral organs, but the analysis of flowers allowed the identification of several metabolites and metabolite groups that differed between the different floral organs. Furthermore, NMR identification of a set of metabolites that showed unexpectedly high accumulation in the leaves of the modified plants led to the discovery of a metabolite class previously uncharacterized in strawberry, i.e., phenylpropanoid glycosides. The phenylpropanoid glycosides bring an interesting new aspect in the research of strawberry phytochemicals, since they are highly bioactive metabolites that have been studied in the context of Asian folk medicine, and have been found to contribute to the therapeutic activities of several plant species.

Universal Decimal Classification: 581.19, 581.2, 581.46, 582.711.712, 602.6, 632.4, 632.911, 634.75
CAB Thesaurus: plant disease control; fungal diseases; disease resistance; *Fragaria ananassa*; strawberries; moulds; *Botrytis cinerea*; bioreactors; in vitro culture; in vitro regeneration; genetic engineering; gene transfer; transgenic plants; tissue culture; plant tissues; stilbenes; resveratrol; phytochemicals; metabolic profiling; metabolomics; secondary metabolites; phenolic compounds; phenylpropanoids; flowers

For Petri, Jaakko, Anni and Tuuli

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Kuopio, May 2008

Kati Hanhineva

ABBREVIATIONS

2-DE	two dimensional electrophoresis
ANS	anthocyanidin synthase
APCI	atmospheric pressure chemical ionization
AS	aureusidin synthase
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CHI	chalcone isomerase
CHS	chalcone synthase
CI	chemical ionization
CoA	coenzyme A
CE	capillary electrophoresis
cv.	cultivar
DMID	7,2'-dihydroxy-4'-methoxyisoflavanol dehydratase
dbp	DNA binding protein
DSDG	dehydroshikimate dehydrogenase
EI	electron ionization
ESI	electrospray ionization
F3H	flavanone hydroxylase
F3'H	flavonoid-3'-hydroxylase
F3',5'H	flavonoid-3',5'-hydroxylase
FAB	fast atom/ion bombardment
fil1	filament1
FLS	flavonol synthase
FNS	flavone synthase
FTMS	fourier transform ion cyclotron mass spectrometry
FTICR	fourier-transform-ion-cyclotron-resonance
GALDI	colloidal graphite-assisted laser desorption/ionization
GC	gas chromatography
GM	genetically modified
HHDP	hexa-hydroxy-di-phenoyl
HPLC	high performance (also: high pressure) LC
IFS	isoflavone synthase
IBA	indole-3-butyric acid
LAR	leucoanthocyanidin reductase
LC	liquid chromatography
LCR	leucoanthocyanidin reductase
MALDI	matrix-assisted laser desorption/ionization
mRNA	messenger RNA
MS	mass spectrometry
MS medium	Murashige-Skoog medium
NMR	nuclear magnetic resonance
PA	proanthocyanidin
PAL	phenylalanine ammonia lyase

PCA	principal component analysis
Phe	phenylalanine
PPM	plant preservative mixture
PTGS	post-transcriptional gene silencing
qPCR	quantitative real-time reverse transcriptase PCR
qTOF	quadrupole time of flight
RNAi	RNA interference
STS	stilbene synthase
TAIL-PCR	thermal asymmetric interlaced PCR
T-DNA	transferred DNA
TDZ	thidiazuron
TIB	temporary immersion bioreactor
UFGT	UDP-glucose flavonol 3-*O*-glucosyl transferase
UPLC	ultra performance liquid chromatography
UV	ultra violet
VR	vestitone reductase
VST	Vitis stilbene synthase promoter

LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following articles, referred to in the text by their Roman numerals. In addition, unpublished results are presented.

I: Hanhineva K, Kokko H, Kärenlampi S (2005) Shoot regeneration from leaf explants of five strawberry (*Fragaria* x *ananassa*) cultivars in temporary immersion bioreactor system *In vitro Cell. Dev. Biol.-Plant* 41: 826-831.

II: Hanhineva K, Kärenlampi S (2007) Production of transgenic strawberries by temporary immersion bioreactor system and verification by TAIL-PCR. *BMC Biotechnology* 7: 11.

III: Hanhineva K, Soininen P, Anttonen M, Siljanen H, Kokko H, Laatikainen R, Rogachev I, Aharoni A, Kärenlampi S: Leaf metabolite profiling of strawberry (*Fragaria* x *ananassa*) transformed with stilbene synthase gene from frost grape (*Vitis riparia*). Submitted for publication.

IV: Hanhineva K, Rogachev I, Kokko H, Mintz-Oron S, Venger I, Kärenlampi S, Aharoni A: Non-targeted analysis of spatial metabolite composition in strawberry (*Fragaria* x *ananassa*) flowers. Under revision.

CONTENTS

1. INTRODUCTION

Genetic modification has provided a tool for plant science over two decades to improve plant quality. The most widely used applications today enhance the agronomic performance of crop plants. In the "second" and "third generation" applications, of particular interest and at the same time very challenging are the approaches aiming at the modification of plant secondary (natural product) metabolism. Plant natural products have enormous structural diversity and also widely varying biological activities that have been exploited by the mankind from ancient times. Genetic engineering offers a means to enhance the production of important metabolites, or to establish the production of a new metabolite in the plant. As the knowledge of the enzymes that function in the secondary metabolism is accumulating, the transfer of whole biosynthetic pathways is becoming feasible.

Genetic engineering of plant secondary metabolism is especially challenging for the reason that very similar enzymes function in closely related metabolic pathways, and that minor changes in the structure of enzymes may result in different catalytic activity. The regulation of parallel secondary metabolism pathways is often interconnected, and thus perturbations in one pathway may be reflected in another one, resulting in a change in the sensitive balance between the pathways. The current advances in the metabolomics-related technologies allow increasingly accurate separation and measurement of highly complex matrices, such as plant tissues, and will aid in the assessment of overall consequences of any modifications of metabolic pathways.

In this thesis, strawberry phenolic metabolism was modified by introducing stilbene synthase gene (*NS-Vitis3*) from frost grape (*Vitis riparia*). The effects of the transgene were studied at the DNA, RNA and protein levels, and by comprehensive metabolite screening. All these approaches together allowed the interpretation of the unexpected consequences of the genetic modification, eventually leading to the characterization of a metabolite class not previously known to be present in strawberry. In addition, a comprehensive metabolite profiling of strawberry floral organs was performed, and several metabolite classes including ellagitannins, flavonols, terpenoids and proanthocyanidins were found to be expressed differentially and with different derivatized forms in the organs. The advanced analytical approach by UPLC-qTOF-MS proved to be feasible in metabolite analysis of rich plant matrices, and could be amenable for resolving plant metabolism related matters when integrated with transcriptomics and proteomics as a part of systems biology approach.

In the review of literature, engineering of the secondary metabolite pathways is discussed in more detail, giving a special emphasis to the biosynthesis of phenolic compounds. In addition, the applicability of metabolomics in plant research is discussed.

2. REVIEW OF THE LITERATURE

2.1. Metabolic engineering

Metabolites are traditionally classified as primary and secondary metabolites. Primary metabolites arise from highly conserved, universal metabolic pathways that the organisms need for basic living processes like growth, development and reproduction; such compound classes are, e.g., carbohydrates, amino acids and fatty acids. Secondary metabolites are natural products, the absence of which does not result in immediate death, but in long term their impairment may lead to malfunction and reduced survivability. Specific secondary metabolites are often restricted to a narrow set of species within a phylogenetic group (Fridman & Pichersky, 2005).

Metabolic engineering aims at modifying cellular metabolite composition in order to produce new compounds, enhance the production of existing compounds, or eliminate undesirable ones. Plant metabolism is modifed either by introducing novel genes or even pathways, or enhancing the expression of endogenous pathways, e.g., by up-regulating transcription factors. Down-regulation of endogenous genes is accomplished by silencing target genes with anti-sense expression, sense expression or RNA interference (RNAi) (Dixon, 2005). The advancements and limitations of genetic modification of plants have been regularly overviewed in numerous reviews and commentaries (Dixon & Arntzen, 1997; Ohlrogge, 1999; DellaPenna, 2001; Broun & Somerville, 2001; Capell & Christou, 2004).

Genetically modified (GM) crops were cultivated in 22 countries in a total area exceeding 100 million hectares by the end of year 2006. The major GM crops were soybean, maize, cotton and canola modified for herbicide and/or pest resistance (James, 2006). Agricultural quality improvement by enhancing resistance to abiotic stresses is one of the highest priorities in plant biotechnology (Zhang et al., 2004; Vinocur & Altman, 2005). Approaches to engineer drought tolerance have been taken as soon as key functional and regulatory genes have been identified (Umezava et al., 2006). Genetic modification is, however, increasingly used for improving various other factors and, especially during the past decade, much attention has been paid to the development of ‘second generation’ GM crops that have traits beneficial for consumers, e.g., increased nutritional value, premium quality or low allergenicity (Yonekura-Sakakibara & Saito, 2006). Approaches for tailor-made, quality-improved foods have been taken by modifying both primary (e.g. improvement of amino acid or fatty acid content; Galili et al.,

2002) and secondary metabolism (for engineering of secondary metabolism, see Chapter 2.1.2.). Important application is also the genetic modification of plants to synthesize therapeutic recombinant proteins (biopharming) and it is regarded as a safe and cost-effective alternative to their production in mammals (Daniell et al., 2001; Ma et al., 2004; Ko and Koprowski, 2005). In addition to whole plants, plant cell cultures are seen as potential matrices for the large-scale production of valuable compounds like drugs, anti-oxidants, vitamins and flavours (Verpoorte et al., 1999; Roberts, 2007; Oksman-Calentey and Inze, 2004). Other applications of plant metabolic engineering include biofuel production (Torney et al., 2007; Chen and Dixon, 2007) and phytoremediation (Chaney et al., 2007).

2.1.1. Metabolic engineering for improved tolerance to pathogens and pests

Enormous progress has been made in the understanding of plant responses to biotic stresses, mainly of plant-pathogen interactions, and several strategies to develop pathogen and pest resistant plants by metabolic engineering are being tackled (Cambell et al., 2002; Grover and Gowthaman, 2003; Tenllado et al., 2004). Especially important are the applications to control pathogens and pests in common crops, and targeted approaches include enhancing the resistance of wheat and barley against fusarium head blight (Dahleen et al., 2001), sugarcane against *Sugarcane mosaic virus* (Gilbert et al., 2005), potato against late blight (Colton et al., 2006) and rice against fungal and bacterial blight (Maruthasalan et al., 2007).

Among the most devastating plant pathogens is the necrotrophic fungus *Botrytis cinerea* that can infect more than 200 plant species including several economically important crops. It causes both pre- and post-harvest disease symptoms characterized by rapid development of necrotic lesions and intensive fungal growth, giving the typical appearance of the gray mould disease (Govrin and Levine, 2000). Means to control *B. cinerea* infections include fungicide and biological control, but since no plants are known to be completely resistant to *B. cinerea*, development of resistant crop varieties by conventional breeding are not often attempted (Sohn et al., 2007). In contrast, intensive research is ongoing in order to genetically engineer *B. cinerea* resistance by several different approaches. Such strategies include the utilization of chitinases, which degrade chitin in fungal cell walls. Several chitinase-enriched transgenic plants have shown enhanced resistance against *B. cinerea*, including cucumber (Tabei et al., 1998; Kishimoto et al., 2004) and tobacco (Terakawa et al., 1997). In addition, combined expression of

chitinase and a lipid transfer protein has been shown efficient against *B.cinerea* infection in transgenic carrot (Jayraj and Punja, 2007).

One of the mechanisms that plants utilize in defense against pathogens is the hypersensitive response leading to programmed cell death. The mechanism is effective against biotrophic bacteria. However, it has been shown that the hypersensitive response triggered by necrotrophic fungi, including *B. cinerea*, actually facilitates the colonization of the fungus utilizing the dead tissue (Govrin and Levine, 2000; Choquer et al., 2007). This has led to genetic engineering strategies that aim at reinforcing other plant defense mechanisms than the hypersensitive response. Attempts include evoking of salicylic acid and jasmonic acid mediated defense mechanisms by a harpin protein in tobacco (Sohn et al., 2007) and the expression of a cell death inhibitor protein (BAX inhibitor-1) in carrots (Imani et al., 2006). In addition to these strategies, the efficient production of plant phytoalexins like resveratrol by the transfer of stilbene synthase (*STS*) gene has been proven efficient in several cases, and is discussed in more detail in chapter 2.2.4.2.

2.1.2. Metabolic engineering of plant secondary metabolism

Although 200.000 different secondary metabolites are estimated to occur in plant kingdom, they all arise from a rather limited number of chemical scaffolds divided into three major groups: terpenoids, alkaloids and phenolic compounds, the last one being further divided into benzoates, central phenylpropanoids and flavonoids (Figure 1). The enormous diversity is based on substrate and/or regio-specific enzymes that decorate the molecular backbones with different chemical modifications by, e.g., hydroxylation, methylation, acylation and glucosylation (Dixon, 2001, 2005). Genetic modification of all major classes of secondary metabolites has been described (the flavonoid pathway is discussed in more detail in Chapter 2.2.).

The nitrogen-containing alkaloids (e.g., morphine, codeine, caffeine, nicotine, cocaine) have been exploited as pharmaceuticals, stimulants, narcotics and poisons. Many of the promising pharmaceuticals are still extracted from their natural plant sources. Alkaloid biosynthesis has also been the target of intensive metabolic engineering, mainly to increase the amount of the target compounds but also to conduct the whole biosynthesis in cell culture systems (Sato et al., 2001; Facchini, 2001).

Terpenoids form the largest group of plant natural products, consisting of over 40.000 compounds. Like alkaloids, terpenoids have pharmaceutical activity and include several important drugs against human ailments such as

cancer (taxanes) and malaria (artemisinin). Mass production of terpenoids by metabolic engineering is thus of great interest (Roberts, 2007). In addition, terpenes are used as flavour and colour enhancers. Plants also use terpenoids in their communication in the environment, and metabolic engineering has been applied, e.g., in *Arabidopsis* to produce a terpenoid that attracts natural enemies to herbivorous pests (Kappers et al., 2005).

Some compounds in the terpenoid pathway, necessary for cellular function and maintenance, are classified as primary metabolites, i.e., carotenoids, gibberellins, sterols and vitamins A and E (Roberts, 2007). Both qualitative and quantitative metabolic engineering of the carotenoid pathway has shown potential to enhance the nutritional quality of food. The 'golden rice' was first developed in 2000 (Ye et al., 2000) and an improved modification with higher provitamin A content was introduced five years later (Paine et al., 2005) to solve one of the serious malnutrition problems of people in developing countries.

Despite the fact that the outcome of genetic modification of carotenoid metabolism has often been unexpected it has given valuable information about the biosynthetic pathway (Sandmann et al., 2006; Galili et al., 2002; Chen et al., 2006). Indeed, a major hindrance in the engineering of secondary metabolism has been the lack of comprehensive understanding of the biosynthetic pathways. Similarly, knowledge of the regulation of the various pathways in the complex metabolic network is limited, and thus insertion of a single gene may have unpredictable effects on the metabolic balance of plant cellular systems (Dixon and Steele, 1999; Forkmann & Martens, 2001; Dixon, 2005). However, progress in molecular genetics and knowledge of plant metabolism will make targeted engineering of both primary (Carrari et al., 2003; Zimmermann and Hurrell, 2002) and secondary (Dixon, 2005) metabolism increasingly feasible. Moreover, as more holistic approaches are taken by systems biology (i.e., integrated transcriptomics, proteomics and metabolomics; see Chapter 2.3.3.), 'predictive metabolic engineering' - process where models are tested to understand the regulatory network of the modified pathway - can be introduced as part of experimental design (Sweetlove et al., 2003).

2.2. Biosynthesis of phenolic compounds

Phenolic compounds include, e.g., flavonoids, ellagitannins, coumarins, furanocoumarins, stilbenes, lignins and lignans (Figure 1). Some 9.000 flavonoids have been identified in higher plants up till now. They have diverse functions in the biochemistry, physiology and ecology of the plants

(Groteweld, 2006). They form the pigments in flowers and fruits, contribute to biotic and abiotic stress tolerance, provide UV shelter and have role in pollen fertility (Martens and Mithöfer, 2005; Winkel-Shirley, 2001). In the diet, flavonoids have health-promoting effects on humans and animals; scientific evidence exists for the antioxidative, antitumor, anti-inflammatory and antiatherosclerotic activities (Parr and Bolwell, 2000). The polyphenolic structure of flavonoids is ideal for radical scavenging activity, and flavonoids have been shown to be several times more effective antioxidants than, e.g., vitamins E and C (Rice-Evans et al., 1997).

2.2.1. General biosynthetic pathway of phenolic compounds

Flavonoids are products of the phenylpropanoid pathway. The central enzyme in phenylpropanoid metabolism that directs carbon from aromatic amino acids to the phenylpropanoids is phenylalanine ammonia-lyase (PAL), which forms cinnamic acid from phenylalanine. This step is regarded as the branch point between the primary metabolism (shikimate pathway) and the secondary metabolism which leads to various natural products including coumarins, phenolic acid esters, lignins and flavonoids (Figure 1; Dixon and Paiva, 1995; Dixon et al., 2002). The biosynthesis of flavonoids occurs in organized multienzyme complexes, 'metabolons', and the transport of flavonoids from the site of synthesis to final destinations such as vacuoles or cell wall, requires specific transferases and membrane transporters (Winkel-Shirley, 2001; Jørgensen et al., 2005).

All flavonoids are composed of flavan nucleus (Figure 2) (Schijlen et al., 2004; Martens and Mithöfer, 2005). Modifications on the central C-ring divide flavonoids to subgroups, e.g., flavanones, isoflavones, flavones, flavonols, flavanols and anthocyanins (Figures 1 and 2). The first enzyme committed to flavonoid biosynthesis is chalcone synthase (CHS), which condensates three acetate units from malonyl-CoA with *p*-coumaroyl-CoA. The resulting 4,2',4',6'-tetrahydroxychalcone (naringenin chalcone) is rapidly converted to naringenin by the enzyme chalcone isomerase (CHI) (Figure 1). These first two enzymes of the flavonoid pathway are found in plants almost ubiquitously. However, the enzymes that catalyze the subsequent steps of flavonoid pathway vary from one plant species to another, giving rise to different flavones, flavonols, anthocyanins and/or proanthocyanidins. For example, strawberry lacks flavonoid 3',5' hydroxylase (Figure 1: crossed) and, consequently, does not have any 5'-hydroxylated flavonoids, such as tricetin, myricetin, delphinidin or 5'-hydroxylated proanthocyanidins (Deng and Davis, 2001). Besides leading directly to anthocyanins, the flavonoid biosynthesis pathway has several side-

branches especially in the early steps, leading to the synthesis of products such as isoflavonoids, pterocarpans and aurones found in a limited number of plant species (Figure 1).
The majority of the key enzymes in the biosynthetic steps leading from one compound class to another along the flavonoid pathway are rather well characterized. Knowledge of the pathways is, nevertheless, continuously being updated and new enzymes and regulatory factors are being characterized. Similarly, in several cases where an enzyme has been assigned to a specific biosynthetic step, it has been later found to contain two or more enzymes responsible for different reaction steps. For example, the synthesis of pterocarpans was originally thought to be catalysed by vestitone reductase (VR) only, but was later shown to include also 7,2'-dihydroxy-4'-methoxyisoflavanol dehydratase (DMID) (Dixon and Paiva, 1995).

Besides the flavonoid biosynthesis enzymes themselves, also their regulation and association with plant biochemical status have been studied extensively. Especially the discovery of the Arabidopsis mutants *transparent testa glabra 1*, *glabrous* and *transparent testa*, deficient in different steps of the flavonoid biosynthesis, has led to the identification of several regulatory elements and the establishment of a model for complex interactions. The majority of the key proteins identified so far belong to the MYB- and bHLH-type transcription factors (Broun, 2005).

One major gap in the characterization of flavonoid biosynthesis remains in the proanthocyanidin (PA) biosynthesis. PAs are synthesized on a branch of the flavonoid biosynthesis pathway, where the leucoanthocyanidins are reduced to catechins by leucoanthocyanidin reductase (LAR) (Figure 1). Catechins serve as initiating units, to which flavan-3,4-diol units are sequentially added to form macromolecules of varying degree of polymerization and varying composition of different units. In spite of extensive research, the genes and enzymes responsible for PA biosynthesis and assembly after LAR catalysis are still mostly uncharacterized (Xie and Dixon, 2005). Overall, although some gaps still remain, modern genomics approaches should help to elucidate the remaining enzymes and regulatory factors, as well as to place flavonid biosynthesis in a more general context of plant metabolism (Winkel-Shirley 2001, 2002).

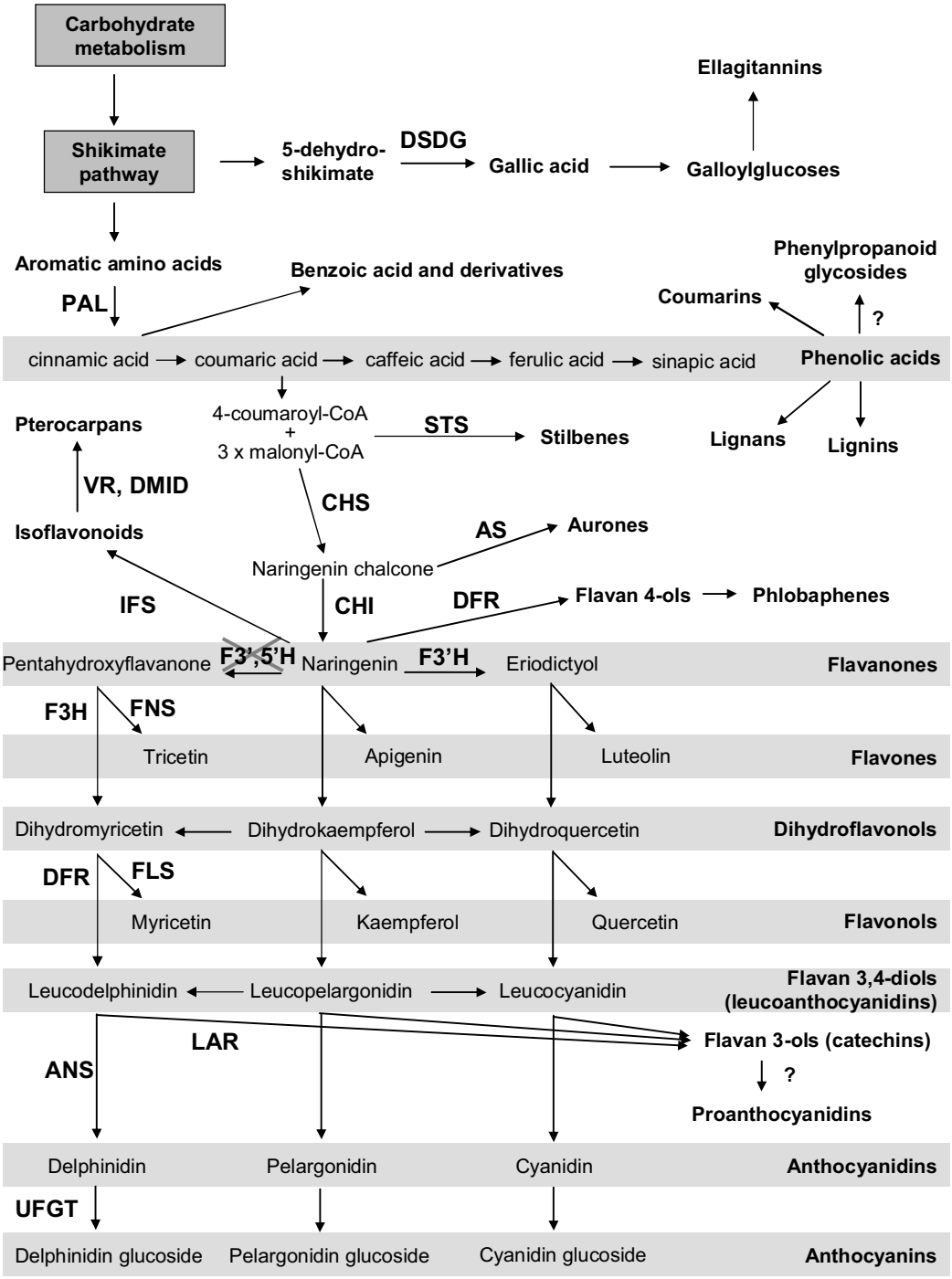

Figure 1. (legend on following page)

Figure 1. (previous page) Biosynthesis of phenolic compounds. PAL, phenylalanine ammonia-lyase; STS, stilbene synthase; CHS, chalcone synthase; CHI, chalcone isomerase; F3',5'H, flavonoid-3',5'-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3H, flavanone hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; ANS, anthocyanidin synthase; IFS, isoflavone synthase; DSDG, dehydroshikimate dehydrogenase; AS, aureusidin synthase; UFGT, UDP-glucose flavonol 3-*O*-glucosyl transferase; VR, vestitone reductase; DMID, 7,2'-dihydroxy-4'metohoxyisoflavanol dehydratase; LCR, leucoanthocyanidin reductase. The flavonoid pathway enzymes marked on the left side downstream from the compound pentahydroxyflavanone function also on the pathways downstream from naringenin and eriodictyol at the corresponding steps.

An interesting group of phenolic compounds that are not ubiquitously present in all plant genera are the gallo- and ellagitannins. They are polyphenols that are not generated through phenylpropanoid pathway, but result directly from the shikimate pathway via 5-dehydroshikimate precursor (Figure 1; Werner et al., 2004). Ellagitannins have been videly reported in the Rosaceae plants, including strawberry (Mullen et al., 2003; Hukkanen et al., 2007). The precursor for a plethora of complex ellagitannin molecules is penta-galloyl-glucose, which is synthetized from glucogallin by a series of strictly position-specific galloylation steps. Subsequent pathways leading to ellagitannins involve further galloylations and laccase-like phenol oxidation to form interlinkages between adjacent galloyl moieties within the ellagitannin structure. Especially oak (*Quercus*) and sumac (*Rhus*) plants have been studied for specific enzymes that catalyse the biosynthesis of ellagitannins (Niemetz and Gross, 2005; Gross, 2007).

2.2.2. Modification of the phenolic backbone

Although the biosynthesis of the backbone of phenolic compounds is among the most comprehensively studied secondary metabolism pathways in plants, the enzymes and regulators processing the various aglycones to different derivatized forms are largely unknown. The most common modifications are hydroxylation, decarboxylation, oxidation/reduction, methylation and addition of larger units such as sugars and acyl moieties. The purpose of the modifications is to fine-tune the functionality of each metabolite, and to allow the physiological control of each metabolite. For example, glycosylation of a flavonoid aglycone may serve as a signal to sequester the molecule in vacuoles until needed in other cellular compartments (Dixon, 2001, 2005). It is possible that each of the differentially modified forms of the same aglycone has a distinct role, since their distribution, e.g., among the different floral organs varies greatly, as was shown in this Thesis (**IV**). Similarly, the composition of flavonoid derivatives varies within a plant

family; characterization of flavonoids in three *Compositae* plants, carried out by LC/DAD-APCI/MS, revealed significant differences in the components and concentrations among the plants (Lai et al., 2007).

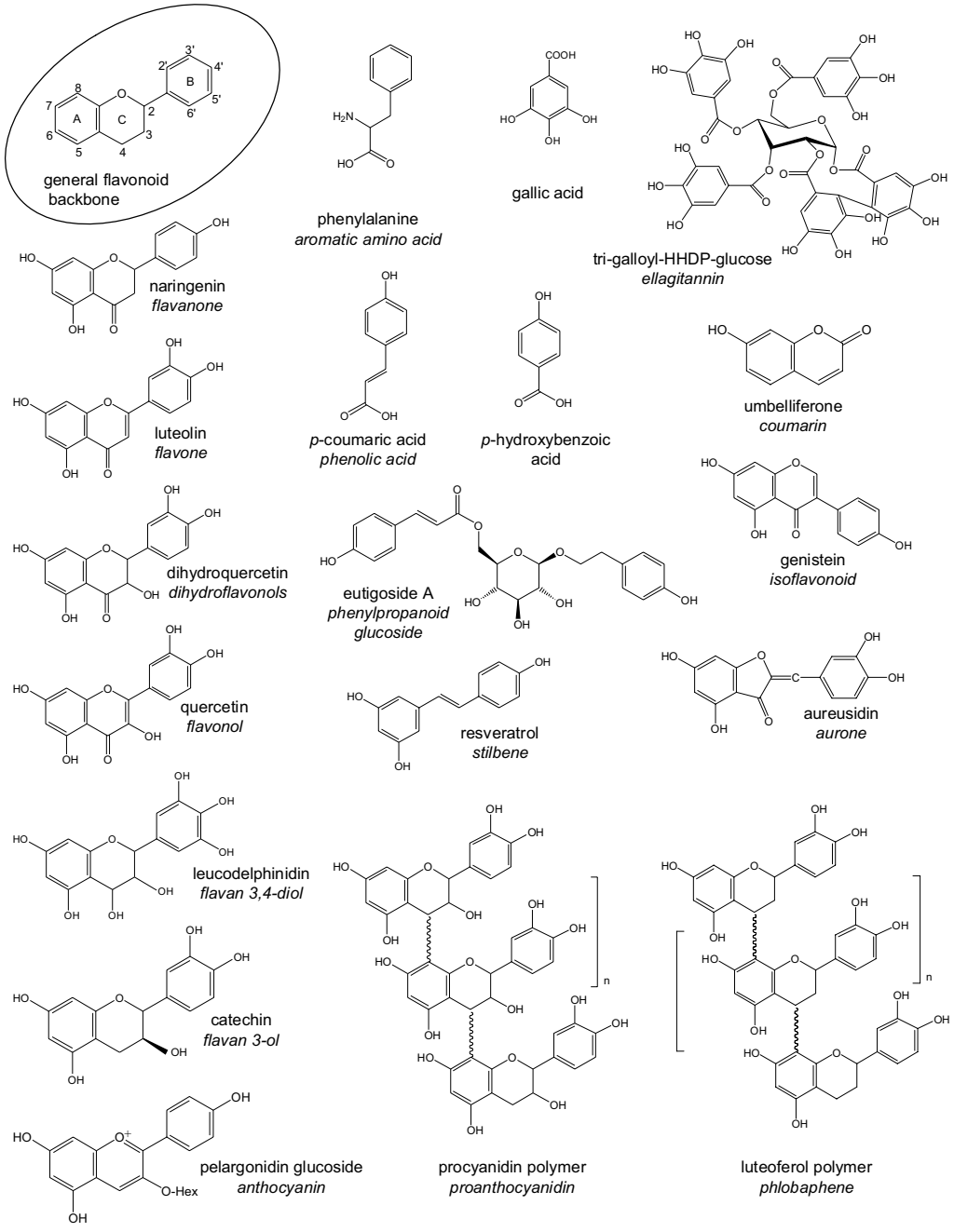

Figure 2. Examples of phenolic compounds. The pathway that produces phenolic metabolites, including those shown here, is depicted in Figure 1.

The enzymes that catalyze the decoration are specialized, both in terms of the aglycone specificity and the moiety used for its decoration. For example, it is estimated that more than 100 different glycosidic linkages are present in the metabolites of a multicellular organism (Keegstra and Raikehel, 2001). Also the repertoire of enzymes needed for glycosylation processes is enormous, and is currently divided into 69 families of glycosyltransferases (Lim and Bowles, 2004). Similarly, the acyltransferases show high catalytic versatility with defined substrate specificities. Acylation is a biochemically significant modification, because the added moieties are biochemically active, and e.g. anthocyanins, flavonols and phytoalexins are commonly acylated (D'Auria, 2006). Acylated flavonoids are important UV-B screening pigments; e.g. in Scots pine the flavonol-3-glycosides doubly acylated by hydroxycinnamic acids are the main UV-B absorbing compounds in the epidermal cell layer (Kaffarnik et al., 2006). Also the acylation of anthocyanins is common and known to contribute to their colour; mainly as more intensive blue colour (Honda et al., 2005). In addition, *in vitro* studies have shown that acylation of flavonoids enhances their antioxidant and antimicrobial capacity (Mellou et al., 2005).

The versatility and exquisite substrate and site specificity of flavonoid modifying enzymes is under investigation in several laboratories. Specialized roles have been assigned for, e.g., flavonol diglycosides which are essential in pollen production of petunia (Vogt and Taylor, 1995), and a specialized enzyme controlling the pollen-specific flavonol glycosylation has been reported (Miller et al., 1999). Other modifying enzymes found to be flavonoid-specific include two glycosyltransferases that link glucose and rhamnose units to distinct positions of flavonols in *Arabidopsis* (Jones et al., 2003), a UDP-glucose:cinnamate glycosyltransferase expressed in the ripening fruit of strawberry (Lunkenbein et al., 2006), and several acyltransferases that perform specific anthocyanin acylations in *Arabidopsis* (Luo et al., 2007).

2.2.3. Genetic modification of the flavonoid biosynthetic pathway

The first report on genetic modification of the flavonoid biosynthetic pathway was published in 1987. The anthocyanin biosynthesis of petunia was modified by a gene from maize enabling pelargonidin-type anthocyanin production (Meyer et al., 1987). This study was followed by two studies on petunia describing the unexpected silencing of the endogenous homologous gene by an additional chalcone synthase (*CHS*) copy, resulting in the loss of flower colour (Napoli et al., 1990) and similar effect was seen also with *CHS* and dihydroflavonol-4-reductase (*DFR*) gene transfer (van der Krol et al.,

1990). Since then, several other studies on the influence of *CHS* silencing on flower pigmentation have been published (Que et al., 1998; Metzlaff et al., 1997; Fukusaki et al., 2004). Besides their role in flower colour, CHS-deficient plants have revealed the importance of flavonoids in pollen development and plant reproduction (Mo et al., 1992; Ylstra et al., 1994; Schijlen et al., 2007). As the key enzyme in the entry to the flavonoid pathway, CHS is the most extensively modified enzyme in the pathway. Nevertheless, also other steps of the pathway have been modified, including the regulation by transcription factors, enhancement of isoflavonoid production, modification of lignin content, and introduction of whole new pathway branches (for reviews, see Dixon and Steele, 1999; Forkmann and Martens, 2001; Schiljen et al., 2004; Dixon, 2005).

For commercial purposes, the most extensively applied area in the modification of flavonoid metabolism is the creation of novel flower colours to existing cultivars (Forkman & Martens, 2001; Tanaka et al., 2005); examples of such flowers, commercially available also within the European Union (Florigene Moonshadow™, Moondust™ and Moonlite™), are carnations modified by adding two genes of the anthocyanin biosynthetic pathway (Tanaka et al., 2005). While similar genes were added to the three commercial varieties, they all have different flower colour shade. Intensive work has also been made on the genetic modification of roses. Rose with blue flower was eventually achieved by several modifications of the anthocyanin biosynthesis that led to delphinidin derivatives (Katsumoto et al., 2007). Metabolic engineering provides also a means to modify both flavonoid content and composition in food plants (Dixon and Steele, 1999; Forkmann and Martens, 2001). As an example, flavonoid-enriched tomatoes have been developed, and their positive effect on health has been shown as reduced cardiovascular risk markers in experimental animals (Rein et al., 2006).

2.2.4. Polyketide enzymes in phenolic metabolism

A group of enzymes that form carbon-carbon bonds using the Claisen condensation are called polyketide synthases, and they belong to a larger thiolase superfamily of enzymes (Haapalainen et al., 2006). Type III polyketide synthase family encompasses a structurally and functionally related, yet highly versatile group of enzymes and include, in addition to CHS, other enzymes that lead to different polyketide varieties; examples of such enzymes are stilbene synthase (STS, Austin et al., 2004), 2-pyrone synthase (Eckermann et al., 1998), bibenzyl synthase (Preizig-Müller et al., 1995), acridone synthase (Junghanns et al., 1995) and valerophenone synthase (Okada and Ito, 2001). In plants, polyketide synthases play

important roles in the synthesis of natural products such as phytoalexins. The common catalytic mechanism of the polyketide enzymes involves CoA-ester from the phenylpropanoid pathway as a substrate, elongation by condensation reactions with acetate units from malonyl-CoA, and folding and aromatization of the polyketide intermediate. As a result, a number of products are formed, such as naringenin chalcone with CHS, and resveratrol with STS (Austin and Noel, 2003; Watanabe et al., 2007; Figure 2). The wide variety of end products of type III polyketide synthases is due to their different preference for phenylpropanoid substrates, the number of condensation reactions performed and differences in the folding mechanism (Morita et al., 2001).

2.2.4.1. Stilbene synthase and its product resveratrol

STS functions in the branch point after phenylpropanoid metabolism, and utilises the same substrates as CHS in catalyzing the synthesis of resveratrol (trans-3,5,4'-trihydroxystilbene) (Figures 1 and 2). *STS* gene is activated by external stimuli, like UV-radiation or pathogen challenge, and contributes to the general defence mechanism through the stilbene-type phytoalexins. Closely related enzymes that synthesize slightly different products such as pinosylvin synthase catalyzing the synthesis of pinosyvin have been identified (Jeandet et al., 2002). Unlike CHS, which is present virtually in all higher plants, STS is restricted to a relatively few genus such as grapevine (Vitaceae), peanut (Cyperaceous) and pine (Pinus), although closely related enzymes are being characterized and the number of plant species containing stilbene-related compounds is steadily increasing (Eckermann et al., 2003, Morita et al., 2001). The amount of stilbene compounds characterized exeeds the number of STS enzymes being studied so far.

Resveratrol is one of the most intensively studied human health-promoting natural products. The best known dietary sources of resveratrol are grapes and derived products like red wine, as well as blueberry, cranberry, mulberry and peanuts (Aggarwal et al., 2004). As plants are being explored more widely, new sources for this health-beneficial compound are continuously reported; most recently, low amounts of resveratrol have been reported to be present in tomato (Ragab et al., 2006) and strawberry (Wang et al., 2007).

The cardioprotective and anticancer properties of resveratrol have been extensively studied (Gusman et al., 2001; Aggarwal et al., 2004). The relationship between moderate consumption of red wine and lowered incidence of cardiovascular diseases, "the French paradox", is believed to be at least partly due to the radical scavenging activity of resveratrol (Ulrich et

al., 2005). Recently, the ability of resveratrol to extend the lifespan of diverse species, including mice on a high caloric diet was reported, suggesting a novel application for resveratrol in treating obesity-related disorders (Baur et al., 2006).

2.2.4.2. Modification of stilbene synthase expression in plants

The positive effects of resveratrol on both plants and humans have inspired efforts to increase its content or to produce it *de novo* by genetic modification. Since STS uses the same precursor metabolites as CHS, introduction of the gene should be quite straightforward and is probably the most widely applied strategy for the modification of plant secondary metabolism. After the hallmark study of Hain and co-workers in 1993 (Hain et al., 1993), in which the production of resveratrol was demonstrated in tobacco, *STS* gene has been introduced into a wide range of plant species. Using the inducible promoter of *STS* or cauliflower mosaic virus 35S promoter, different outcomes have been demonstrated (Table I). The most common source of *STS* has been the grapevine *Vitis vinifera*, but also other *STS* genes have been used such as those from Concord grapevine *Vitis labrusca,* frost grape *Vitis riparia* (Kobayashi et al., 2000) and silvervein creeper *Parthenocissus henryana* (Liu et al., 2006), and the gene encoding pinosylvin synthase of Scots pine *Pinus sylvestris* (Seppänen et al., 2004). In addition to endogenous STS and 35S promoters, e.g., PR-10 (Coutos-Thévenot et al., 2001) and ubquitin promoters (Fettig & Hess, 1999) have been used to control *STS* expression. Typically the most successful applications of *STS* gene transfer have been those in which the gene is transferred with its endogenous promoter, and resistance against pathogens like *Botrytis cinerea* (Hain et al., 1993; Leckband and Lörz, 1998), *Phytophtora infestans* (Thomzik et al., 1997), *Phytophtora palmivora* (Zhu et al., 2004) and *Puccinia recondite* (Serazetdinova et al., 2005) was demonstrated (Table I). The outcome of the genetic modification is, nevertheless, highly dependent on the host plant in question, as for example *STS*-modified kiwifruit showed no improvement in resistance against grey mould (Kobayashi et al., 2000), even though improved resistance to the same fungus was achieved in wheat by using the same approach (Leckband and Lörz, 1998).

Table I. Modification of stilbene synthase expression in plants

Recipient plant	Donor of transgene	Promoter	Observations	Ref.
tobacco	*V. vinifera*	VST	increased resistance to *Botrytis cinerea*	Hain et al., 1993
rice	*V. vinifera*	VST	increased resistance to *Pyricularia oryzae* (rice blast)	Starc-Lorenzen et al., 1997
tomato	*V. vinifera*	VST	accumulation of trans-resveratrol, increased resistance to *Phytophtora infestans*, but not to *Botrytis cinerea*, *Alternaria solani*	Thomzik et al., 1997
tobacco	*V. vinifera*	35S	altered flower morphology, male sterility	Fisher et al., 1997
wheat	*V. vinifera*	VST	increased resistance to *Botrytis cinerea*	Leckband and Lörtz, 1998
wheat	*V.vinifera*	ubiquitin	detection of resveratrol	Fettig and Hess, 1999
wheat	*V. vinifera*	enhanced VST	increased resistance to powdery mildew	Liang et al., 2000
alfalfa	*Arachis hypogaea*	35S	accumulation of piceid, increased resistance to *Phoma medicagnis*	Hipskind and Paiva, 2000
grapevine	*V. vinifera*	PR10	accumulation of resveratrol, increased tolerance to *Botrytis cinerea*	Coutos-Thevenot et al., 2001
apple	*V. vinifera*	VST	accumulation of unidentified resveratrol-glycoside	Szankowski et al., 2003
kiwi	*V. vinifera, V. labrusca and V. riparia*	VST	detection of piceid, but no increase in resistance to *Botrytis cinerea*	Kobayashi et al., 2003
wheat	*V. vinifera, Pinus sylvestris*	VST	detection of unknown stilbenes, increased resistance to *Puccinia recondita*	Serazetdinova et al., 2005
poplar	*V. vinifera*	35S	detection of piceid, no increase in resistance to *Melampsora pulcherrima*	Giorcelli et al., 2004
aspen	*P. sylvestris* (pinosylvin synthase)	4x35S	increased resistance to *Phellinus tremulae*, accumulation of stilbenes not detected	Seppänen et al., 2004

papaya	*V. vinifera*	VST	increased resistance to *Phytophtora palmivora*	Zhu et al., 2004
Brassica napus seeds	*V. vinifera*	napin	accumulation of piceid, no difference in other agronomic traits	Husken et al., 2005
tomato	*V. vinifera*	35S	increased antioxidant activity	Giovinazzo et al., 2005
pea	*V. vinifera*	VST	inducible resveratrol accumulation	Richter et al., 2006
apple fruit	*V. vinifera*	VST	accumulation of piceid in the fruit	Ruhmann et al., 2006
Arabidopsis	*Sorghum bicolor*	35S	accumulation of *cis*-resveratrol	Yu et al., 2006
lettuce	*Parthenocissus henryana*	35S	detection of resveratrol	Liu et al., 2006
hop	*V. vinifera*	35S	accumulation of *trans*- and *cis*-piceid, resveratrol and astringin	Schwekendiek et al., 2007

VST, promoter of *Vitis* stilbene synthase, i.e., the endogenous, inducible promoter of the gene

2.3. Plant metabolomics

2.3.1. Metabolomics: definition and application

The technical landmark in the analysis of metabolite contents of different matrices was the development of chromatographic separation methods, which enabled the initial detection of individual metabolites (Unger, 2004; Fritz 2004). Historically, the analysis of metabolites has been focused on small group of compounds, like in the studies on glycolysis and respiration. As the knowledge about novel compound classes and new metabolite pathways has increased, it has been realized that metabolic pathways do not act in isolation, but comprise extensive networks, which sets the need for more comprehensive approaches (Ryan and Robards, 2006; Fiehn, 2002). Today, the term metabolomics is used in parallel with transcriptomics and proteomics that are focused on mRNA and protein profiling, respectively, and is characterized as the systematic quantitative and qualitative study of the complement of metabolites in a particular biological system. Similarly, as transcriptome and proteome define the mRNA and protein complement of the system, the set of metabolites constitute its metabolome. The terminology related to metabolite analysis is diverse, and different terms have been used interchangeably. The most common terms used in current literature are listed in Table II (Dettmer et al., 2007; Hall, 2006; Krishnan et al., 2005; Seger and Sturm, 2007).

Table II. Metabolomics related terminology

Term	Definition
Metabolite	Small molecules that participate in general metabolic reactions and are required for the maintenance, growth and normal function of a cell
Metabolome	The complete set of metabolites in an organism
Metabolomics	Unbiased identification and quantification of all metabolites in a biological system, or a complete set of metabolites in a cell or tissue type
Metabonomics	A term not used in plant science, although sometimes used interchangeably with metabolomics. Mostly used in disease diagnostics and toxicology as the quantitative measurement of the dynamic response of living systems to pathophysiological stimuli or genetic modification
Metabolite (or metabolic) profiling	Quantitative analysis of a set of metabolites in a selected biochemical pathway or a specific class of compounds; biased/targeted approach which addresses only limited number of often structurally closely related analytes due to methodological limitations of the analytical platforms used
Metabolic fingerprinting	High-throughput, rapid global analysis; unbiased global screening approach to classify samples based on metabolite patterns or "fingerprints" that change in response to disease, environmental, or genetic perturbations, not necessarily involving analyte identification and quantification
Metabolic footprinting	Fingerprinting analysis of extracellular metabolites in cell culture medium as a reflection of metabolite excretion or uptake by cells

An alternative approach in the terminology of metabolite-related research avoids the omics-related buzzwords and clearly divides the metabolite analysis into targeted analysis (biased identification and quantification), and metabolite (or metabolic) profiling (screening the metabolite profile of the analyst with selected approach), and is used also in this Thesis (Seger and Sturm, 2007; Villas-Boas et al., 2005). In a concept where metabolite profiling is discussed as part of the understanding of plant metabolism in general, it is often referred to as 'metabolic phytochemistry' (Sumner et al., 2003; Petersen, 2007; Fernie, 2007).

The importance of metabolites lies in their fundamental role as regulatory components of the biochemical pathways, and as end products of various cellular processes. The information flow from DNA sequence over messenger RNA to proteins usually leads to the synthesis of metabolites catalyzed by the proteins. Metabolite profiling can give an instantaneous snapshot of the physiological condition of the target and is thus the most predictive one among the various profiling levels (Seger and Sturm, 2007; Ryan and Robards, 2006; Fernie et al., 2004).

Plant metabolomics is a particularly interesting field of research not least because plants have the ability to synthesize an enormous diversity of secondary metabolites; current estimation of the number of different compounds present in plant kingdom is 200.000 or higher. Behind the diversity of plant chemicals is the fact that plants are, among all living organisms on earth, unique in several aspects; they are sessile autotrophs that are obliged to respond chemically to survive in continually changing and often hostile environment, as well as to allure pollinators and seed dispersals for their reproduction (Saito et al., 2006; Hall, 2006). Further interest in plant metabolomics arises from the fact that several important attributes significant for human health also originate from plant metabolites, such as food quality (e.g., carotenoid content; Fraser and Bramley, 2006) and drug production (e.g., taxol-producing suspension cultures; Ketchum and Croteau, 2006).

Metabolomics is currently one of the fastest growing areas in plant science, and is most likely also the field where major scientific breakthroughs remain to be made. Perspective to this can be drawn from the fact that even though *Arabidopsis thaliana* genome has been completely sequenced, over 30% of its genes still lack functional classification, and metabolomics is expected to play a key role in the elucidation of the whole molecular phenotype (*Arabidopsis* genome initiative, 2000; Fiehn, 2002). Plant kingdom still holds unexplored resources, which can be revealed by “bioprospecting“ for pharmaceutically active compounds from wild endemic species. In order to be able to exploit the natural resources efficiently but soundly, a comprehensive picture of plant metabolism is needed, implying a better coverage of pant metabolome as well as a better understanding of its function; these are tasks where metabolomics plays an essential role (Fernie, 2007).

2.3.2. Analytical technologies in plant metabolomics

2.3.2.1. Sample preparation

Delicate sample preparation is the most important step in metabolite analysis. Sample types encountered in plant metabolite research include versatile types of tissues extending from soft fruit to rigid root material. Like the sample type, also the compounds to be analysed and the analytical tools of choice determine the extraction method and the solvent to be used, and need to be adapted on a case-by-case basis (Seger and Sturm, 2007). Factors having the most significant effect on metabolite profiling with gas-chromatography-mass-spectrometry (GC-MS) were evaluated on sample preparation of potato tubers (Shepherd et al., 2007). In addition to reaction conditions used to prepare the GC-MS-detectable derivatives, also sampling methods had effect on the results. The use of freeze-dried material gave highly linear and repeatable results

2.3.2.2. Analytical platforms

Recent advances made in the tools applied for metabolite analysis has paved the way for simultaneous monitoring of multitude of targets and enabled the development of the metabolomics concept. Ample number of detailed reviews on various aspects of metabolite analysis is available (Seger and Sturm, 2007; Dettmer et al., 2007; Glinski and Weckwerth, 2006; Dunn et al., 2005; Villas-Boas et al., 2005). Different mass-spectrometric (MS) technologies are currently the key detection methods for small molecules (Dunn et al., 2005). Critical instrumental parameters are 1) the ionization technology, i.e., electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption/ionization (MALDI) or fast atom/ion bombardment (FAB); and 2) the type of the mass analyzer, i.e. quadrupole, triple-quadrupole, ion-trap or time of flight (TOF) (Villas-Boas et al., 2005). The samples can be analyzed directly by MS or may be separated by different chromatographic techniques; the choice of method depends on the metabolites of interest. GC-MS has the longest history in metabolite detection, providing the advantage of well-established protocols and instrumental set-up as well as chromatogram evaluation and interpretation (Fernie et al., 2004). GC-MS has a very high separation efficiency that can resolve complex biological volatile mixtures. Sample preparation for GC-MS may involve solvent-free preparation by, e.g., headspace sampling, or requires volatile solvent and derivatization techniques to increase analyte volatility (Seger and Sturm, 2007; Dettmer et al., 2007). Liquid-chromatography coupled MS (LC-MS) allows the separation and detection of

different groups of metabolites according to the column and eluent applied, and thus enables to study versatile metabolite classes in single analysis. When accompanied by targeted purification and chromatographic optimization it is suitable for accurate measurement and classification of a particular metabolite class present even in a very complex matrix (Dunn et al., 2005). The column technology for the liquid phase separation is also rapidly evolving, and the performance of the columns is moving from the high (HPLC) to ultra-high (UPLC) level. The advantage of UPLC is the enhanced retention time reproducibility and better separation performance accompanied with faster chromatographic runs. The method has been applied successfully to measure plant-derived metabolites (Chan et al., 2007) and was used also in the present Thesis (**III, IV**). Capillary-electrophoresis-mass-spectrometry (CE-MS) is a highly sensitive method that can detect low-abundance metabolites with good analyte separation, and potentially will be applied in metabolomics studies in the future; until now the research with CE-MS has focused on the optimization of operational parameters (Villas-Boas et al., 2007; Dunn et al., 2005). Fourier-transform-ion-cyclotron-resonance-MS (FTICR-MS) is a mass spectrometric method that relies solely on extremely high mass accuracy detection, allowing the determination of empirical formulae for thousands of metabolites. The pitfall of the method is, however, the lack of chromatographic separation, which renders it incapable of discriminating between metabolite isomers (Fernie et al., 2004). Nuclear-magnetic-resonance spectroscopy (NMR) is one of the most widely applied methods to study organic molecules, and has been used for over 20 years for metabolomics research. It is a non-destructive method and offers a variety of detection schemes, being highly versatile and adjustable technique for any chemical analysis. NMR is the ultimate tool to unravel the identity of a compound (Ward et al., 2007; Krishnan et al., 2005; Dunn et al., 2005), and was also used in the present Thesis (**III**).

In spite of the extensive development of analytical methods for metabolome research, the ultimate goal of plant metabolomics, i.e. gaining a complete overview of the metabolite complement of a plant in one or small series of analyses is currently inconceivable. Sample preparation technologies and analytical tools do not yet meet the demands, but continuous improvement in the technologies, including combined platforms of NMR and GC or LC-MS, should bring further advances in the field (Hall, 2006; Ryan and Robards, 2006).

2.3.2.3. Data processing

Multiple experimental platforms applied in metabolomics research generate complex data matrices which involve laborious handling and have great impact in the extent and quality of interpretation of the analytical results (Katajamaa & Oresic, 2005, 2007). A typical LC-MS raw dataset contains information about recorded histograms from the mass detector, including retention time, *m/z* value and ion intensity for each signal. Several processing steps such as filtering, feature detection, alignment and normalization need to be applied to purify and condense the data so that they are accessible for data mining tools and multivariate analyses such as clustering, or for discovering important differences between groups of samples. Bioinformatics and biostatistics are among the most essential disciplines in metabolome research, and automated computational techniques and algorithms are currently being developed to aid in metabolite identification, quantification and differential profiling. Furthermore, as increasing amounts of data accumulate, there is a need for standards in order to keep the data accessible and comparable between the laboratories. A metabolomics standards initiative has been taken by the metabolomic society, and minimal reporting standards have been recently proposed on metabolomics data (Fiehn et al., 2007). The accumulating information on plant metabolomics also necessitates the organization of metabolic information into currently accessible databases, and initiatives are taken both by individual laboratories as well as broader scientific community, including the KEGG (Kanehisa et al., 2002), KNApSAcK (Shinbo et al., 2006) and MoTo databases (Moco et al., 2006).

2.3.3. Metabolomics as part of functional genomics and systems biology

Metabolite profiling is increasingly used in connection with other methods such as transcriptomics and proteomics, and it has become a key tool for the overall understanding of biological systems. When applied together with other profiling methods, metabolomics provides a means to resolve interactions among various pathways (Sumner et al., 2003; Bhalla et al., 2005; Schauer and Fernie, 2006). Combination of metabolomics with functional genomics has recently been applied to find the link between genes and their function in plant metabolic network (Hall et al., 2002; Bino et al., 2004; Fiehn et al., 2000; Goossens et al., 2003). For a comprehensive understanding of the behaviour of cellular systems, the response to perturbations need to be measured and combined at all levels of expression, through transcriptomics, proteomics to metabolomics; i.e. at systems biology level (Bino et al., 2004; Weckwerth, 2003).

A great challenge is the computational combination of measurements at different biological levels including transcriptomics, proteomics and metabolomics for the interdisciplinary approach. The full interpretation of global datasets is unfeasible for science today, but efforts on organizing hierarchical networks and integrating information for both bottom-up (reductionistic) and top-down (holistic) approaches are undertaken (Lange, 2006; Morgenthal et al., 2006; Damian et al., 2007).

In addition to the limitations in computational handling, the current analytical applications are not yet at their full potential for systems biology purposes, but are merely in the level of feasibility studies and hardware setup. Nevertheless, the potential of current technologies to fulfill the aim of comprehensive understanding of biological processes is impressively proven (Seger and Sturm, 2007).

2.3.4. Metabolomics in the context of genetic modification

Targeted analysis is usually the method of choice in the characterization of GM plants, as the direct effect of the transgene is studied at the metabolite, RNA and/or protein level. A targeted metabolite analysis was carried out on GM potato tubers that had been altered in primary carbohydrate metabolism, polyamine biosynthesis and glycoprotein processing (Shepherd et al., 2006). No consistent differences were revealed between GM and parental lines, when soluble carbohydrates, glycoalkaloids, vitamin C, total nitrogen, fatty acids and trypsin inhibitor were assayed. Results on non-targeted metabolite profiling of GM plants are also emerging. Roessner et al. (2001) showed that metabolic profiling may be used together with other profiling techniques for genotyping of GM potato lines altered in sucrose metabolism. Defernez and co-workers (2004) described metabolite profiles of 40 GM potato lines modified in primary carbon metabolism, starch synthesis, glycoprotein processing or polyamine/ethylene metabolism. The main differences in NMR and HPLC-UV profiles between the GM and parental varieties were found in polyamine metabolism. Metabolite profiles of GM and parental wheat cultivars were analyzed by NMR and GC-MS in a study where substantial equivalence was determined (Baker et al., 2006). Metabolic changes in spermidine and spermine accumulating GM tomato line have been studied in extensive metabolite profiling by NMR (Mattoo et al., 2006). Also flavonoid enriched GM-tomatoes have been studied by LC/NMR and LC/MS metabolite profiling, revealing 10-fold increase in flavonoid glycosides in two GM lines (Le Gall et al., 2003a).

Metabolite profiling may elucidate also differences in metabolome beyond those intended to be achieved by incorporation of the transgene, e.g. those resulting from integration site or somaclonal variation that takes place during the *in vitro*-regeneration process (Filipecky and Malepszy, 2006). Such variation has been demonstrated e.g. on potato tubers, as differences in individual compounds between parental and vector-control, as well as between non-transformed cell-culture-derived lines were found (Shepherd et al., 2006). Similarly, NMR metabolite profiling study of GM tomatoes revealed significant changes other than those anticipated from the gene transfer, although the changes were determined to be within natural variation of the field-grown crop (Le Gall et al., 2003b).

In addition to the integration site-specific changes and somaclonal variation, genetic modification often results in pleiotropic effects that limit the usefulness of the technology, and are encountered especially when secondary metabolism is modified (Filipecki & Malepszy, 2006). Pleiotropic effects shown so far include, e.g., silencing of the flavonoid pathway eventually leading to male sterility (Napoli et al., 1990, Van der Meer et al., 1992). Metabolite profiling can, however, facilitate the interpretation of the effects of the genetic modification, as has been demonstrated, e.g., in GM potato profiling (Defernez et al., 2004), in NMR analysis of GM peas (Charlton et al., 2004) and in the present Thesis (**III**). One of the reasons for unexpected effects is that the modified pathways are still insufficiently characterised, which does not support predictive metabolic engineering (Sweeltove et al., 2003; Trethewey, 2004).

Metabolite profiling has even been suggested as an integral part of the assessment of the safety of foods derived from GM crops (Kuiper et al., 2001) in addition to the extensive test regime which includes careful monitoring of the key nutrients, toxins, allergens, anti-nutrients and biologically active substances known to be associated with the crop (König et al., 2004).

2.3.5. Application of metabolomics in agriculture

Metabolomics, preferentially as a part of comprehensive systems biology approach, has the potential to aid in trait development through breeding. Comprehensive unbiased metabolite profiling of complex plant material by the most commonly applied methods, i.e. GC-MS (Tikunov et al., 2005), LC-MS (von Roepenack-Lahaye et al., 2004) and NMR (Mattoo et al., 2006) has proven feasible and, if applied in parallel, provides a rather complete picture of the composition of food and feed products and may thus be applied to guide the improvement of diet and health (Dixon et al., 2006).

2.3.6. Profiling of strawberry secondary metabolites

Strawberry (*Fragaria* × *ananassa*) is among the most lucrative agricultural crops worldwide and its consumption has doubled since 1980 (FAOSTAT database). Like several other fruits including apples, pears, plums, peaches and raspberries it belongs to the subfamily Rosoideae of the Rosaceae family. These fruits are known to have exceptionally rich secondary metabolite composition and, in particular, the phenolic composition is well studied in several Rosaceae fruits, including strawberry (Koponen et al., 2007; Puupponen-Pimiä et al., 2006; Hannum, 2004). In addition to flavonoids, anthocyanins and other widely occurring phenolic compounds, remarkable amounts of ellagic acid and ellagitannins are present in plants of Rosaceae family (Kähkönen et al., 2001). Because the fruit is rich in bioactive phytochemicals, especially phenolic compounds with high antioxidant capacity, strawberry as a part of daily diet could be beneficial for human health (Hannum, 2004).

Strawberry contains a wide array of phenolic compounds such as phenolic acids, flavonols (kaempferol and quercetin derivatives), anthocyanins (cyanidin and pelargonidin derivatives), proanthocyanidins, galloylglucoses and ellagitannins (Table III). The metabolite composition varies during fruit development (Halbvirth et al., 2006; Kosar et al., 2004; Aharoni et al., 2002). The comopsition is different in fruit flesh and achenes (Aaby et al., 2005), and achene content also contributes to the total phenolic compound content of strawberry purees (Aaby et al., 2007b). Elevated ozone (Oertel et al., 2001), pathogen challenge (Terry et al., 2004) and other preharvest conditions (Wang et al., 2007; Hukkanen et al., 2007) influence the metabolite composition.

Several metabolite profiling studies, including identification by LC-MS, have been published both on strawberry fruit and leaves (Aaby et al., 2007a; Hukkanen et al., 2007; Seeram et al., 2006; Määttä-Riihinen et al., 2004). Strawberry metabolites have also been subjected to the new metabolite profiling techniques such as fourier transform ion cyclotron mass spectrometry, FTMS (Aharoni et al., 2002) and colloidal graphite-assisted laser desorption/ionization MS, GALDI (Zhang H et al., 2007). Specific compound classes have been characterised in strawberry, such as phenolic acids (Mattila & Kumpulainen, 2002), ellagitannins (Okuda et al., 1992; Cerda et al., 2005), anthocyanins (Nyman & Kumpulainen 2001; Lopes-da-Silva et al., 2002; Koponen et al., 2007), proanthocyanidins (Gu et al., 2003) and flavonols (Häkkinen & Auriola, 1998). Several studies have concentrated on one or a few strawberry metabolites identified by NMR and include compounds phlorizin (Hilt et al., 2003), 1-*O*-trans-cinnamoyl-*β*-D-

glucopyranose (Latza et al., 1996), E-cinnamic acid derivatives (Cheel et al., 2005), ellagic cid derivatives (Heur et al., 1992), cytochrome P450 inhibitors (Tsukamoto et al., 2004), 5-carboxypyranopelargonidin (Andersen et al., 2004), anthocyanin-flavan-3-ol metabolites (Fossen et al., 2004) and taxifolin 3-arabinoside (Ishimaru et al., 1995). A comprehensive list of strawberry metabolites is presented in Table III. The new information gained on strawberry flower and leaf metabolite composition in the original studies **III** and **IV** of this Thesis are also included in the table.

Table III. Secondary metabolites of strawberry reported in the literature and in this Thesis.

COMPOUND	MW	UV	organ	ref
Benzoic acid derivatives				
benzoic acid	122		Fr	6
hydroxybenzoylhexose	300	262	Fr	2
hydroxybenzoic acid	138		Fr	6
vanillic acid	168		Fr	6
dihydroxybenzoquinone	140		Fr	6
Cinnamic acid derivatives				
p-coumaric acid glucoside	326	264, 293	Fr, F, L	1, **III, IV**
p-coumaroyl hexose	326	236, 300sh,310	Fr	1, 2, 3, 5
p-coumaroylhexose-4-*O*-hexoside	488	312	Fr	2
p-coumaroyl-ester	356	235, 330	Fr	3
di-coumaroyl hexose	472		F	**IV**
caffeoylglucose, caffeic acid hexose	342	264, 300sh, 330	Fr, F	1, **IV**
caffeate	180		Fr	6
ethyl cinnamate	176		Fr	6
methyl cinnamate	162		Fr	6
hydroxyferulate	210		Fr	6
4-coumarate	164		Fr	6
sinapyl alcohol	210		Fr	6
cinnamate glucose	310		Fr	6
cinnamoyl-xylopyranoside	280	284	Fr	10
cinnamoyl-rhamnopyranoside	294	284	Fr	10
cinnamoyl-xylofuranosyl-glucopyranose	442	284	Fr	10
cinnamoyl-glucopyranoside		287	Fr	14
chlorogenic acid	354	sh-323	F, L	**III, IV**
ferulic acid hexose	356	sh-328	F	**IV**
galloyl caffeoyl hexose	494	252, 367	F	**IV**
galloyl coumaroyl hexose	478		F	**IV**
coumaroyl quinic acid	338		F	**III**

Phenylpropanoid glucosides				
eutigoside A				
2-(4-hydroxyphenyl)ethyl-(6-O-(E)-coumaroyl)-β-D-glucopyranoside	446	311	L	**III**
2-(4-hydroxyphenyl)ethyl-(6-O-(Z)-coumaroyl)-β-D-glucopyranoside	446		L	**III**
4-(2-hydroxyethyl)phenyl-(6-O-(E)-coumaroyl)-β-D-glucopyranoside	446		L	**III**
Grayanoside A;				
2-(4-hydroxyphenyl)ethyl-(6-O-(E)-feruloyl)-β-D-glucopyranoside	476		L	**III**
2-(4-hydroxyphenyl)ethyl-(6-O-(E)-caffeoyl)-β-D-glucopyranoside	476		L	**III**
Gallic acid, ellagic acid				
ellagic acid 4-pentoside	435	252, 362	Fr	1
ellagic acid pentoside	434	254, 360	Fr, L, F	2, 5, **IV**
ellagic acid	302	252, 368	Fr, L, F	1, 2, 3, 5, 12, **IV**
ellagic acid acetylpentoside	476	254, 358	Fr, L	1
ellagic acid deoxyhexoside	448	254, 362	Fr, L, F	2, 5, **IV**
methyl-ellagic acid pentose	448	250, 370	Fr	3
ellagic acid hexose	464	n.a.	L, F	5, **IV**
glucogallin, galloylglucose	332	276	Fr, F, L	6, **III, IV**
galloylquinic acid	344	270	F, L	**III, IV**
di-galloylquinic acid	496		F	**IV**
di-galloylglucose	484	276	F	**IV**
tri-galloylglucose	636	272	F, L	**III, IV**
tetra-galloylglucose	788	278	F	**IV**
penta-galloylglucose	940	277	F, L	**III, IV**
Ellagitannins				
HHDP-glucose	482	slope	F	**IV**
bis-HHDP-glucose	784	232, slope	Fr, F, L	2, **III, IV**
galloyl-HHDP-glucose	634	232, slope	Fr, F, L	2, **III, IV**
HHDP-galloyl-glucose	634	300sh, 284	Fr	2
galloyl-bis-HHDP-glucose	936	234	Fr, L	2, **III**
di-galloyl HHDP glucose	786	270	F	**IV**
sanguiin H6	1870	260, 345	Fr	3
sanguiin H10	1568	230, 280sh	L	5
tri-galloyl-HHDP glucose	938		F, L	**III, IV**
di(HHDP-galloylglucose)-pentose	1416	225	L	5
casuarictin	936	225, 280sh	L, F	5, **IV**
trigalloyl-triHHDP-diglucose	1718	230, 280sh	L	5
potentillin	936	230, 260sh, 280sh	L, F	5, **IV**
agrimoniin	1870	230, 260sh, 280sh	L, F	5, **IV**

pedungulagin			R	7
Flavanones				
dihydrokaempferol (aromadendrin)	288		Fr	6
dihydroquercetin (taxifolin)	304		Fr	6
taxifolin 3-arabinofuranoside	436		R	7
Flavones				
apigenin	270		Fr	16
apigenin glucoside	432		Fr	16
Flavan-3-ols, proanthocyanidin				
(+)-catechin	290	278/280	Fr, R, F, L	1, 2, 3, 6, 7, **III, IV**
(-)-epicatechin	290	278	Fr	1
(+)-afzelehin-catechin			R	7
dimer B2	578	278	Fr, F, L	1, 5, **III, IV**
proanthocyanidin B1	578	310,286	Fr	2
proanthocyanidin B3	578	312sh, 284	Fr, R	2, 7
procyanidin tetramer	1154	277	F	**IV**
procyanidin pentamer	1442	277	F	**IV**
propelargonidin dimer	562	277	F	17**, IV**
proanthocyanidin trimer(EC-4,8-EC-4,8-C)	866	284	Fr, F, L	2, **III, IV**
propelargonidin trimer	850	276	F	17**, IV**
procyanidin B6			R	7
Flavonols				
quercetin 3-glucoside, quercetin hexose	464	354, 285	Fr, F	1, 3, 6, **IV**
quercetin di-hexose	626		F	**IV**
quercetin hexose glucuronide	640	260, 353	F	**IV**
quercetin pentose glucuronide	610	255, 353	F, L	**III, IV**
quercetin 3-glucuronide	478	354, 258	Fr, L, F	1, 2, 3, 5, **III, IV**
quercetin 3-glucurone-deoxyhexoside	624	254, 300sh, 354	Fr	1
quercetin-3-malonylglucoside, quercetin malonylhexose	550	256, 354	Fr, F	2, **IV**
quercetin-rutinoside (rutin)	610	255, 355	Fr	3
quercetin-deoxyhexose-hexose (not rutin)	610	255, 295sh, 350	L	5
kaempferol 3-glucuronide	462	348, 265	Fr, F, L	1, 3, 5, **III, IV**
kaempferol-3-glucoside, kaempferol hexose	448	266, 348	Fr, F	2, **IV**
kaempferol 3-malonylglucoside, kaempferol malonylhexose	534	266, 346	Fr, F	2, 5, **IV**
kaempferol 3-coumaroylglucoside (tiliroside)	594	268, 314/ 250	Fr, F, L	2, 3, 11, **III, IV**
kaempferol di-hexose glucuronide	786	264, 345	F	**IV**
kaempferol di-pentose hexose glucuronide	888	265, 345	F	**IV**

kaempferol di-hexose	610		F	**IV**
kaempferol hexose glucuronide	624	264, 344	F, L	**III, IV**
kaempferol pentose glucuronide	594	265, 345	F, L	**III, IV**
isorhamnetin 3-glucuronide	492	254, 300sh, 354	Fr, F	**IV**
isorhamnetin sophorose hexose	802		F	**IV**
isorhamnetin di-hexose	640	253, 362	F	**IV**
isorhamnetin rutinose	624		F	**IV**
isorhamnetin hexose malonylhexose	726	253, 360	F	**IV**
leucocyanidin	306		Fr	6
Anthocyanins				
cyanidin 3-glucoside	449	280, 516	Fr	1, 2, 3, 4, 6
cyanidin hexoside	449	280, 516	Fr	1
cyanidin hexose- deoxyhexoside	595	280, 516	Fr	1
cyanidin 3-sophoroside	611	280, 516	Fr	1
cyanidin 3-(2^G-glucosylrutinoside)	757	280, 516	Fr	1
cyanidin 3-rutinoside	595	280, 516	Fr	1, 4
cyanidin 3-malonylglucose-5-glucose	697	524	Fr	4
pelargonidin 3-glucoside	433	276, 504, 428sh	Fr	1, 2, 3, 4, 5, 6, 8,9
pelargonidin 3-rutinoside	579	276, 504	Fr	1, 3, 4
pelargonidin 3-malonylglucoside	519	276, 504, 430sh	Fr	1, 2, 5
pelargonidin 3-succinylglucoside	533	276, 504	Fr	1
5-pyranopelargonidin-3-glucoside	501	492, 358, 262sh	Fr	2
pelargonidin 3-malonylrhamnoside or 3-succinylarabinoside	503	280, 430sh, 506	Fr	2
pelargonidin diglucoside	594/595	275, 520 / 500	Fr	3, 4
pelargonidin 3-malylglucoside	549	503	Fr	4
pelargonidin hexose pentose acylated with acetic acid	607	503	Fr	4
pelargonidin 3-acetylglucoside	475	504	Fr	4
catechin-4,8-pelargonidin-3-glucoside	721	518, 438	Fr	8
epicatechin-4,8-pelargonidin-3-glucoside	721	518, 433	Fr	8
afzelechin-4,8-pelargonidin-3-glucoside		516, 434	Fr	8
epiafzelechin-4,8-pelargonidin-3-glucoside	705	520, 432	Fr	8
5-carboxypyranopelargonidin-3-glucoside	501	360, 496	Fr	9
5-carboxpyranocyanidin-3-glucoside	517	278, 351, 505		
Others				
L-(+)-ascorbic acid	176	244	Fr	2
quinic acid	192	225, 270	L, Fr	5, 6
gentisic/protocatechuic acid	154		Fr	6
N-propyl carbazole	209		Fr	6
3-methylcatechol	124		Fr	6
1,4-benzoquinone	1008		Fr	6
2-glucopyranosyloxy-4,6,-dihydroxyisovalerophone	372	225, 286	Fr	11

trans-resveratrol	228	320	Fr, A	12
cis-resveratrol	228	288	Fr, A	12
dihydrochalcone glycoside (phloridzin)	436	285	Fr	
Several sesquiterpenoid, triterpenoid and spermidine derivatives			F	**IV**

References: **Metabolite profiling studies: 1**. Määttä-Riihinen et al., 2004¸**2**. Aaby et al., 2007; **3**. Seeram et al., 2006; **4**. Lopes-da-Silva et al., 2002 (focus on anthocyanins); **5**. Hukkanen et al., 2007; **6**. Aharoni et al., 2002; **III, IV**. **Targeted analyses including NMR: 7**. Ishimaru et al., 1995; **8**. Fossen et al., 2004; **9**. Andersen et al., 2004; **10**. Cheel et al., 2005 (*F.chiloensis*); **11**. Tsukamoto et al., 2004; **12**. Heur et al., 1992; **13**. Wang et al., 2007; **14**. Latza et al., 1996; **15**. Hilt et al., 2003; **16**. Zhang et al., 2007 (no NMR); 17 Gu et al., 2003; **IV.** Abbreviations: Fr, fruit; L, leaf; A, achene; F, flower; R, root.

2.4. Towards metabolic engineering of strawberry

In spite of wide cultivation of strawberry, relatively few studies demonstrate the successful genetic modification of this plant. The main reason why strawberry is not easily amenable for genetic modification or conventional breeding is the complex octoploid genome of cultivated strawberry. However, breeding through genetic modification offers a more straightforward strategy than does conventional breeding by allowing the direct introduction of dominant traits to the parental cultivar. Once stably integrated into strawberry genome, the transgene remains in the subsequent rounds of vegetative propagation through runners. The transgene is thus not lost from the complex genome, as might be the case in sexual propagation.

Since the first report on the *in vitro* regeneration of strawberry, resulting in the large-scale commercial micropropagation of a crop plant for the first time (Boxus, 1974), several optimized protocols for *in vitro* techniques used on different strawberry cultivars have been developed (Liu and Sanford, 1988; Passey et al., 2003; **I**). Also the *Agrobacterium*-mediated transformation of the octoploid strawberry has been well documented (Nehra et al., 1990; Nyman and Wallin, 1992; Mathews et al., 1995; Schaart et al., 2004; Gruchala et al., 2004; Folta et al., 2006; Mezzetti and Constantini, 2006; **II**). Since diploid strawberry (*Fragaria vesca*) has been suggested as a model for functional genomics research in Rosaceae, high-efficiency gene transfer protocols also for this variety have been developed (Alsheikh et al., 2002; Oosumi et al., 2006). A technical advancement that allows rapid RNA-based gene silencing in strawberry fruit was achieved by injection of Agrobacterium directly to developing receptacles (Hoffmann et al., 2006). As a very rapid approach, the method is suggested to aid in the studies of gene function in combination with metabolite profiling.

In spite of the extensive technical reports, relatively few studies are published on successful genetic modification of any traits in strawberry, some rare examples being, e.g., better firmness of ripe fruit gained by introducing antisense sequence of strawberry pectate lyase (Jiménez-Bermúdez et al., 2002), and enhanced freezing tolerance by expression of the acidic dehydrin protein from wheat (Houde et al., 2004). Enhanced resistance to grey mould has been achieved by the expression of thaumatin II defence protein originating from *Thaumatococcus daniellii* (Schestibratov and Dolgov, 2005). Resistance against another fungus, *Verticillium dahliae*, was similarly obtained in a study demonstrating the expression of a chitinase gene from *Lycopersicon chilense* in strawberry (Chalavi and Tabaeizadeh, 2003). Alteration of the secondary metabolism of strawberry by genetic modification has so far been reported only in two studies demonstrating the silencing of *CHS* gene (Hoffmann et al., 2006; Lunkenbein et al., 2006a) and in this Thesis describing the effects of *STS* gene transfer (**III**). Other modifications done on strawberry secondary metabolism include enhancement of proanthocyanidin content (Bovy et al., in press).

3. AIMS OF THE PRESENT STUDY

The aims of the present Thesis were:

1. To optimize conditions for the *in vitro* tissue culture of strawberry in liquid medium in temporary immersion bioreactors (**I**) and to establish genetic modification system utilizing the bioreactor (**II**);

2. To develop genetically modified strawberry plants by transferring *STS* gene under the control of floral (fil1) and general (35S) promoters in order to inhibit grey mould infection (**II**, **III**);

3. To verify transgene integration by TAIL-PCR (**II**) and to analyse transgene expression by qPCR (**III**);

4. To conduct metabolite profiling of the leaf (**III**) and floral (**IV**) tissues of the genetically modified and parental strawberries.

4. MATERIALS AND METHODS

4.1. Nomenclature

The nomenclature in this Thesis is based on the instructions for authors of the journals Plant Cell and Plant Physiology. Proteins are abbreviated by capital letters (STS) and corresponding genes by italics capitals (*STS*). In case a specific gene is annotated, PubMed database accession number is provided together with a specific name for the gene in question, if available (*NS-Vitis3*). Promoter names mentioned in the text are in roman type (35S), but when fused to a coding region of a gene, the complex is abbreviated in italics and the promoter and coding sequence are separated by a colon (*35S:GUS*). Genus names are written in italics, except when the genus name is the accepted common name, the name is in roman type lowercase (first letter uppercase), e.g. Arabidopsis is common name for *Arabidopsis thaliana* and Agrobacterium for *Agrobacterium tumefaciens*.

4.2. Tobacco transformation, PCR analysis and histochemical staining

In order to achieve flower/filament specific gene expression, several vector constructs were developed on the pCAMBIA plant gene transfer vectors (Cambia org., Canberra, Australia). The expression pattern of four heterologous promoters was studied using tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) as the model plant. The promoters were fused to *uidA* (referred to as GUS in the following) reporter gene from *Escherichia coli* encoding β-glucuronidase (GUS) in the vector pCAMBIA1391Z containing hygromycin selectable marker gene under the control of *Cauliflower mosaic virus* 35S (35S) promoter. Two of the promoters, fil1 and fil2, originate from genes related to floral development of *Antirrhinum majus*. The other two promoters ('424' and '570') are synthetic and made from regulatory elements derived from the promoter of *Arabidopsis thaliana APETALA3* gene combined with minimal 35S promoter (Table IV). All the constructs contained NOS terminator in the vector backbone. The constructs were transferred to *Agrobacterium tumefaciens* LBA4404 using a standard freeze-thaw method (Chen et al., 1994).

Table IV. Gene constructs used in the promoter analysis.

Construct	Promoter size, bp	Source	Reference
PCAMBIA2301			Cambia, org.
pCAMBIA1391Z			Cambia, org.
fil1:GUS[a]	1414	*A. majus*	Nacken *et al.* 1991
fil2:GUS[a]	1486	*A. majus*	Steinmayr *et al.* 1994
424:GUS[a]	542	synthetic	Tilly *et al.* 1998
570:GUS[a]	542	synthetic	Tilly *et al.* 1998

[a] Constructs were made on pCAMBIA1391Z vector backbone and contain the promoters in front of the *GUS* gene with NOS terminator.

Transgenic tobacco plants were obtained by Agrobacterium-mediated transformation following a standard method (Horsch et al., 1985) with hygromycin selection (15 mg/l) on MS-based medium containing BAP (1 mg/l) and NAA (0.1 mg/l) as regeneration hormones, and cefotaxime (500 mg/l) to control the growth of Agrobacterium. Histochemical assays were performed as described by Jefferson (1987).

After testing the expression, the *GUS* marker gene in the T-DNA region of pCAMBIA1391Z vector was replaced by the coding region of *Vitis riparia* stilbene synthase gene termed *NS-Vitis3* (AF128861, Goodwin et al., 2000). In addition, the vector pCAMBIA2301 with the original 35S promoter was similarly changed to contain *NS-Vitis3* coding region. The NOS terminator was retained in all the constructs. The resulting *NS-Vitis3* -containing vectors, termed *fil1:NS-Vitis3*, *fil2:NS-Vitis3* and *35S:NS-Vitis3* were used for strawberry transformation.

4.3. Strawberry micropropagation in liquid culture

Young, folded leaves of greenhouse-grown strawberry (*Fragaria* x *ananassa*) cultivars Bounty, Jonsok, Korona, Polka and Zephyr were collected and surface-sterilized with 70% ethanol and sodium hypochlorite (1.5%). The leaves were chopped and placed in the temporary immersion system (RITA® bioreactors, Cirad, France) or on a semi-solid medium in petri plates. MS medium (Murashige and Skoog, 1962) was supplemented with the growth regulators thidiazuron (TDZ, 9 μM) and indole-3-butyric acid (IBA, 2.5 μM) together with Plant Preservative Mixture (2 ml/l) (PPM™, Plant Cell Technology, Washington, USA). For cultivation on semi-solid medium, the regeneration medium was solidified with 0.8% Plant Agar (Duchefa, Haarlem, The Netherlands). For cultivation in a TIB system, the containers were connected to the automatic pressure system with the

immersion frequency of 5 min every 4 h. The light intensity in the room was 36 $\mu Em^{-2}s^{-1}$ for the Petri dishes and 20 $\mu Em^{-2}s^{-1}$ inside the TIB container (19 h at 22°C). Both systems were subcultured every 2 weeks. Regeneration frequencies were calculated as the number of leaf explants producing adventitious shoot regenerants versus total number of leaf explants, and the statistical analysis of the results was carried out using a binomial model.

4.4. Agrobacterium-mediated gene transfer of strawberry

Plant material from strawberry (*Fragaria* × *ananassa*) cv. Jonsok was sterilized and chopped. The leaf pieces were incubated in *fil1:NS-Vitis3* or *fil2:NS-Vitis3* plasmid-bearing Agrobacterium/MS suspension for 30 min, briefly dried on Whatman filter paper and co-cultivated on MS agar plate overnight. For regeneration, the pieces were placed into (TIB) containers together with the regeneration medium. Cefotaxime (200 mg/l) was added for the first two weeks in order to eliminate Agrobacterium. The selective antibiotic hygromycin (10 mg/l for the first two to four weeks followed by 15 mg/l for one to two weeks) was applied as the regenerating shoots started to emerge. Shoots that continued developing in the presence of hygromycin were transferred into another container containing MS medium for rooting. The acclimatized GM strawberry events, together with the wild-type controls, were grown in greenhouse in the following conditions: temperature at daytime 20ºC and at night 18ºC; humidity 60 to 70% and period of daylight 16 h. The plants were grown in 12-cm pots in a peat-sand mixture (3:1) and fertilized weekly with Superex-9 fertilizer (N 19%, P 5%, K 20%) supplied with micronutrients (Kekkilä, Finland). To induce flowering, a 5- to 6-week short daylight (13 h) period was applied. The *35S:NS-Vitis3* strawberry line was developed essentially as described by Schaart et al. (2002) on solid medium with kanamycin selection.

4.5. T-DNA junction analysis by TAIL-PCR

The left border T-DNA junction sequences were determined by the TAIL-PCR method (Liu and Whittier, 1995). Genomic DNA was extracted from young folded leaves with DNeasy® Plant Mini Kit (Qiagen). All PCR reactions were carried out in Go Taq® Green Master Mix (Promega) according to manufacturer's instructions in iCycler iQ™ (Bio-Rad). The specific PCR products from the tertiary TAIL-PCR reactions were extracted from agarose gel with QIAquick® Gel Extraction Kit (Qiagen). The DNA fragments were sequenced using Thermo Sequenase CY5 Dye Terminator Kit (Amersham Biosciences) and automated sequencer A.L.F. express DNA sequencer (Amersham Biosciences).

4.6. Quantitative real-time reverse transcriptase PCR (qPCR)

Total RNA from floral organs and leaves was extracted according to Bowtell and Sambrook (2003). The cDNA was synthesized from DNaseI-treated (Fermentas UAB, Vilnius, Lithuania) total RNA by using the M-MuLV reverse transcription reagents (Fermentas UAB, Vilnius, Lithuania). For the determination of the inserted *NS-Vitis3* and endogenous chalcone synthase (*CHS*) expression levels, Real Time SYBR Green quantitative PCR was set up using DyNAmo HS SYBR Green qPCR kit F-410 (Finnzymes, Espoo, Finland) in the iCycler iQ instrument (Bio-Rad). The expression levels of *NS-Vitis3* and *CHS* genes were measured by comparing target gene Ct values to an endogenous internal control gene, i.e., DNA binding protein *DBP* (Schaart et al., 2002). Expression levels were calculated from the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). The expression of each target gene was compared to the lowest expression level (set to 1) in the sample series.

4.7. Protein extraction and two-dimensional Western analysis

Strawberry leaf proteins were extracted as described by Koistinen et al. (2002). Proteins were dissolved in 2-DE sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% (v/v) Bio-Lyte 3/10 ampholyte (Bio-Rad), and the total protein concentration was analyzed using Bio-Rad Protein Assay Dye reagent. The 2-DE analysis was performed as described by Lehesranta et al. (2005), with the exception that the first dimension isoelectric focusing was performed using 7 cm immobilized pH gradient (4-7) strips (Amersham Biosciences). The second dimension was performed in 12% SDS-PAGE (Bio-Rad Minigel II apparatus). After 2-D focusing, the proteins were transferred to PVDF membrane (Immobilon P, Millipore) and visualized by SYPRO Ruby protein blot stain (Bio-Rad). Gel images were acquired with FLA-3000 fluorescent image analyzer (Fuji Photo Film) using excitation and emission wavelengths of 473 and 580 nm, respectively. After staining, the same membranes were used for Western blotting (Koistinen et al., 2002), with a rabbit polyclonal antiserum raised against recombinant STS protein (Giovinazzo et al., 2005) in 1:1000 dilution, and alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Zymed) as secondary antibody.

4.8. Metabolite profiling by UPLC-qTOF-MS

Semipolar metabolites were extracted by methanol from the whole flowers, the separated floral organs, and young and mature leaves. Methanol (80%) was added at a ratio of 0.75 ml/500 mg ground sample for the flowers, and at the ratio of 1.5 ml/500 mg for the leaves. Mass spectrometric analysis was

carried out by UPLC-UV(PDA)-qTOF (Waters Premier qTOF, Milford, MA, USA). Separation of metabolites was performed on a 150 x 2.1 mm i.d., 1.7 µm UPLC BEH C18 column (Waters Acquity). The UV spectra (Waters Acquity PDA detector) were recorded between 210 and 500 nm, or the UV trace was measured at 240 nm (Waters Acquity UV detector). The electrospray ionization was performed in positive and negative modes. MassLynx software version 4.1 was used to control all instruments and to calculate the accurate mass. The data analysis was done by MarkerLynx 4.1 software for mass signal extraction and alignment, PCA analysis, and metabolite marker list creation. Putative annotation of the metabolites was based on mass fragmentation spectra and the molecular formula postulated by the MassLynx software based on the accurate mass (in 3 to 5 ppm accuracy range) and isotopic pattern recognitions. The putative metabolites were screened from the Dictionary of Natural Products (Chapman & Hall/CRC) and SciFinder Scholar databases and verified by MS and MS/MS fragmentation.

4.9. Isolation of compounds for NMR analysis with semipreparative HPLC

Semipreparative HPLC was used to isolate and concentrate selected compounds for NMR profiling. Metabolite extraction was performed from the old leaf samples similarly to that for the UPLC-MS analysis but on a larger scale. The extracts were concentrated by solid phase extraction (Waters Oasis HLB Plus; Waters Corporation, Milford, MA, USA). The HPLC apparatus was HP 1090 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with diode array detector and operated with HP Chemstation Rev. A. 10.02 software. Compounds were separated on Discovery HS C18 column with 250 x 10 mm i.d. and 5 µm particle size equipped with respective 10 x 10 mm i.d. guard column (Supelco, Bellefonte, BA, USA) using gradient elution with 1% (v/v) formic acid (A) and acetonitrile (B). The flow rate was 3.0 ml/min, and the gradient program was as follows: 0-10 min, 5-20% B; 10-40 min, 20-35% B; 40-55 min, 100% B; and 55-62 min, 100-5% B. Compounds were detected at a wavelength of 280 nm. Spectroscopic data were recorded between 240 and 600 nm with 2 nm steps. Altogether 14 fractions were collected from all the extracts, pooled and evaporated to dryness in vacuum centrifuge at room temperature. A sample from each fraction was taken to UPLC-MS/MS analysis in parallel with the NMR profiling to confirm the purity and identity of each fraction.

4.10. NMR spectroscopy

Spectral assignment and structure identification was done by NMR spectroscopy for selected fractions based on the purity of the fraction using Bruker AVANCE DRX 500 (Bruker BioSpin GmbH, Germany) spectrometer operating at 500.13 MHz for proton and 125.77 MHz for carbon equipped with 5 mm inverse triple resonance probe. The samples obtained from preparative HPLC were dissolved in 190 µl of methanol-d_4 (CD_3OD, 99.96% D, Euriso-Top SA, France). Chemical shifts were referenced to solvent residual peak at 3.31 ppm for proton and 49.00 ppm for carbon. One-dimensional spectra were processed and analyzed using PERCH NMR Software (Laatikainen et al., 2007). The spectral parameters were solved first by applying the integral-transform method (Laatikainen et al., 1996), after which the parameters were refined by the total-line-shape fitting mode of the PERCHit iterator.

4.11. Fluorescence microscopy

A fresh 1 x 1 cm disc of strawberry leaf was mounted on glycerol on a glass slide and enclosed with a cover slip. The sample was squashed between the glasses with an apparatus occupying two parallel metal discs, and immediately viewed with a Nikon Microphot-FXA microscope equipped with fluorescence filter allowing excitation at 330 to 380 nm and emission above 420 nm. The autofluorescent images were captured with RS Photometrix CoolSNAP digital camera and processed with the CoolSNAP software.

4.12. Botrytis inoculation

Botrytis cinerea Pers.: Fr strain B.05.10 (Quidde et al., 1999) was used in the inoculation experiment. The strain was maintained on potato dextrose agar as described by Muckenschabel et al. (2002). The attached strawberry leaves were placed on Petri dish containing water agar (0.8%). The leaf surface was wounded from three places by punching with syringe needle and a 3 µl droplet of the conidial suspension ($1x10^5$ conidia/ml) was placed on the wound. The plates were sealed with parafilm and kept at room temperature in natural light. The leaves were photographed and the lesion areas were measured by ImageJ software (Rasband, 1997-2007).

5. RESULTS AND DISCUSSION

5.1. Suitability of temporary immersion bioreactors for strawberry tissue culture (I)

Temporary immersion bioreactors (TIB) are used to propagate plants *in vitro* in an apparatus where the cultivated material is immersed in the liquid medium in fixed intervals. One advantage of the system is the easy maintaining, since the renewal of the medium is accomplished easily without handling of the plant material. In addition, the problems often faced *in vitro* in liquid cultures, such as asphyxia and hyperhydricity, are avoided since the culture is exposed to air between the immersions (Etienne and Berthouly, 2002).

Suitability of the TIB system for *in vitro* propagation has been reported for several plant species. In the present study it was tested for five strawberry cultivars, i.e., Bounty, Jonsok, Korona, Polka and Zephyr. TIB was proven as an effective shoot regeneration environment for all of the tested cultivars; after eight weeks of cultivation, all the containers showed as high regeneration frequency as in the conventional semi-solid media controls (**I**, Figures 1 and 2). The regeneration medium (MS, TDZ 9.0 μM, IBA 2.5 μM) used in this study proved to be particularly effective for strawberry leaf disc regeneration, since all the cultivars achieved high frequency of regeneration (70 to 90%) (**I**, Table I). Further advantage of the TIB system was that rooting of the regenerated explants was achieved directly in the TIB containers simply by changing the medium to basic MS without the growth regulators. A particularly efficient root formation was achieved for the cultivars Jonsok and Korona (**I**, Figure 3), while the other cultivars showed moderate rooting. The rooting was strong enough for the plantlets to be moved directly into the soil, and the acclimatization of the plantlets was successfully achieved for all cultivars, since all planted explants survived.

The TIB system was proven as a convenient method for leaf regeneration of several strawberry cultivars. One of the main advantages of the method is that the active components of the medium become into direct contact with all of the plant material and that control of the cultivation regime is straightforward with a simple renewal of the medium. The system enables working with large quantity of plant material, since there is no need for handling individual leaf pieces; the method was thus chosen for the generation of the GM strawberries.

5.2. Strawberry transformation by stilbene synthase-encoding gene (II, III)

5.2.1. Gene transfer strategy

Plants have been genetically modified for enhanced defense against pathogens, including the grey mould causing fungus *Botrytis cinerea*, by introducing stilbene synthase (*STS*) gene (see Chapter 2.2.4.2.). STS catalyzes the synthesis of the multi-functional phytoalexin resveratrol that has beneficial effects both on the plant and on humans. In this study, the coding region of *STS* gene *NS-Vitis3* from frost grape (*Vitis riparia*) was transferred into the strawberry cultivar Jonsok, which has good frost tolerance (Sonsteby and Karhu, 2005). The *B. cinerea* infection is believed to enter the strawberry at the flowering stage *via* the filaments of stamen, and remain latent at the base of the flower, i.e., in the receptacle. During fruit maturing the symptoms develop in favorable environmental conditions (Powelson, 1960). In most cases, the stress-inducible promoter of *STS* gene has been used, but other promoters such as the 35S and ubiquitin promoters have also been applied. In this work, the goal was to modify strawberry to produce resveratrol in the infection route of the grey mould fungus, i.e., in the filaments of the flower.

5.2.2. Promoter testing in tobacco model

In order to achieve flower/filament-specific gene expression, four vector constructs were assembled and tested in the model plant *Nicotiana tabacum*. Two of the promoters originated from *Antirrhinum majus* (Nacken et al., 1991; Steinmayr et al., 1994) and two were synthetic promoters containing regulatory elements from the APETALA3 promoter of Arabidopsis (Tilly et al., 1998). All the promoters were fused in front of the *GUS* coding region for reporter expression. Localization of the expression was monitored by GUS staining, and examined visually from the flowers and leaves of tobacco (Table V). The flowers were studied at six different developmental stages.

Table V. Typical GUS expression pattern observed in the inflorescences of GM tobacco. The developmental stages were numbered from early bud to mature open flower on a scale 1 to 6. +, faint; ++, significant; and +++, strong staining.

	fil1:GUS			***fil2:GUS***			***424:GUS***			***570:GUS***		
	1-2	3-4	5-6	1-2	3-4	5-6	1-2	3-4	5-6	1-2	3-4	5-6
receptacle	-	-	-	-	+	-	+	++	+	+	+	+
sepal	-	-	-	-	+	+	+	++	+	++	++	++
petal	-	-	+	-	-	-	++	++	++	++	++	++
anther	-	+	+	-	+++	+	-	-	++	-	-	++
filament	-	-	+	-	++	+	-	-	++	-	-	+++
stigma	-	-	+	-	+	++	++	+++	++	+++	++	++
style	-	-	++	-	++	++	+	++	+	++	++	++
leaf		-			-			++			++	

The two natural promoters of *A. majus* (fil1 and fil2) were capable of directing *GUS* expression in tobacco flowers. The *fil1:GUS* construct showed expression mainly at the later stages of flower development, being strongest in the anthers where the expression was detectable already at the developmental stages 3 to 4. With the construct *fil2:GUS* the expression was stronger at stages 3 to 4 than at the later stages. In the mature flowers, the only staining detected was in the pistils and stamens, and very faintly in the sepals. For the two constructs containing synthetic promoters, the expression was very strong throughout the development, and staining was also detected in the leaf tissue. In *A. majus,* the *fil1* gene participates in floral development (Nacken *et al*., 1991) as a putative target gene for the homeotic MADS-box transcription factor DEFICIENCE. The strongest expression is present in the filaments and at the base of petals, weaker expression signals being observed in the tapetum and ovules. In the GM tobacco, the expression pattern followed the one in *A. majus* by being localised in the floral organs (mainly stamen and pistil), but the temporal and exact spatial pattern was slightly different. Restriction of the transient transgene expression to the flower suggests that the transgenic fil1 promoter is recognized in the tobacco flower by the homeotic transcription machinery which is in charge of the floral development. Another putative target gene of DEFICIENS in *A. majus* is *fil2* (Steinmayr *et al.,* 1994). Its expression is weak in the petals, highest in the stamens and is also present in the pistils. In tobacco, fil2 promoter was found to act in a similar way to that in *A. majus*. The *fil2:GUS* expression was strongest in the stamens, being strongly localized to the anther part of the organ throughout the development.

5.2.3. Strawberry transformation in temporary immersion bioreactor system is feasible

Based on the promoter testing in tobacco, two of the plasmid constructs providing floral expression (*fil1:GUS, fil2:GUS*) were modified by replacing the *GUS* reporter gene with the coding region of the *NS-Vitis3* gene. In addition to these flower-specific constructs, a plasmid containing *NS-Vitis3* under the control of 35S promoter was included in the study. The *Agrobacterium*-mediated transformation of strawberry was done in parallel on the semi-solid medium and in the TIB system.

The Agrobacterium-inoculated leaf pieces were placed in the TIB containers, and the Agrobacterium was removed by 200 mg/l of kefotaxime. The TIB system allows a very efficient removal of the Agrobacterium, since flushing with the liquid medium removes all traces of Agrobacterium from the surface of the explants within two weeks (**II**, Figure 3). The possibility of completing kefotaxime selection as rapidly as possible is particularly important for strawberry shoot regeneration from leaf discs, since a prolonged exposure to this antibiotic was shown to inhibit regeneration in a test performed in semi-solid medium (**II**, Figure 2). The same inhibitory effect of kefotaxime on regeneration has been observed also for the diploid strawberry cultivars (Alsheikh et al., 2002).

For the selection of transformed plants among the regenerating shoots, the antibiotic regime was adjusted to 10 mg/l of hygromycin, which had been used previously in strawberry transformation (Mathews et al., 1995; Nyman and Wallin, 1992). The suitability of kanamycin as a selective antibiotic was also tested in the TIB transformation system, but the selection by hygromycin was visually more easily discernible, and was thus used in further transformations (**II**, Figure 1).

The leaf discs were subjected to antibiotic selection at a stage where efficient shoot regeneration started (**II**, Figure 1), and the shoots that continued developing and turned green in the antibiotic were transferred to another container with basic MS medium, and subsequently planted in the soil.
From the experiments performed with the *fil1:NS-Vitis3* construct, several events developed in the TIB system (**II**), but the fil2 promoter-driven construct gave no transformants. The *35S:NS-Vitis3* construct was stably integrated in strawberry genome in one transformant regenerated from the semi-solid medium (**III**).

The advantages of the TIB system for strawberry transformation were mainly in the convenient controlling of the constituents in the culture media. The

TIB system allowed rapid change of the antibiotic regime, as well as adjustment of the growth regulators simply by changing the medium without the need for laborious handling of individual leaf pieces. In addition, unlike in the semi-solid medium, the liquid medium comes to immediate contact with all parts of the explants, which further enhances the effect of medium changes. Strawberry is regarded as rather recalcitrant plant in terms of genetic modification, although several methods describing the optimization of the protocols for specific cultivars have been published (Schaart et al., 2004; Gruchala et al., 2004; Folta et al., 2006). The TIB method was proven to be suitable for the transformation of strawberry cv. Jonsok, and this may be true for the transformation of other plant species as well, but this remains to be proven, since the transformation is usually highly species and cultivar specific, and the efficiency of the transformation was rather low for strawberry in our study.

5.2.4. Sequencing of the left border of the transgene integration site by TAIL-PCR differentiated between the different transformation events

Strawberry leaf is extremely rich in phenolic compounds and polysaccharides which interfere with DNA purification. Several reports have been published on the purification of strawberry DNA, but none has proven efficient as a general procedure (Porebski et al., 1997; Mercado et al., 1999; Medina-Escobar et al., 1997). Since verification of transgene integration in the host genome by conventional Southern analysis requires relatively high amount of pure DNA (10-20 μg), an alternative approach was taken in order to verify the transgene in the strawberry genome as well as to differentiate between the different transgenic events. Thermal asymmetric interlaced PCR (TAIL-PCR), originally described by Liu and Whittier (Liu and Whittier, 1995; Liu et al., 1995) is used to clone the DNA in the T-DNA insertion site. The strategy involves the use of nested, T-DNA border-specific primers together with a shorter arbitrary degenerate primer for the unknown genomic DNA region flanking the insertion site. Such priming creates both specific and unspecific products, whose relative amplification efficiencies can be thermally controlled. In three serial PCR reactions, the unspecific products are gradually diluted out and in the final reaction the specific products are detectable in the gel by the slight shift in size due to the nested oligonucleotides in the T-DNA region. Since its development, TAIL-PCR has become an extremely valuable and versatile tool in all research involving recovery of unknown genomic sequences adjacent to known sequences, and it has been utilized in functional genomics (Sessions et al., 2002; Settles et al., 2004), characterization of promoter sequences (Terauchi & Kahl, 2000) and also in the detection of GM material in food (Hernandez et al., 2003;

Yang et al., 2005). Application of TAIL-PCR for the initial screening of transgenic events was first published in the original article **II** of this Thesis.

A set of primers consisting of T-DNA-specific and arbitrary oligonucleotides (**II**, Table I) was used to analyse the regenerants grown in soil. The analysis showed that several of the regenerated shoots apparently originated from the same gene transfer event, since the junction sequences were identical. Eventually, three different GM lines with differing T-DNA border sequences could be identified (**II**, Figure 4). The TAIL-PCR approach was proven useful for analysing strawberry DNA since the small amount (10-100 ng) of pure DNA needed as template can be easily isolated with commercial plant DNA extraction kits.

The utilization of TAIL-PCR in transgene verification is a convenient alternative to Southern analysis, since it may be performed already from a very small plant material. In the development of GM plants, the first indication of modification is usually obtained at the early stage by conventional PCR, and the verification of separate gene transfer events is only possible at a later stage, when there is enough plant material for the isolation of sufficient amount of DNA for Southern analysis. Since TAIL-PCR can be performed at a very early stage, different transgenic events can be sorted immediately based on the sequence information about the integration site.

5.3. Comparison of flowers from the GM and parental strawberries (III, IV)

5.3.1. Expression of *NS-Vitis3* transgene in the flowers of the GM strawberries

The expression of the transgene was studied at the mRNA level by quantitative real-time reverse transcriptase PCR (qPCR). The RNA was extracted from the floral organs, i.e., petal, sepal, stamen (filament and anther separated), pistil and receptacle dissected from the flowers of the three GM lines referred herein as J47/1 (*fil1:NS-Vitis3*), J47/2 (*fil1:NS-Vitis3*) and *35S:NS-Vitis3* together with the parental non-modified strawberry. The qPCR analysis was conducted by using oligonucleotides specific for the exon region of *NS-Vitis3*. The expression levels were normalized to the expression of the endogenous control gene of DNA binding protein (*DBP*) that has been used as qPCR reference in strawberry leaves (Schaart et al., 2002). The gene was tested for expression levels in floral organs, and it proved to function rather uniformly in the different organs (Figure 3).

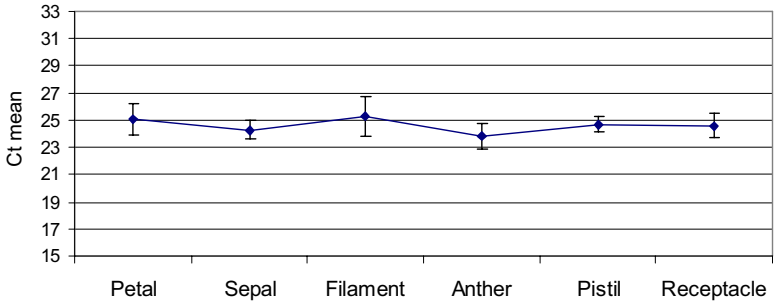

Figure 3. RT-qPCR analysis of *dbp* expression in strawberry floral organs.

Accumulation of *NS-Vitis3* mRNA was detected in all three GM lines but not in the parental strawberry (**III**, Figure 3). In the floral organs, the highest *NS-Vitis3* expression was detected in the filament and pistil of the J47/1 line, being higher than in the *35S:NS-Vitis3* line in both organs. The expression level of *NS-Vitis3* in the pistil of the J47/1 line was 4-fold compared with the mean expression in all flower parts. The other line with fil1 promoter (J47/2), however, had nearly no expression in the filament and pistil (**III**, Figure 3), which indicates transgenic event-specific expression. Both fil1-promoter containing lines (J47/1 and J47/2), however, showed transgene mRNA accumulation also in the young and old leaves (**III**, Figure 3). One likely reason for the non-specific expression of the transgene may be that the pCAMBIA plasmids that were used in the construct assembly have a strong 35S promoter which drives the expression of the *hptII* marker gene in the T-DNA, and this promoter has been shown to interfere with other genes in the construct in several gene transfer studies (http://www.cambia.org /daisy/bios/585.html). In the TAIL PCR analysis, line J47/1 was found to contain at least two copies of the transgene, whereas J47/2 showed only one integration (**II,** Figure 5B). However, it can not be ruled out that also other integration sites not found in TAIL-PCR may exist, and Southern analysis would be required for the reliable determination of copy number. The difference in transgene copy number as well as the transgene position in the genome affect the outcome of gene transfer (Filipecki and Malepszy, 2006), but since only a few transgenic lines were obtained in the study, no definitive conclutions can be drawn from the results regarding the suitability of the fil1 promoter for directing flower-specific expression in strawberry.

5.3.2. Metabolites in strawberry flowers

5.3.2.1. Metabolite profiles of the whole strawberry flowers in the GM and parental strawberries

After verifying the floral expression of the *NS-Vitis3* transgene, the metabolite composition was studied for the presence of the STS enzyme product resveratrol. Metabolites were extracted with 80% methanol from the flowers of the three GM lines (J47/1, J47/2 and *35S:NS-Vitis3*) together with the parental strawberry cultivar, and analyzed by UPLC-qTOF-MS. The presence of resveratrol in the GM lines was studied particularly carefully by comparing to commercial resveratrol standard and by screening for the most common derivatives of resveratrol (Püssa et al., 2006). No expected metabolites were found when extraction and analysis methods comparable to those reported for other *STS*-modified plants which produce resveratrol or its glucosylated derivative were applied (Thomzik et al., 1997; Starck-Lorenzen et al., 1997; Hipskind & Paiva, 2000). None of the metabolites observed appeared to be unique to the GM lines. Kruskal-Wallis analysis of the metabolites between the three GM lines and the parental strawberry showed no statistically significant differences. In addition, principal component analysis (PCA) did not clearly differentiate between the different strawberry lines (Figure 4B).

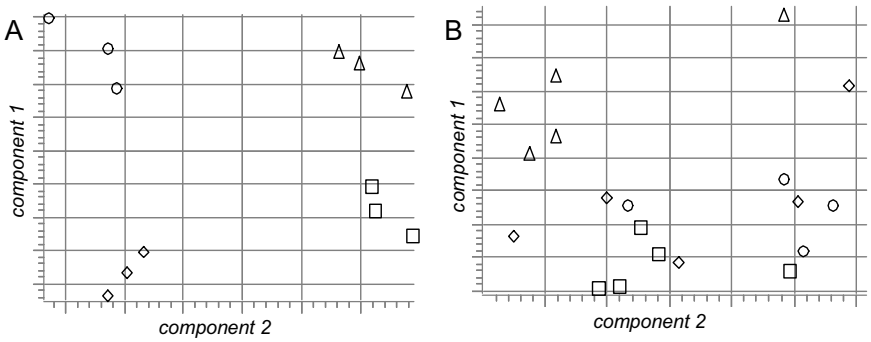

Figure 4. Principal component analysis of the mass signals in ES (-) in (A) Leaves of the *35S:NS-Vitis3* and wild type plants, ◊ = WT old leaves; ○ = *35S:NS-Vitis3* old leaves; □ = WT young leaves; Δ = *35S:NS-Vitis3* young leaves (B) Whole flowers, ◊ = WT; Δ = J47/2; ○ = J47/1; □ = *35S:NS-Vitis3*.

The *STS* gene (*NS-Vitis3*) used in this study originates from *Vitis riparia* (Goodwin et al., 2000). Another Vitis riparia STS gene (termed pBSRIP) has been previously used at least in kiwifruit transformation and the plants were found to produce glucosylated resveratrol (Kobayashi et al., 2000). The most

likely reason for the unaltered metabolite profiles of the GM strawberry flowers is that the transgene expression levels were not high enough to have detectable impact in the metabolite content of the GM plants, in contrast to the leaves of the *35S:NS-Vitis3* line, where *NS-Vitis3* expression levels were higher and the metabolites clustered clearly according to genotype (and age) in PCA (Figure 4A; Chapter 5.4.).

5.3.2.2. Metabolite profiles of the different floral organs showed differential localization of several metabolite classes

In addition to the whole flowers, also the separated floral organs were studied for metabolites. The UPLC-qTOF-MS analysis allowed the tentative identification and the localization of metabolites belonging to various chemical classes (e.g., ellagitannins, proanthocyanidins, flavonols, terpenoids and spermidine derivatives) to the different flower organs. The highly sensitive chromatographic separation by the UPLC and the accurate mass measurement by qTOF-MS facilitated the characterization of several compounds not previously detected in strawberry, e.g., the galloylglucose derivatives (**IV**, Table I). The measurement of accurate mass was inevitable also for the annotation of the neutral losses of various sugars and acyl groups, since several detachable units have very similar molecular weights, e.g. caffeoyl (162.0317 amu) and hexose (162.0528 amu) (**IV**, Table II). In this work, also these losses could be distinguished in the fragmentation pattern, as exemplified by caffeoyl-di-coumaroyl-spermidine and pentose-hexose-glucuronide-kaempferol in Figure 5.

The secondary metabolite composition of strawberry flowers was shown to exhibit high versatility both in terms of different compounds produced, as well as in the amount of specific compounds or even compound classes in the different flower organs. The vast majority of the tentatively identified metabolites were phenolic compounds, among which the most abundant were ellagitannins that accumulated in all five floral organs (**IV**, Figure 7). Extensive variation in the accumulation of different ellagitannin derivatives in the organs was observed. While the role of ellagitannins in strawberry flower is mostly unknown, the present results that show the complexity and variation in ellagitannins between the various floral organs should stimulate further investigations. The vast array of flavonols tentatively identified in this study, altogether 23 derivatives, suggests an important role for this metabolite class in strawberry flower physiology. Also flavonols exhibited differential pattern in the different organs (**IV**, Figure 7). The end products of the flavonoid pathway, proanthocyanidins, were almost exclusively detected in the receptacle and sepals (**IV**, Figure 7).

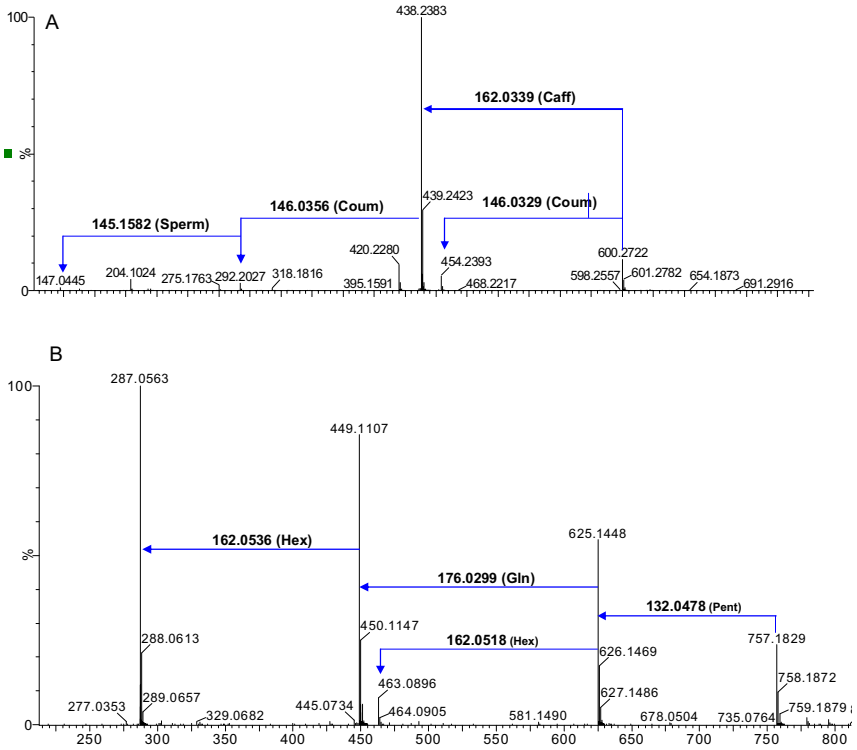

Figure 5. Mass fragmentation in positive ESI in 30 ev of A. caffeoyl-di-coumaroyl-spermidine and B. pentose-hexose-glucuronide-kaempferol. The accurate mass measurement allows the detection of the difference in the loss of hexose (monoisotopic mass 162.0528 amu) and caffeoyl (monoisotopic mass 162.0317), as well as the difference between deoxyhexose (monoisotopic mass 146.0579) and coumaroyl (monoisotopic mass 146.0368). Sperm, spermidine; Coum, coumaroyl; Caff, caffeoyl; Hex, hexose; Gln, glucuronide; Pent, pentose.

Two metabolite classes that have not been previously reported in LC-MS analysis of strawberry were detected in the floral organs, namely spermidines and terpenoid derivatives. Spermidines (esterified with phenolic acids) were present uniquely in the stamen and pistil (**IV**, Figure 7). Acyl-conjucated spermidines have been studied previously in Rosaceae flowers (Starck et al., 1990) and are known to participate in the floral induction of strawberry (Tarenghi and Martin-Tanguy, 1995). The presence of polyamines is essential for flower development (Kumar et al., 1997), but their precise function is not defined.

Terpenoids were detected as triterpenoid saponins (glycosylated triterpenoids) and sesquiterpenoid derivatives, most of them with highest levels in the sepals. Saponins are suggested to serve as antimicrobials and antifeedants, although their precise role is not determined (Osbourn, 2003; Sparg et al., 2004). Mono- and sesquiterpenes are essential components of floral scent mixtures of various species (Pichersky et al., 1994; Aharoni et al., 2005), and may contribute to the fragrance of strawberry flower as well.

Up till now, relatively few studies have been performed on the differences in metabolite composition between the floral organs, the majority of flower-related studies being concentrated on resolving the regulatory factors controlling floral development. The metabolite profiling studies published so far are either non-targeted analyses of the whole flowers (Li et al., 2006; Spitaler et al., 2006) or focused on specific metabolite classes such as flavonoids (Nielsen et al., 2005; Lai et al., 2007) or phenolic acids (Clifford et al., 2006; Lai et al., 2007). As transcriptomics does not indicate the actual biochemical status of the floral organs, metabolite profiling is fundamental for a comprehensive understanding of their physiology. This work provides the basis for a more focused investigation of the metabolic networks in individual floral organs of strawberry.

5.4. Comparison of the leaves of the GM and parental strawberries (III)

Leaves of line *35S:NS-Vitis3* had approximately 10-fold *NS-Vitis3* expression levels compared to the floral organs when analysed by qRT-PCR (Figure 6). Because the impact of the *NS-Vitis3* transgene in the floral organs remained undetectable, the leaves with stronger transgene expression were taken into further characterization.

5.4.1. Metabolite profiles of the leaves clearly differed between the GM and parental strawberries

Metabolite analysis was performed on the leaves of the GM strawberry line *35S:NS-Vitis3*. Both young, pale green folded leaves and mature, dark green fully opened leaves were subjected to the analysis. Similarly to the flowers, the leaves showed no metabolite signals that could be interpreted as resveratrol or any of its derivatives. However, unlike in the flowers, the GM leaves showed significant changes in their metabolite profiles compared to the parental strawberry. The two leaf types from the *35S:NS-Vitis3* line and parental strawberry formed four distinct clusters in PCA analysis (**III**, Figure 4).

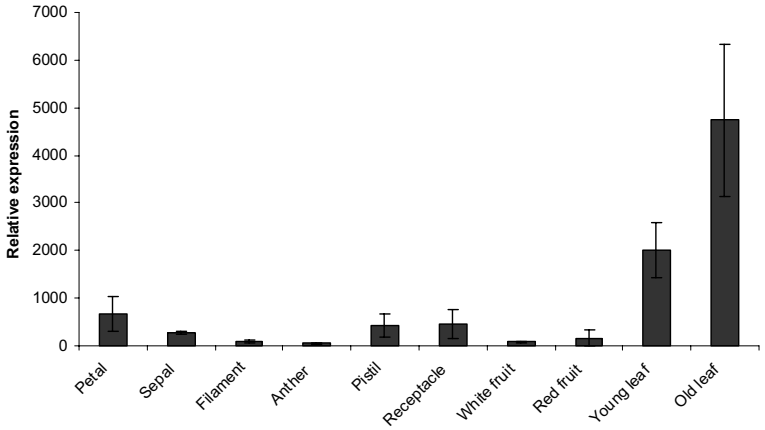

Figure 6. *NS-Vitis3* expression in various organs of GM strawberry line *35S:NS-Vitis3*. The results shown are means of three biological samples with the standard deviation.

A clear pattern was observed in the metabolite profiles of the four samples (young and old, WT and GM). Six derivatives of kaempferol and two quercetin metabolites were identified (Figure 7; **III**, Table I). In line *35S:NS-Vitis3*, the levels of all these flavonol derivatives were decreased significantly compared to the old leaves of the parental line, the acylated kaempferol-coumaroyl-glucosides also in the young leaves (Figure 7). In contrast, phenolic acid derivatives accumulated in the leaves of *35S:NS-Vitis3* at higher levels than in the parental strawberry. Coumaric acid hexose, coumaroyl quinic acid and chlorogenic acid were detected at elevated levels in both young and old leaves of *35S:NS-Vitis3* (Figure 7; **III**, Table I). In addition to the tentatively identified phenolic acid derivatives, several compounds that could not be directly assigned by the MS analysis accumulated in the *35S:NS-Vitis3* line, and were annotated as F1 to F14 (**III**, Table I). These compounds were isolated with semi-preparative HPLC and subjected to structural analysis by NMR (Chapter 5.4.6.).

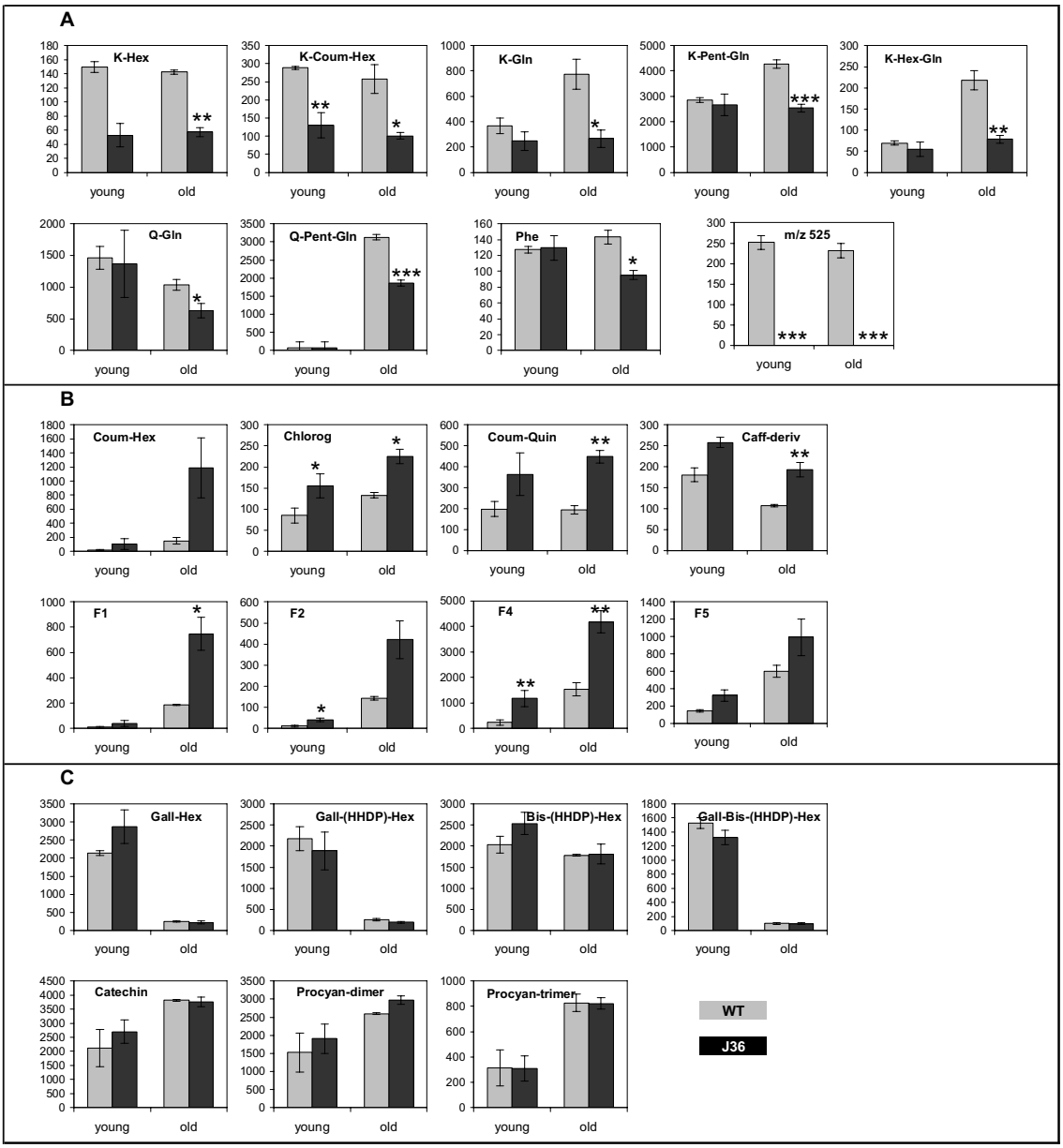

Figure 7. Metabolites identified in strawberry leaves. **A**: Metabolites found at reduced levels in the GM strawberry *35S:NS-Vitis3*. **B**: Metabolites found at elevated levels in *35S:NS-Vitis3*. **C**: Metabolites found at constant levels in both lines. The amounts of metabolites are expressed as relative peak response areas of the molecular ion in three replicates. Significance levels are marked as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Abbreviations: K, kaempferol; Hex, hexose; Coum, coumaroyl; Gln, glucuronide; Pent, pentose; Q, quercetin; Phe, phenylalanine; Chlorog, chlorogenic acid; Quin, quinic acid; Caff, caffeic acid; Gall, galloyl (continue on following page)

Figure 7. (continue) HHDP hexa-hydroxy-di-phenoyl; Procyan, proanthocyanidin. F1, F2, F4 and F5 represent metabolites isolated with semi-preparative HPLC and resolved by NMR analysis (see 5.4.6.), *m/z* 525 indicates a non-identified metabolite (or fragment).

5.4.2. The mRNA analysis revealed down-regulation of chalcone synthase in the leaves of GM strawberry

Since the phenylpropanoid and flavonoid levels in the leaves of *35S:NS-Vitis3* were clearly affected, the mRNA levels of chalcone synthase (CHS), which acts at the interface of these two pathways, was analysed by qPCR (for the pathway see Figure 1). The oligonucleotides used as primers were designed to a region with no homology to the *NS-Vitis3* transgene. The analysis showed that in the old leaves of line *35S:NS-Vitis3* the expression of *CHS* was less than 8% of that in the parental line, indicating a strong down-regulation (**III**, Figure 2B). The results suggested that the inserted *NS-Vitis3* gene caused silencing of the endogenous *CHS*, even though the overall DNA homology between *Fragaria* × *ananassa CHS* (AY997297) and *Vitis riparia NS-Vitis3* (AF128861) genes is only 69% (**III**, Supplemental figure S2).

Post-transcriptional gene silencing (PTGS) is usually triggered by double-stranded RNA formation, referred to as RNA interference (RNAi), and is widely applied to resolve functions of the silenced genes (Kusaba, 2004). In RNAi the formation of double stranded RNA triggers gene silencing *via* degradation of mRNAs of both the inserted gene and the endogenous homologous counterpart. In this study the *NS-Vitis3* mRNA was accumulating in the old leaves of the *35S:NS-Vitis3* line where the *CHS* was most strongly down regulated (**III**, Figure 2A). A theoretical explanation could be that the transgene mRNA is present in the *35S:NS-Vitis3* leaves in such an exess, that it is visible still even part of the mRNA is degraded with the *CHS* mRNA.

The recently obtained results of the presence of resveratrol in certain strawberry cultivars indicate that also other polyketide-type enzymes than *CHS* are present in strawberry, although not yet characterized. Therefore it is also possible that the insertion of the *NS-Vitis3* has silenced an endogenous stilbene synthase gene in strawberry and eventually led to silencing of *CHS* via spreading of RNA silencing, a phenomenon known to occur in plants (Van Houdt et al., 2003). In the case of spreading of RNA silencing, the expression of a secondary target gene may be reduced even if there is no homology to the initial silencing inducer. In this study the silencing by the spreading phenomenon is likely, as the *STS* sequences between different species may be highly homologous (over 99%, Goodwin et al., 2000),

whereas similarities between *STS* and *CHS* are generally much smaller (69% between the genes used in this study, see above).

The transcriptional regulation of genes at the phenylpropanoid-flavonoid branch in strawberry is not well studied. As the inserted gene is similar to strawberry endogenous genes, it may be that insertion of the *NS-Vitis3* gene has resulted in changes at the gene expression regulation and eventually led to the lower transcript levels of CHS.

Several studies have been published on the RNAi mediated silencing of *CHS* by an additional sense copy of the same gene, including reports also for strawberry fruits (Hoffmann et al., 2006; Lunkenbein et al., 2006). The changes in metabolite levels observed in the present study are in line with the effect of *CHS* silencing on strawberry fruits, the flavonoid levels decreasing and phenolic acid levels increasing. The outcome of the insertion of a homologous gene is not always predictable, as has been shown also on the flavonoid pathway, e.g. transfer of *CHS* gene has caused silencing of both the transferred gene and endogenous homolog of petunia (Napoli et al., 1990), whereas insertion of chalcone isomerase (*CHI*) has resulted in an increase in functional protein, leading to enhanced flavonoid content in tomato (Muir et al., 2001).

5.4.3. 2D-Western analysis of leaf showed no major differences between the GM and parental strawberry

The levels of the *NS-Vitis3* transcripts were strikingly high in the *35S:NS-Vitis3* leaves in spite of silencing of *CHS*. Therefore, the STS protein content of the GM and parental leaf material were compared in a two-dimensional Western analysis by using an antibody raised against recombinant STS protein (Giovinazzo et al., 2005). The protein profile was very similar in the two lines. The STS antibody showed extensive cross-reaction with several endogenous proteins in strawberry leaves (**III**, Supplemental figure S1), which has been detected also in GM *STS*-poplar with the same antibody (Giorcelli et al., 2004). A minor protein spot was observed solely in the *35S:NS-Vitis3* line, which could be interpreted as STS protein based on its location. However, the amount was very low, and not amenable for MS identification, and apparently not sufficient for the production of detectable amounts of metabolites (**III**, Supplemental figure S1).

5.4.5. Leaf inoculation indicated decreased resistance against grey mould in the GM strawberry

Since the metabolite profile of the *NS-Vitis3*-transformed strawberry was significantly altered, albeit not in the expected manner, it was of interest to test the susceptibility of the plant to fungal infection. A well-characterised strain of grey mould causing fungus *Botrytis cinerea* Pers.: Fr strain B.05.10 (Quidde et al., 1999) was used to infect mature leaves of the *35S:NS-Vitis3* line and parental strawberries. Pairwise comparison indicated that the lesion areas were statistically significantly larger ($p=0.017$) in the leaves of *35S:NS-Vitis3* than in the parental (**III**, Figure 9B), which suggested that the depletion of flavonol content resulted in increased susceptibility to *Botrytis* infection. The mechanisms responsible for the biochemical responses of strawberry upon pathogen or other environmental stress challenge are rather poorly understood and the general view of the defence response is far from clear. The natural products from flavonoid pathway are widely known to contribute to the plant defence (Grotewold, 2006), also in strawberry (Terry et al., 2004; Halbwirth et al., 2006; Hukkanen et al., 2007). In addition, other individual metabolites or metabolite classes have been suggested to participate in the defence response of strawberry, e.g., antifungal proteins (Martinez et al., 2005; Zhang and Shih, 2007), inducible phytoalexins including triterpenoids (Grayer et al., 2001; Hirai et al., 2000), volatile compounds (Arroyo et al., 2007) and the preformed antimicrobial compound, fragarin (Filippone et al., 1999). In our study, the identified compounds that were decreased in line *35S:NS-Vitis3* were all representatives from the flavonol group, which suggests that their role in the defence is crucial.

5.4.6. Phenylpropanoid glycosides accumulated in GM strawberry leaves

Transformation with *NS-Vitis3* had clear effect on the metabolite profile of strawberry leaves. It was, therefore, of particular interest to determine the structure of the unidentified compounds that accumulated at high levels in the leaves of *35S:NS-Vitis3*. For this purpose, a semi-preparative HPLC system was set up and the individual compounds were collected and purified for NMR analysis, accompanied by UPLC-qTOF-MS/MS. Altogether 14 peaks (F1 to F14) were collected and subjected to the analysis by NMR (**III**, Figure 8A). All of the characterized compounds, except for two flavonol derivatives (F8, F9), were phenylpropanoid glucosides, a compound class that has not been previously reported in strawberry (**III**, Table II). All the compounds contained coumaric-, caffeic- or ferulic acid moiety esterified to hexose sugar with additional phenylpropanoid residue. The putative metabolites found in the collected fractions are summarized in table VI.

Table VI: Summary of the metabolites identified in the NMR and UPLC-qTOF-MS/MS analysis. Fractions that were identified unambiguosly by NMR are marked bold, the others are assigned based on the fractionation in MS/MS analysis.

Fraction	*m/z* (ES-)	Elemental composition	Tentative identification
F1, F4, F6	445.15	$C_{23}H_{26}O_9$	hydroxyphenylethyl-coumaroyl-glucopyranoside
F3, F7	471.13	$C_{24}H_{24}O_{10}$	di-coumaroyl-glucopyranoside
F11	477.1	$C_{22}H_{22}O_{12}$	coumaroyl-galloyl-glucopyranoside
F5	475.16	$C_{24}H_{28}O_{10}$	hydroxyphenylethyl-feruloyl-glucopyranoside
F13, **F14**	461.15	$C_{23}H_{26}O_{10}$	hydroxyphenylethyl-caffeoyl-glucopyranoside
F8, F9	593.13	$C_{30}H_{26}O_{13}$	kaempferol-coumaroyl-glucopyranoside
F2, F10	several		related phenylpropanoid compounds
F12	several		unidentifiable, no typical phenylpropanoid mass fragments

Phenylpropanoid glucosides have been studied in Asian folk medicine and have been characterized from species like *Akebia trifoliata* (Gao & Wang, 2006), *Eurya tigang* (Khan et al., 1992), *Eurya emarginata* (Park et al., 2005a,b), *Orobanche caerulescens* and *Boschniakia rossica* (Lin et al., 2006.) Among the compounds identified from *35S:NS-Vitis3* leaves in this study, the *m/z* 445.15 compound assigned as Eutigoside A (for structural formula, see Figure 2) was among the most abundant ones present in the *35S:NS-Vitis3* leaf extract (**III**, Figure 5B, Table II).

None of the compounds characterized from the transgenic *35S:NS-Vitis3* leaves showed similarity to any polyketide structures that might have resulted from the action of functional STS protein. The conclusion is that the accumulation of phenylpropanoid compounds resulted from the redirection of secondary metabolism after down-regulation of endogenous *CHS* (**III**, Figure 1). The strong accumulation of the newly identified phenylpropanoid glucosides suggests that these compounds are important factors in the phenolic metabolism of strawberry, although their role is yet unknown. Phenylpropanoid glycosides like Eutigoside compounds have undergone extensive pharmacological analysis, and show inhibitory effects on inflammatory mediators (Park et al., 2005a). In addition, eutigosides are cytotoxic for HL-60 promyelocytic leukaemia cells (Park et al., 2005b) and induce their apoptosis (Park et al., 2004). The *35S:NS-Vitis3* leaves accumulated these bioactive compounds at high levels, and might thus serve as an interesting material for pharmacological studies.

5.5. Verification of the transgene effect with the *fil1:NS-Vitis3* lines (III)

Following the detailed analysis of the strong transgene expression possessing leaf tissue of *35S:NS-Vitis3* line, a new runner propagation of all the transgenic lines (*35S:NS-Vitis3*, J47/1, J47/2) together with the parental cultivar was produced to verify the gene transfer effect in the *35S:NS-Vitis3* line and to study whether similar effect is present in the two other transgenic lines.

In the qPCR analysis the *CHS* down regulation was verified for the *35S:NS-Vitis3* line (**III**, Figure 7). In addition, the results were parallel also for the J47/1 and J47/2 lines (**III**, Figure 7), although not as striking as for *35S:NS-Vitis3*, which is in accordance with the lower expression levels obtained for *NS-Vitis3* in the J47/1 and J47/2.

On the metabolite level increased intensities in metabolite signals for phenolic acid containing metabolites were observed in the J47/1 and J47/2 lines similarly as in the *35S:NS-Vitis3* line. Most prominent differences were in coumaric acid hexose (**III**, Figure 6B) and the newly identified coumaroyl-galloyl-glucopyranoside in F11 (**III**, Figure 6C). On the flavonol levels of the J47/1 and J47/2 lines the gene transfer impact was not as pronounced as it was for the line *35S:NS-Vitis3*, as high fluctuation on the levels of individual compounds was observed. Both isomers of kaempferol coumaroyl glucoside were observed in lower levels in all of the transgenic lines than the WT controls (**III**, Figure 6D), but in contrast, the levels obtained for the quercetin pentose glucuronide showed enhanced levels in the two J44 lines, whereas a slight reduction was visible again in the *35S:NS-Vitis3* line (**III**, Figure 6E). Similar effect was observed also for the flavonol derivative kaempferol pentose glucuronide.

6. CONCLUSIONS

The secondary metabolism of strawberry was altered by introducing the gene encoding stilbene synthase (*NS-Vitis3*). Instead of resulting in the production of resveratrol, the *NS-Vitis3* gene unexpectedly down-regulated the endogenous gene encoding chalcone synthase (*CHS*). Down-regulation was clearly observed in the part of the plant where transgene expression was strongest, i.e., in the leaf of line *35S:NS-Vitis3*, but similar trend was observed in the two other GM lines. Until now, strawberry transformation by *STS* has not been reported, but in several other plant species the introduction of *STS* has resulted in successful synthesis of resveratrol.

Comprehensive metabolite profiling by UPLC-qTOF-MS combined with unambiguous identification by NMR was used to study the consequences of the *NS-Vitis3* gene transfer on strawberry secondary metabolism. Flavonoid levels were decreased, and the central phenylpropanoid pathway accumulated phenolic acid derivatives which normally function as precursors of flavonoids. The alterations of phenylpropanoid metabolism suggested down regulation of the endogenous *CHS* and the phenomenon was eventually verified by qPCR analysis of the *CHS* mRNA levels. Metabolite profiling in this study was the key for the understanding of the unintended effect of the genetic modification. Metabolomics might in the future serve as a useful tool to characterise GM crops, complementing the targeted analysis of nutrients, antinutrients and toxins.

In addition to previously identified compounds, several phenylpropanoid derivatives accumulated at high levels in the GM strawberry leaves, and were subsequently assigned as phenylpropanoid glucosides by NMR analysis. Interestingly, these compounds have been previously reported mainly in the context of Asian folk medicine, contributing to the pharmacological activity of the plant extracts. Therefore, the present study could open new avenues for the phytochemical analysis of strawberry. As the analytical methods continue to provide tools for increasingly accurate investigation of the natural resources, it is highly likely that also the presently well-characterised plant species will be shown to contain new compounds, as was demonstrated for strawberry in this Thesis.

In addition to differences in secondary metabolite composition observed between the parental and GM strawberry lines, also significantly different metabolite profiles were characterized among the different floral organs of strawberry. The secondary metabolite composition of strawberry floral

organs was shown to exhibit high versatility in the amount of specific compounds or even compound classes in the different organs, as well as between the different derivatives observed within each metabolite class. Especially abundant in terms of different compounds were the ellagitannins that were shown to be present in all floral organs, but with particular structural diversity in the reproductive organs (stamen and pistil). The role of ellagitannins in floral biochemistry is mostly unknown, but the present results should stimulate more targeted profiling of these compounds in strawberry floral organs. The vast array of flavonols tentatively identified in this study, altogether 23 derivatives, also suggest an important role for this metabolite class in strawberry flower physiology. The comprehensive metabolite picture of strawberry floral secondary metabolism obtained in this Thesis may serve as an important adjunct to studies on other levels of profiling including functional genomics and proteomics, and aid in elucidating floral physiology in general.

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