Metabolic Engineering of Flavonoid Biosynthesis in Apple by Genetic Transformation

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Abstract

Flavonoids are a large family of polyphenolic compounds with manifold functions in plants including pathogen defence. Present in a wide range of vegetables and fruits, flavonoids form an integral part of the human diet and confer multiple health benefits. Modifying flavonoid biosynthesis in fruit crops such as apple offers the opportunity to increase plant resistance against pathogens and the health benefit potential of the fruit. Both overexpression and RNAi-based suppression strategies were used to modify flavonoid biosynthesis in apple. Introducing the maize *Lc* transcription factor gene, responsible for controlling the expression of structural genes of the flavonoid biosynthetic pathway in maize, into *Malus domestica* Borkh. cv. 'Holsteiner Cox' resulted in enhanced anthocyanin accumulation in regenerated shoots. Five independent *Lc* lines were investigated for integration of *Lc* into the plant genome by Southern blot and PCR analyses. The *Lc*-transgenic lines contained one or two *Lc* gene copies and showed increased mRNA levels for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavanone 3 beta-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin reductases (LAR), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR). HPLC-DAD and LC-MS analyses revealed higher levels of the anthocyanin idaein (12-fold), the flavan 3-ol epicatechin (14-fold), and especially the isomeric catechin (41-fold), and some distinct dimeric proanthocyanidins (7 to 134-fold) in leaf tissues of *Lc*-transgenic lines. The levels of phenylpropanoids and their derivatives were only slightly increased. In a second approach the consequences of RNAi silencing of the anthocyanidin synthase (*ANS*) gene to induce a shift towards flavan-3-ols were examined. Five 'Holsteiner Cox' and four 'TNR31-35' transgenic lines were investigated for integration of *ANS* RNAi constructs into the plant genome by Southern blot and PCR analyses. All of the *ANS* RNAi transgenic lines showed decreased mRNA levels for anthocyanidin synthase (ANS). In transgenic 'TNR31-35', the mRNA levels of leucoanthocyanidin reductases (LAR) increased slightly. HPLC-DAD and LC-MS analyses revealed higher levels of the flavonol, the flavan 3-ol catechin and epicatechin in leaf tissues of *ANS* RNAi transgenic

lines. Especially, the levels of anthocyanidin in transgenic ‘TNR31-35’ were significantly decreased. Thus, transformation of *ANS* RNAi in ‘TNR31-35’ resulted in enhanced biosynthesis of flavonol, catechin and epicatechin, which play important roles in phytopathology.

Keywords:

Apple, favonoid, genetic transformation, *Lc* gene, RNA interference.

Zusammenfassung

Eine große Familie sekundärer Pflanzuenstoffe stellen die Flavonoide dar, die sich in mehrere Klassen unterteilen lassen: v.a. Flavanone, Flavone, Flavonole, Flavanole, Anthocyanine. Die Flavonoide üben unterschiedlichste Funktionen in der Pflanze aus (UV-Schutz, Anlockung potentieller Bestäuber, Signal zur Etablierung symbiotischer Beziehungen) und spielen eine herausragende Rolle bei der Pathogenabwehr. Als Bestandteile von Obst und Gemüse stellen sie eine wichtige Komponente der menschenliche Ernährung dar und besitzen zahlreiche gesundheitsförderliche Eigenschaften. Ziel der Studie war durch *Metabolic Engineering* eine generelle Erhöhung der Flavonoid-Akkumulation bzw. eine Erhöhung bestimmter Flavonoide in Apfel zu erzielen. Weiterhin sollte untersucht werden, inwieweit sich eine verstärkte Anthocyanakkumulation als Marker zur Selektion transgener Zellen eignet. Mittels des *Agrobacterium tumefaciens*-vermittelten Gentransfers wurde das *Lc*-Gen aus Mais in die Apfelsorte `Holsteiner Cox` mittels übertragen und überexprimiert. Das Gen kodiert für einen *basic-helix-loop-helix* (bHLH) Transkriptionsfaktor, der die Transkription der Strukturgene des Flavonoidstoffwechsels reguliert. In den transgenen Pflanzen wurde eine verstärkte Transkription fast aller Strukturgene des Flavonoidstoffwechsels erzielt. Metabolitanalysen ergaben, dass besonders Anthocyane sowie Flavan 3-ole und Proanthocyanidine stark in den transgenen Pflanzen akkumulierten. In einem zweiten Ansatz wurde mittels der RNA-Interferenz das ANS-Gen, welches für die Anthocyan-Synthase kodiert, herunterreguliert, um den konkurrierenden Weg zur Synthese der Flavan-3-ole zu begünstigen. Transformiert wurde die grünlaubige Apfel ‘Holsteiner Cox’ und der rotlaubige Apfel ‘TNR31-35’. Fünf transgene ‘Holsteiner Cox’ und vier transgene ‘TNR31-35’ Linien wurden regeneriert und hinsichtlich der Integration und Expression analysiert. Alle *ANS*-RNAi transgenen Linien zeigten verminderte mRNA Levels für die Anthocyanidin-Synthase (ANS). In den transgenen ‘TNR31-35’ Pflanzen, war der mRNA Level der Leucoanthocyanidin Reductase (LAR) leicht angestiegen. HPLC-DAD und LC-MS Analysen zeigten, dass die

Anthocyanakkumulation in dem rotlaubigen Apfel ‘TNR31-53’ starkt vermindert war. Die Reduktion der Expression des *ANS*-Gens führte zu einer verstärkten Biosynthese von Flavonol, Catechin und Epicatechin, die möglicherweise eine Rolle bei der Pathogenabwehr spielen.

Schlagworte:

Apfel, Flavonoid, genetische Transformation, *Lc* Gen, RNA Interferenz.

Abbreviations and terms

ANS	Anthocyanidin synthase
ANR	Anthocyanidin reductase
BAP	6-Benzylaminopurine
CaMV	Cauliflower mosaic virus
cDNA	complementare DNA
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	CoenzymA
Cy	Cyanidin
cv.	Cultivar
DAD	Diode array detection
DFR	Dihydroflavonol 4-reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphat
EDTA	Ethylene-diaminetetraacetic acid
F3'H	Flavonoid 3'-hydroxylase
F3'5'H	Flavonoid 3'-5'-hydroxylase
FGT	UDP-Glucose flavonoid 3-O-glucosyltransferase
FHT	Flavanon 3 beta-hydroxylase
FLS	Flavonol synthase
FNS	Flavone synthase
GA	Gibberellic acid
GUS	X´-GlcA-5-Brono-4-Chlor-3-Indolyl-ß-D-Glucuronicacid
HPLC	High performance liquid chromatography
HC	Holsteiner Cox
IBA	Indole-3-butyric acid
LAR	Leucoanthocyanidin reductase
Lc	Maize leaf colour
LC-MS	Liquid chromatography/mass spectroscopy

MeOH	Methanol
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
NADPH	ß-Nicotinamid-adenin-dinucleotid-Phosphat
NLS	Nuclear localization signal
NZ	Niedzwetzkyana
OD_{600}	Optimal density of 600 nm
PAL	Phenylalanin ammonia-lyase
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	round per minute
TDZ	Thidiazuron
TLC	Thin layer chromatography
Tris	Trishydroxylaminomethane
YEP	Yeast extracts pepton

1 Introduction

1.1 Background and aim of the work

Flavonoids are a large family of polyphenolic compounds with manifold functions in plants. Present in a wide range of vegetables and fruits, flavonoids form an integral part of the human diet and confer multiple health benefits. In fruit crops such as apple flavonoids are of utmost importance as they impart much of the colour and contribute to the flavour of the fruit.

The aim of the work was metabolic engineering of flavonoid biosynthesis in apple by genetic transformation in order to enhance accumulation of specific flavonoid classes, which play important roles in both phytopathology and human health. In this respect it was aimed to transform apple by the *Agrobacterium tumefaciens* mediated gene transfer technology, which is the most commonly used method for the transformation of dicotyledonous plants.

Metabolic engineering of the flavonoid biosynthetic pathway was performed by overexpression of a flavonoid synthesis regulator gene as well as by RNA interference (RNAi). The maize regulatory gene *Lc* was used to enhance flavonoid biosynthesis. Effects on the expression of phenylpropanoid and flavonoid genes and on the metabolite levels of anthocyanins, monomeric and polymeric flavan 3-ols (catechins, proanthocyanidins), flavonols, dihydrochalcones, hydroxycinnamic acids and hydroxybenzoic acids were considered in detail. Down regulation of structural genes of the flavonoid synthesis pathway by RNA interference was used to enhance synthesis of specific flavonoids.

As a side effect, investigations were performed to use the anthocyanin induction as a marker for the selection of transgenic cells.

1.2 Apple

Apple (*Malus domestica* Borkh.) belongs to the *Pomoideae* subfamily *Rosaceae*, along with pear (*Pyrus communis*), plum (*Prunus domestica*) and cherry (*Prunus avium*). The common domesticated apple is believed to have originated in the Heavenly Mountains on the border of Western China, in the former USSR and in Central Asia (Korban and Skirvin, 1984; Phipps et al., 1990). It is one of the most widely cultivated tree fruits. Nowadays more than 7.000 varieties have been described. Different cultivars are available for temperate and subtropical climates. Apples do not flower in tropical climates because they have a chilling requirement (Ghariani and Stebbins 1994).

Figure 1: Apple *(Malus domestica* Borkh.*)*

Apple trees take four to five years to produce their first fruit. Forty-five million metric tonnes of apples were grown worldwide in 2002 (Ministry of Agriculture, Food and Fisheries, British Columbia, 2004). China produced almost half of this. Argentina is the second leading producer, with more than 15% of the world production. Apples account for 50% of the world's deciduous fruit tree production.

The estimated apple crop for 2005 is about 234 million bushels of apples. That is about 79 apples per person (Mattern et al., 2006).

Apples are propagated by two methods: grafting or budding. Apples are self-incompatible and must be cross-pollinated to develop fruit. Pollination management is an important component of apple culture. The basic amount of chromosomes is X=17. Diploid and triploid forms are known.

Apples are affected by a large number of diseases, including apple scab, fire blight, mildew, and bacterial cancer. The diseases are caused by different pathogens, such as fungi, bacteria, nematodes, viruses and phytoplasmas, or can be physiological, genetically or have an unidentified basis. Economic losses are variable since some can kill trees, while others may infect fruits, making them unmarketable.

Apple scab, caused by *Venturia inaequalis*, is the most damaging fungal disease of commercial apple orchards. It presents a wide spread distribution, occurring nowadays in all regions where apple is cultivated and can cause losses of about 80% where the summer presents high humidity and mild temperatures. The apple scab symptoms appear as light, olive-coloured spots on the abaxial surface of sepals of young leaves of the flower buds. The lesions then become olive grey with velvety surface and finally acquire a metallic black colour. Occasionally, small scab spots are produced on twigs and blossoms. Infected fruits are scabby and sometimes cracked. If the fruits are infected early, they become misshapen and frequently drop prematurely. When fruits are infected approaching maturity, the disease causes only small lesions. The control of this disease is normally chemical, and over 20 fungicide applications can be necessary per season (Kollar, 1997).

1.3 Flavonoids

Flavonoids are plant polyphenolic compounds derived from the phenylpropanoid pathway. On the basis of their structure they can be classified into the classes flavanones, flavones, isoflavones, dihydroflavonols, flavonols, leucoanthocyanidins, flavan 3-ols (catechins and the polymeric proanthocyanidins), and anthocyanins. Representatives of most of these classes

are also found in *Malus* (Figure 5). Chalcones are direct precursors of all flavonoids. The prominent dihydrochalcones present in *Malus* are chemical derivatives of them. Flavonoids have manifold functions in plants such as UV-protection, attraction of pollinators, warranty of pollen fertility, regulation of polar auxin transport, establishment of microbial symbioses, and pathogen defence (Treutter, 2005). In fruit crops such as apple, flavonoids are of utmost importance as they impart much of the colour and contribute to the flavor of the apple fruit e.g. by astringency of proanthocyanidins.

The flavonoids are the most important flower pigments in nature because of their tremendous variety and spreading. The role of flavonoids as the major red, blue, and purple pigments in plants has gained these secondary products a great deal of attention over the years. From the first description of acid and basic effects on plant pigments by Robert Boyle in 1664 to the characterization of structural and regulatory genes in the late 20th century, a wealth of information has been collected on the structures, chemical activities, and biosynthesis of these compounds.

Flavonoids represent an important class of natural products occurring in all vascular plants, and also in some mosses. In a single species, dozens of different flavonoids may be present. To date, more than 4000 flavonoid compounds have been isolated and identified. Flavonoids exhibit a wide range of functions in biochemistry, physiology, and ecology of plants, for example in the coloration of flower petals, the fertility and germination of pollen, and the activation of bacterial nodulation genes, which are involved in the nitrogen fixation process, in warding off pathogenic microorganisms (phytoalexins), and in protection against ultraviolet light (UV-B screening pigments). Furthermore, flavonoids can act as plant growth regulators, enzyme inhibitors, insect antifeedants as well as potential anticancer agents in humans.

1.3.1 Functions of flavonoids

1.3.1.1 Functions of flavonoids for the plants

1.3.1.1.1 Plants pigment

The flavonoids are the most important flower pigments. They express colours ranging from white over yellow, red and violet up to the deepest blue. They belong to the genetically, chemically, biochemically and molecular-biologically best-researched chemical compounds of the secondary metabolism (Forkmann and Heller, 1999).

1.3.1.1.2 Defence against diseases in apple

The presence of preformed flavanols in apple leaves may account for efficient defence against the fungus *Venturia inaequalis* (Treutter and Feucht, 1990). The constitutive level of flavanols is involved in defence since: after scab infection, a few cells surrounding the infection site accumulate flavanols (Mayr and Treutter, 1998). It was furthermore shown, that the inhibition of the enzyme phenylalanine ammonia-lyase led to severe scab symptoms on leaves of a resistant cultivar (Mayr et al., 1997). Environmental conditions (N fertilisation) favouring the growth of apple trees inhibited their flavonoid biosynthesis and increased the susceptibility to the pathogen (Rühmann et al., 2002; Rühmann and Treutter, 2003; Leser and Treutter, 2005). A transient inhibition of the flavanone 3-hydroxylase (FHT) in apple leaves by treatment with the dioxygenase inhibitor Prohexadione-Ca® induced several changes in their flavonoid composition. The most pronounced change is an accumulation of flavanones, which are further metabolised through an unusual pathway towards the 3-deoxycatechins (Römmelt et al., 2003; Halbwirth et al., 2003). Concomitant with this altered flavonoid pathway, the susceptibility of apple leaves to scab (*Venturia inaequalis*), as well as to the pathogenic bacterium *Erwinia amylovora*, decreased (Bazzi et al., 2003).

1.3.1.1.3 Auxin transport inhibitors

A number of lines of experimentation have suggested that specific classes of flavonoid compounds may act as auxin transport inhibitors *in vitro*. The idea that phenolic compounds might block auxin transport was first proposed in the 1970s (Marigo and Boudet, 1977). Plants grown on quinic acid accumulated phenolic compounds, including but not limited to flavonoids, and had reduced auxin transport. Also, some flavonoids reduce polar auxin transport in zucchini

hypocotyls (Jacobs and Rubery, 1988). A range of flavonoid compounds have been screened for their ability to block binding of a synthetic auxin transport inhibitor, naphthylphthalamic acid (NPA) and to inhibit auxin movement from hypocotyl segments (Jacobs and Rubery, 1988). One report (Fischer et al., 1987) indicates that quercetin produces developmental alterations that are similar to those produced by NPA in wheat embryos. *In vivo* data demonstrate that changes in endogenous flavonoid concentration lead to changes in auxin transport that strengthen the hypothesis that flavonoids are endogenous regulators of auxin transport.

1.3.1.1.4 UV- protection

Evidence for a UV-protective role of flavonoids were listed by Reuber et al. (1996), Shirley (1996), and Rozema et al. (1999): epidermal flavonoids which absorb UV radiation protect the internal tissues of leaves and stems; sensitivity of flavonoid deficient mutants of maize and *Arabidopsis thaliana* to UV-B radiation is increased; flavonoids are potent scavengers of reactive oxygen species and thus prevent peroxidation of lipids. It is assumed by several authors that flavonoids may also provide antioxidant functions in response to excess light (Gould et al., 2002; Rozema et al., 2002; Tattini et al., 2004). Harmful UV radiation leads to the formation of flavonoids, which accumulate in the epidermis and protect plants against photo-oxidative damage. This protection prevents dangerous photo lesions within the range of photo system II, in the DNA, with enzymes such as RUBISCO and the peroxidation of membrane-lipids. Just like by the cooxidation of carotinoids during the radical chain process, the flavonoids can act as radical inhibitors (radical scavenger) and by inactivating reactive oxygen species reduce or remove oxidative damages caused on tissues (Feucht and Treutter, 1989).

1.3.1.1.5 Antioxidant

Flavonoids are the quantitatively most frequent and most effective anti-oxidative compounds in vegetable food. They are active both in hydrophilic and in lipophilic systems due to their chemical structure. For its anti-oxidative effect

number and position of the free hydroxyl groups as well as the appearance of a carbon double connection at the C-ring play substantial roles (Feucht and Treutter, 1989).

1.3.1.2 Functions of flavonoids for the human health

1.3.1.2.1 Anti-cancer

There are numerous references to anti-cancer effects of flavonoids *in vivo*. When humans absorb a large number of flavonoids through vegetables and fruits, the stomach-, large intestine- and breast-cancer risk is reduced (Watzl and Leitzmann, 1999). Among dietary factors, certain phytochemicals particularly those in the daily diet, have marked cancer chemopreventative properties. Genistein, a flavonoid abundant in soy, shows anticancer activity against human breast cancer cells (Nakagawa et al., 2001). Resveratrol, a naturally occurring product found in grapes and wine, is another dietary phytochemical that inhibits human breast cancer cell growth (Mgbonyebi et al., 1998; Nakagawa et al., 2001). Among people with high dietary intakes of quercetin and other major flavonoids, studies show lower rates of breast, lung, pancreatic, and stomach cancers (Knekt et al., 2002).

1.3.1.2.2 Enzyme inhibition

Numerous *in vitro* attempts as well as observations at living organisms showed an immune-modulator effect of the flavonoids. These secondary metabolites, which are widespread in vegetables, possibly influence the immunological equilibrium. The effect of the flavonoids depends on their chemical structure. The predominant number of the available studies occupies an immune-suppressive effect of the flavonoids, whereby quercetin was particularly intensively examined.

Certain flavonoids affect directly the protein kinases (which give the signal to different proteins in the cells for intensive growth through catalysis of the phosphorylation), by usually restraining the activity of these enzymes. Quercetin also blocks the enzyme phospholipase A2, which is needed for the synthesis of prostaglandins from the unsaturated fatty acid arachnidan acid. Apart from their

influence on proteinases flavonoids further modulate regulation mechanisms of the immune system. Certain flavonoids e.g. quercetin, myricetin as well as kaempferol, block the histamine release and hinder the activity of the lipoxygenase (Watzl and Leitzmann, 1999).

1.3.1.2.3 Anti-thrombotic

Different flavonoids can influence the blood clotting. Their effect is due to the inhibition of the arachnidan oxygen metabolism, whose final products are the prostaglandin and the thromboxan. Flavonoids can direct inhibit enzymes activity of the lipoxygenase systems and thus lead to a reduction of thromboxan-A2 formation. Otherwise because of their anti-oxidative characteristic effect the blood clotting will be restrained (Watzl and Leitzmann, 1999).

1.3.2 Structure of flavonoids

Early work in structure elucidation of anthocyanins, flavones, and flavonols revealed that flavonoids have a basic c6-c3-c6 skeleton structure in common, consisting of two aromatic rings (A and B), and a heterocyclic ring (C) containing one oxygen atom (Figure 2, Forkmann, 1999).

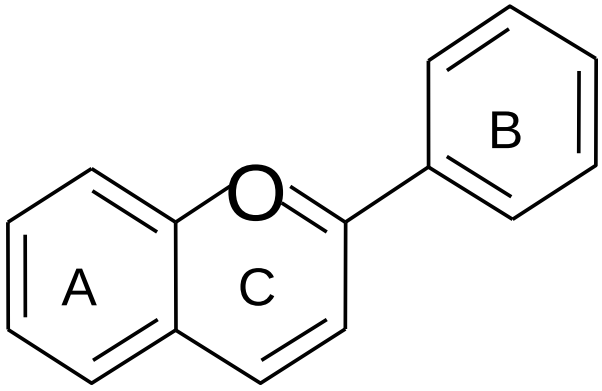


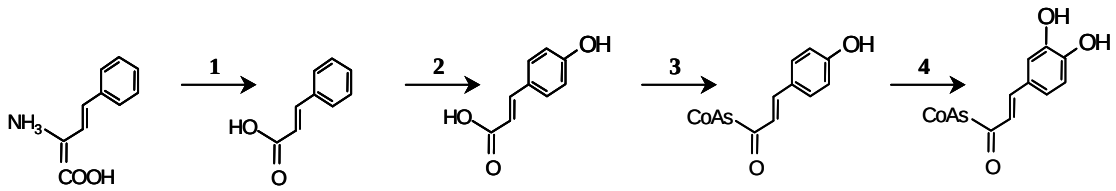
Figure 2: Basic structure of flavonoid

1.3.3 Flavonoid biosynthesis pathway

The overall flavonoid synthesis pathway can be dissected into three blocks, which can produce different sets of specialized products.

1. Pre-steps

Most of the biosynthesis of the chemical compounds in this stage is formed from phenylalanine, and phenylalanine-ammonium lyase (PAL) is the key enzyme for flavonoid synthesis. The first four steps in the pathway are common to a wide range of secondary metabolites. This is the core phenylpropanoid pathway (Figure 3).



Phenylalanine Cinnamate Coumarate P-coumaroyl-CoA Caffeoyl-CoA

Figure 3: Biosynthesis of the hydroxy cinnamon acid ester.

1. Phenylalanin-Ammonia-Lyase (PAL). 2. Cinnamate 4-Hydroxylase (C4H).

3. p-Cumarate-CoA-Ligase (4CL). 4. p-Cumaroyl-CoA-Hydroxylase (CC3H).

2. Main steps

The main steps of flavonoids synthesis lead to synthesis of anthocyanidins. There is a series of reactions, ending with the addition of glucose to the anthocyanidin. In this step, anthocyanidins are synthesized, there are 3 types: delphinidin, pelargonidin and cyanidin. The three anthocyanidins and their intermediate products show different colour.

The chalcone synthase (CHS) catalyzes the sequential condensation of three acetate units from malonyl-CoA with a suitable hydroxycinnamic acid CoA ester giving chalcones as the central C_{15} intermediates from which all flavonoids originate (Figure 4, Forkmann 1999).

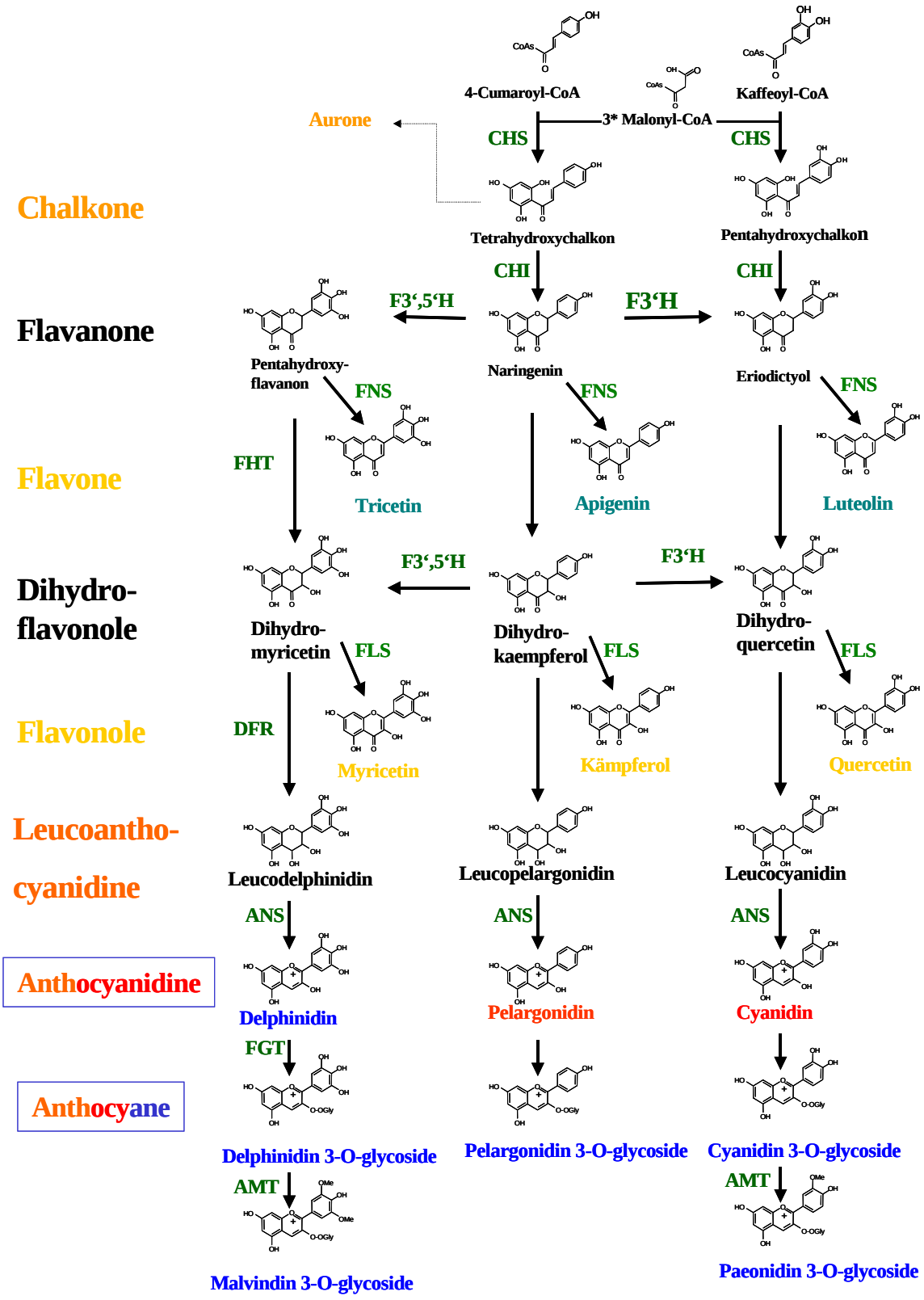


Figure 4: Main steps of flavonoid synthesis.

Chalcone isomerase was the first enzyme of the flavonoid pathway detected. It catalyzes the stereospecific cyclization of chalcones to flavanones, which were found to be the exclusive substrates for the reactions to other flavonoid classes.

There are two different flavone synthases, FNS I and FNS II. FNS I is apparently not very widespread, since it is only found in some members of the plant family *Apiaceae*. In contrast, FNS II has been observed in many plant species. The FNS II reaction has an absolute requirement for NADPH and molecular oxygen. The distribution of FNS I and FNS II in the plant kingdom might therefore be of taxonomic significance and might also be important in considering the evolution of flavonoid biosynthesis (Forkmann, 1999).

Flavonol synthase catalyzes the introduction of a double bond between C-2 and C-3 of dihydroflavonols.

Flavanone hydroxylase catalyzes the hydroxylation of flavonones to dihydroflavonols. It was classified as a 2-oxoglutarate-dependent dioxygenase according to its requirement of 2-oxoglutarate and ferrous iron in the enzyme assay.

With NADPH as a cofactor, dihydroflavonol 4-reductase (DFR) catalyzes the reduction of dihydroflavonols to the leucoanthocyanidins.

Anthocyanidin synthase (ANS) gives flavan-3, 4-diol a double bond between C-2 and C-3 and leads to anthocyanidin synthesis. UDP-Glucose: flavonoid 3-*O*-glucosyltransferase (FGT) catalyzes the transfer of glucose to the hydroxyl group in position 3 of flavonols and anthocyanidins (Forkmann, 1999).

3. Modification steps

Modification by hydroxylation, methylation, glycosylation, acylation, and prenylation occurs within virtually all flavonoid classes. However, some other modifications, such as C-glycosylation and sulfation, are restricted to a few classes as known so far. Hydroxylation of both aromatic rings and methylation of hydroxyl groups lead to the formation of the different aglycones within each flavonoid class. Glycosylation of the hydroxyl group of flavonoid aglycones not only increases water solubility but also provides new substrates for further glycosylation leading to di- and even oligoglycosides, and for acylation with both

aliphatic and aromatic acids. Various enzymes have been described catalyzing hydroxylation, methylation, glycosylation, acylation, and other reactions on flavonoids (Forkmann, 1999).

1.3.4 Flavonoid biosynthesis in apple

The phenolic compounds, so far isolated and identified from apple tissues, and their synthesis pathway in apple, are given in Figure 5.

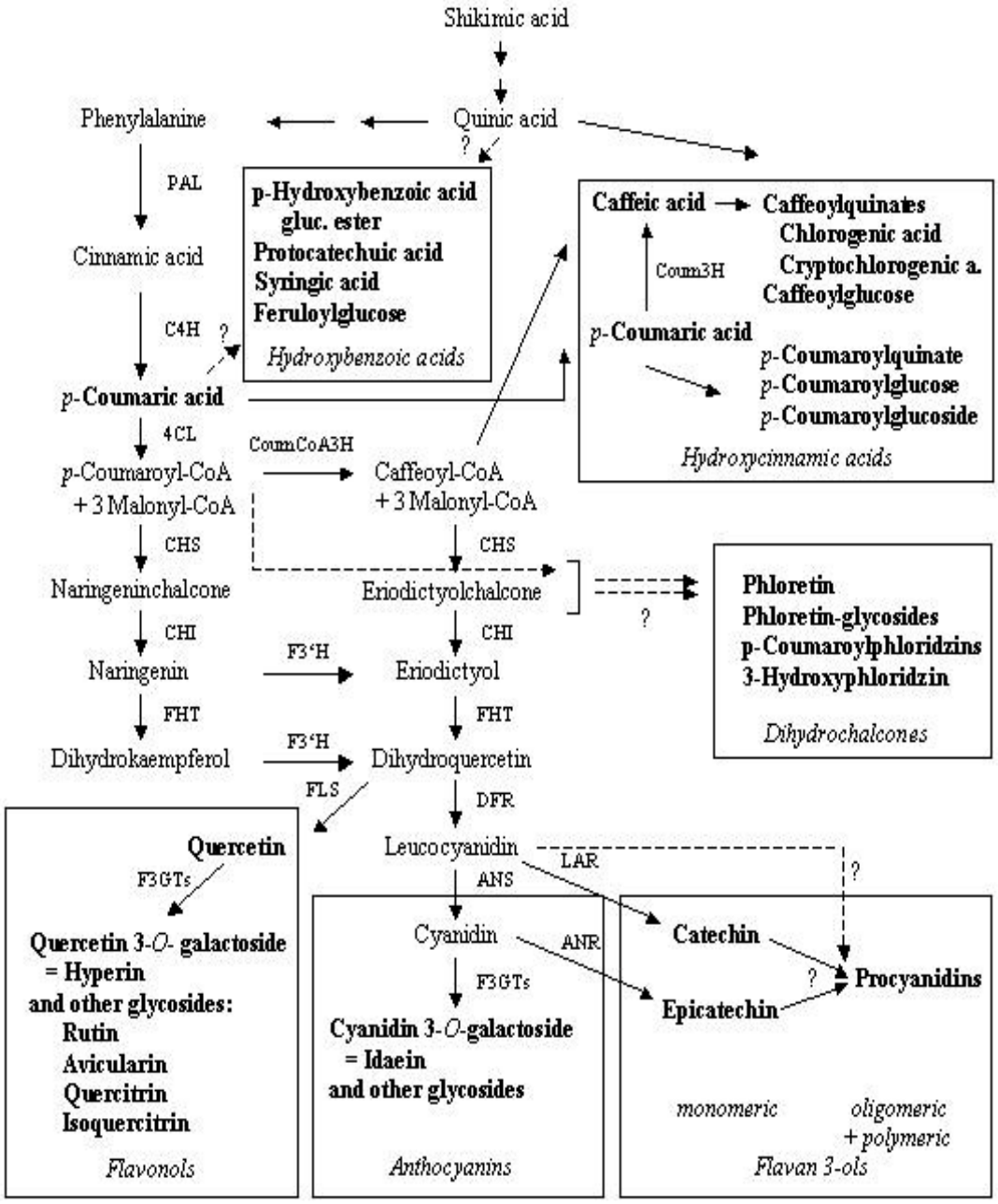


Figure 5: Biosynthetic Pathways of Phenolic Compounds in Apple (Szankowski, personal communication**).**

1.4 Metabolic engineering of flavonoid biosynthesis

Nowadays, the flavonoid biosynthetic pathway has been almost completely elucidated. Many of the structural and some of the regulatory genes have been cloned from several model plants, including maize, Antirrhinum, tobacco, Petunia and Arabidopsis (Holton and Cornisch, 1995) and have been expressed in genetically modified model plants and micro-organisms (Dixon and Steele, 1999; Forkmann and Martens, 2001). Today, standard molecular tools are available to genetically modify plants including several world-wide important crops such as maize, potato, tomato, sugar beet and wheat.

1.4.1 Regulators controlling the flavonoid pathway

Coordinate transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the final levels of secondary metabolites in plant cells. This regulation is achieved by specific transcription factors. These DNA binding proteins interact with promoter regions of target genes and modulate the rate of initiation of mRNA synthesis by RNA polymerase II (Ranish and Hahn, 1996). Regulatory genes, in particular those controlling pigmentation intensity and pigmentation pattern through influencing the expression of many different structural genes, have been identified in many plants (Holton and Cornisch, 1995).

Several of the anthocyanin *myc* and *myb* regulatory genes have been tested for their ability to improve anthocyanin accumulation when expressed in heterologous plants. However, up to now the success of this strategy has been highly variable. Amongst the most well characterized regulatory plant genes is the maize leaf colour (*Lc*) gene belonging to the MYC type *R* gene family and the MYB type *C1* (colourless) gene. Already more than a decade ago, activation of anthocyanin production was achieved in Arabidopsis and tobacco plants by the introduction of the maize regulators *R* and *C1*. In both plant species, expression of the *R* regulatory genes resulted in enhanced anthocyanin pigmentation of tissues that normally produce anthocyanins, whereas in Arabidopsis also an increased trichome production was observed. The *C1* gene alone had no visible effect. Accumulation of anthocyanins in tissues that normally do not contain any

anthocyanins was observed in hybrid transgenic Arabidopsis plants expressing both *C1* and *R* (Lloyd et al., 1992).

In many dicot species, expression of 35s- *Lc* resulted in enhanced anthocyanin pigmentation. For example, in *Lc* over-expressing cherry tomato plants, anthocyanins accumulated in leaves, stems, sepals, petal, in the main vein and, to a lesser extent, in developing green fruits (Goldsbrough et al., 1996). Not only anthocyanins, but also other classes of flavonoids have been reported to accumulate when *Lc* and *C1* are ectopically expressed. For example in red ripe tomato fruits, which normally produce only small amounts of the flavonols kaempferol and quercetin in the fruit peel tissue, the introduction and co-ordinate expression of the maize regulatory genes *Lc* and *C1* under the control of a combination of general and fruit specific promoters, was sufficient to up-regulate the flavonoid pathway in the fruit flesh, a tissue that normally does not produce flavonoids (Bovy et al., 2002).

There are numerous reports of the regulation of genes in the anthocyanin pathway by transcription factors (TFs), and collectively these have established that the components of the regulatory complex controlling anthocyanin biosynthesis are conserved in all higher plants (Holton and Cornish, 1995). MYB TFs have been shown to play an important role in transcriptional regulation of anthocyanins. Plant MYBs have been implicated in controlling pathways as diverse as secondary metabolism (including the anthocyanin pathway), development, signal transduction and disease resistance (Jin and Martin, 1999). Over-expression of *MdMYB10* in apple generated a strong phenotype with strong pigmentation due to enhanced levels of anthocyanins. The effect of *MdMYB10* over-expression on the pigmentation of fruit cortex will be apparent when *MdMYB10* transgenic plantlets have fruited (Espley et al. 2006).

1.4.2 Modifying of the structural genes

Down-regulation or over-expression of structural flavonoid genes in transgenic plants have shown to be useful tools to elucidate the function of flavonoid pathway genes. Furthermore, over-expression of structural genes can be used in metabolic engineering strategies to overcome rate-limiting enzymatic steps in the pathway. In this way, the flux through an already existing pathway of the

hostplant can be increased, which may lead to enhanced levels of specific flavonoids or even new flavonoids. As outlined below, this approach has been used extensively to increase the flavonoid content of tomato fruit, in order to improve the food quality of this important crop (Schijlen et al., 2004).

1.4.2.1 Over-expression of structural flavonoid genes

Structural flavonoid genes can be over-expressed by introducing structural genes. In potato, a *Solanaceous* crop, several attempts have been made to increase the flavonoid production in the tubers by introducing structural genes. Over-expression of *chs* cDNA from *Petunia* resulted in an increase in petunidin and pelargonidin-type anthocyanins in potato tubers. This result could not be obtained, however, with all *chs* genes. For example the cDNA encoding CHS from barley was not functional in transgenic potato (Lukaszewicz et al., 2004). Transgenic potato plants over-expressing a *Petunia* cDNA encoding DFR resulted in an increase of tuber anthocyanins, namely a 3-fold increase in petunidin and a 4-fold increase in pelargonidin derivatives (Lukaszewicz et al., 2004).

1.4.2.2 Down regulation of structural flavonoid genes with anti sense constructs

Antisense molecules interact with complementary strands of nucleic acids, modifying expression of genes. A significant decrease in anthocyanin levels was observed when the plants were transformed with a corresponding anti-sense *DFR* construct (Lukaszewicz et al., 2004). Introduction of a constitutive antisense full-length chalcone synthase (CHS) cDNA gene in petunia can result in an inhibition of flower pigmentation (Alexander et al., 1990).

1.4.2.3 Down regulation of structural flavonoid genes with RNA interference

RNA interference was an efficient method for the down regulation of structural flavonoid genes. Flavonoid biosynthesis pathway was blocked by down-regulation of the using RNA interference (RNAi)-mediated suppression of chalcone synthase (CHS), the first gene in the flavonoid pathway. In *CHS* RNAi plants, total flavonoid levels, transcript levels of both *CHS*1 and *CHS*2, as well as

CHS enzyme activity were reduced by up to a few percent of the corresponding wild-type values (Schijlen et al., 2007). Isoflavone synthase (IFS) is a key enzyme for the formation of the isoflavones. The silencing of both copies of IFS genes in roots initiated from soybean cotyledons leads to the effective spread of silencing throughout the nontransformed cotyledon tissues. Distal silencing effected a nearly complete suppression of mRNA accumulation for both the *IFS1* and *IFS2* genes and of isoflavone production induced by IFS RNA interference (Subramanian et al., 2005).

1.5 *Lc* gene

The leaf colour (*Lc*) gene from maize is one of the best characterized regulatory genes (Ludwig et al., 1989). This gene, belonging to the *myc* type R gene family, encodes a protein with the basic helix-loop-helix (bHLH) motif. *Lc* is one of the transcription factors which determine the amount, location and timing of anthocyanin accumulation in maize. The heterologous expression of *Lc* or combinations of *Lc* along with the *myb* type *C1* (colourless) gene resulted in increased anthocyanin pigmentation in *Nicotiana tabacum* L., Arabidopsis (Lloyd et al. 1992), *Lycopersicon esculentum* Mill (Goldsbrough et al. 1996), a Petunia hybrid (Bradley et al., 1998), *Medicago sativa* L. (Ray et al., 2003) and *Caladium bicolor* (Li et al., 2005). Flavonoids of other classes have also been reported to accumulate after heterologous *Lc* expression. In the Petunia hybrid, an up-regulation of anthocyanin contents occurred accompanied by up-regulation of gene expression of most but not all enzymes of the flavonoid pathway (Bradley et al., 1998). Flavonol contents were positively and negatively influenced in a tissue-specific way. The coordinate expression of the maize regulatory genes *Lc* and *C1* in transgenic *L. esculentum* resulted in an increased production of anthocyanins (in leaves) and of the flavonol kaempferol (in leaves and fruits; Bovy et al. 2002). In *M. sativa*, the transgenic *Lc* gene caused induction of anthocyanins and proanthocyanidins correlated with a reduction of flavone concentrations (Ray et al., 2003).

1.6 RNA interference

RNA interference is a RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute (Bagasra and Prilliman, 2004).

1.6.1 Functions of RNA interference

RNA interference is a vital part of the immune response to viruses and other foreign genetic material, especially in plants where it may also prevent self-propagation by transposons. Plants such as *Arabidopsis thaliana* express multiple dicer homologs that are specialized to react differently when the plant is exposed to different types of viruses (Blevins et al., 2006). Even before the RNAi pathway was fully understood, it was known that induced gene silencing in plants could spread throughout the plant in a systemic effect, and could be transferred from stock to scion plants via grafting. This phenomenon has since been recognized as a feature of the plant adaptive immune system, and allows the entire plant to respond to a virus after an initial localized encounter (Palauqui et al., 1997). In response, many plant viruses have evolved elaborate mechanisms that suppress the RNAi response in plant cells. These include viral proteins that bind short double-stranded RNA fragments with single-stranded overhang ends, such as those produced by the action of dicer. Some plant genomes also express endogenous siRNAs in response to infection by specific types of bacteria. These effects may be part of a generalized response to pathogens that downregulates any metabolic processes in the host that aid the infection process (Mérai et al., 2006).

Endogenously expressed miRNAs, including both intronic and intergenic miRNAs, are most important in translational repression and in the regulation of development, especially the timing of morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types such as stem cells (Carrington and Ambros, 2003). The role of endogenously expressed miRNA in

downregulating gene expression was first described in *C. elegans* in 1993 (Lee et al., 1993). In plants this function was discovered when the "JAW microRNA" of Arabidopsis was shown to be involved in the regulation of several genes that control plant shape. In plants, the majority of genes regulated by miRNAs are transcription factors; thus miRNA activity is particularly wide-ranging and regulates entire gene networks during development by modulating the expression of key regulatory genes, including transcription factors as well as F-box proteins (Jones-Rhoades et al., 2006). In many organisms, including humans, miRNAs have also been linked to the formation of tumors and downregulation of the cell cycle. Here, miRNAs can function as both oncogenes and tumor suppressors (Zhang et al., 2007).

1.6.2 Mechanism of RNA interference

Eukaryotic cells use a specialized type of RNA degradation as a defence mechanism to destroy foreign RNA molecules, specifically those that can be identified by virtue of their occurrence within the cells in double-stranded form. Termed RNA interference (RNAi), this mechanism is found in a wide variety of organisms, including single-celled fungi, plants, worms, mice and probably humans-suggesting that it is an evolutionarily ancient defence mechanism. In plants RNA interference protects cells against viruses. The presence of free, double-stranded RNA triggers RNAi by attracting a protein complex containing an RNA nuclease and an RNA helicase. This protein complex cleaves the double –stranded RNA into small (approximately 23 nucleotide pair) fragments which remain associated with the enzyme. The bund RNA fragments then direct the enzyme complex to other RNA molecules that have complementary nucleotide sequences, and the enzyme degrades these as well. These other molecules can be either single- or double- stranded (as long as they have a complementary strand). In this way, the experimental introduction of a double-stranded RNA molecule can be used by scientists to inactivate specific cellular mRNAs (Figure 6).
Each time it cleaves a new RNA, the enzyme complex is regenerated with a short RNA molecule, so that an original double-stranded RNA molecule can act

catalytically to destroy many complementary RNAs. In addition, the short double-stranded RNA cleavage products themselves can be replicated by additional cellular enzymes, providing an even greater amplification of RNA interference activity. The amplification ensures that once initiated, RNA interference can continue even after all the initiating double-stranded RNA has been degraded or deleted out. For example, it permits progeny cells to continue carrying out RNA interference that was provoked in the parent cells. In addition, the RNA interference activity can be spread by the transfer of RNA fragments from cell to cell. This is particularly important in plants, because it allows an entire plant to become resistant to an RNA virus after only a few of its cells have been infected (Schijlen et al., 2007).

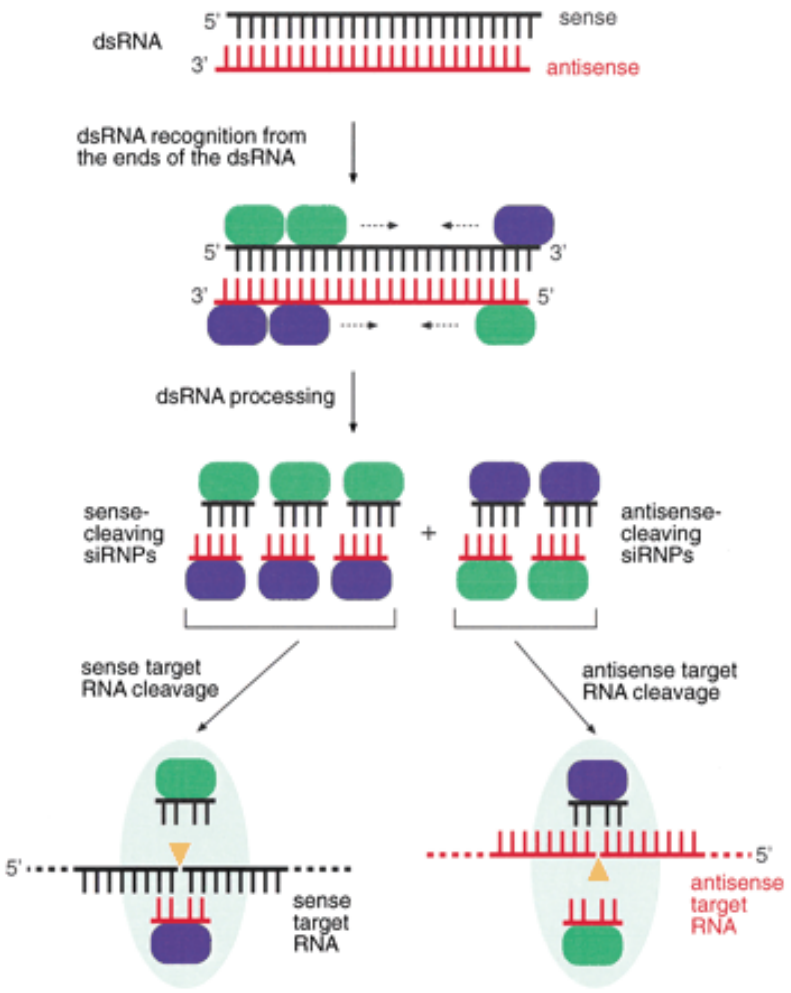


Figure 6: RNA cleavage through RNA interference (Schijlen et al., 2007)

1.7 Genetic transformation

In theory, plant transformation provides the opportunity to transfer any gene from any source into plant cells. These cells can then be regenerated to produce transgenic plants that contain the new genetic information in every cell. Transgenic plants have now been produced from a surprisingly large number of mono- and dicotyledonous species.

1.7.1 Biology of *A. tumefaciens*

Agrobacterium is a plant pathogenic gram-negative bacterium and often found in soil. Crown gall disease can be induced in most dicotyledonous plants by *Agrobacterium tumefaciens*. The bacterium is a parasite that affects a genetic change in the eukaryotic host cell, with consequences for both parasite and host. It improves condition for survival of the parasite and causes the plant cell to grow like a tumor. In a natural infection the *Agrobacterium* infects the plant and inserts a part of its own DNA, the so-called T-DNA (transfer DNA) into the chromosomal DNA of the infected plants cells. On the T-DNA oncogenes alter the plants endogenous production and the sensitivity to the growth hormones auxin and cytokinin and also the production of opines. Opines are amino acids that the bacteria use as carbon and nitrogen sources but cannot be metabolized by the plant (Figure 7). Different bacterial strains are classified according to the opine which they produce directly, namely mannopine, octopine, succinamopine or argenine. The T-DNA from *A. tumefaciens* causes galls while the T-DNA from *A. rhizogenes* causes roots with abnormal growth (Odenbach et al., 1997).

Agrobacterium-mediated transformation of plants has been adapted to many dicotyledonous plant species. The wide applicability of this transformation method is a result of several factors: the broad host range of *Agrobacterium*, the regeneration responsiveness of many different tissues, and the utility of a wide range of selectable marker genes (Odenbach et al., 1997).

1.7.2 Ti-plasmid

The T-DNA is located on a large Tumor inducing (Ti) plasmid (> 100 kb) which contains two additional classes of genes: *Vir* and Opine catabolism. *Vir* genes encode the proteins needed for the formation of T-DNA, T-pilus and

transport of the T-DNA to the host cells nucleus (Michielse et al., 2004). While the Opine catabolism genes encodes the enzymes needed for the utilization of the opine compound released by the plant host following successful transformation. The boundaries of the T-DNA region is defined by two imperfect repeats (25 bp long) called right and left border, RB and LB respectively (Figure 7) (Frandsen et al., 2006).

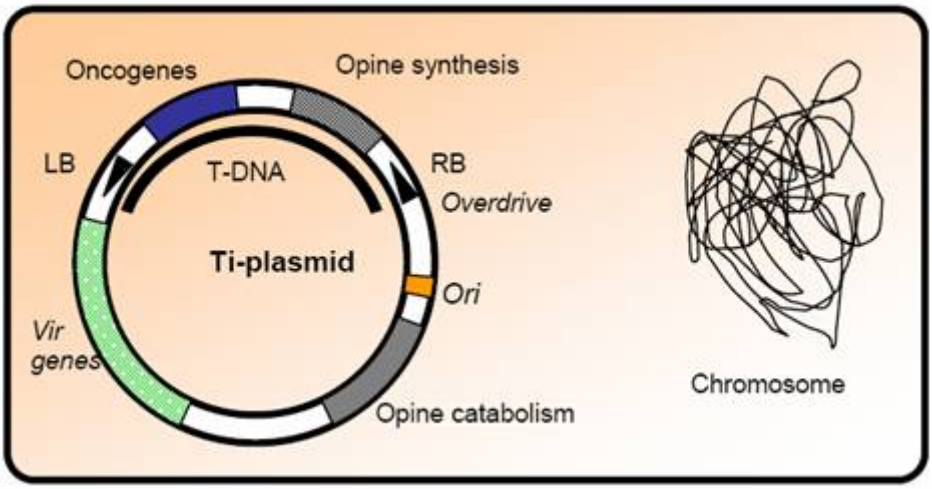


Figure 7: Structure of Ti-plasmid. The T-DNA stretches from RB to LB, including oncogenes and opine synthesis genes (Frandsen et al., 2006).

1.7.3 The mechanism of gene transfer

Agrobacterium typically infect wounded plants. The release of plant saps, containing amino acids, sugars and organic acids, attracts the bacteria to the wound by positive chemotaxis. Once the bacteria reach the wound, it attaches itself to the plant surface by synthesizing cellulose fibres. In addition to the mentioned metabolites wounding plants also produce a wide range of phenolic compounds, such as coniferyl alcohol and acetosyringone. These compounds induce the bacteria to generate T-DNA, by a two component signalling system (VirA/VirG). Acetosyringone activates VirA, a membrane bond receptor, which activates the VirG (transcription factor). The activated VirG can then interact with activator elements found in the promoters of the virA, virB, virC, virD, virE and virG operons, resulting in elevation of their expression levels (Figure 8).

VirC and virD (both nicking endonucleases) bind to the RB/overdrive sequence and cut the ssT-DNA region out of the Ti plasmid. VirE2 binds to the ssT-DNA, protecting it from degeneration by nucleases and self-annealing. VirB2-B11 forms a T-pilus through which the VirE2 coated ssT-DNA is transferred from the bacteria into the targeted plant cell.

Inside the host cell, a C-terminal located NLS in VirE2 directs the DNA into the nucleus, where host factors are believed to facilitate its integration into the genome, possibly mediated by the DNA repair system. If no great sequence similarity exists between the plant genome and the introduced T-DNA, the T-DNA integrates randomly into the plant nuclear genome (Michielse et al 2004).

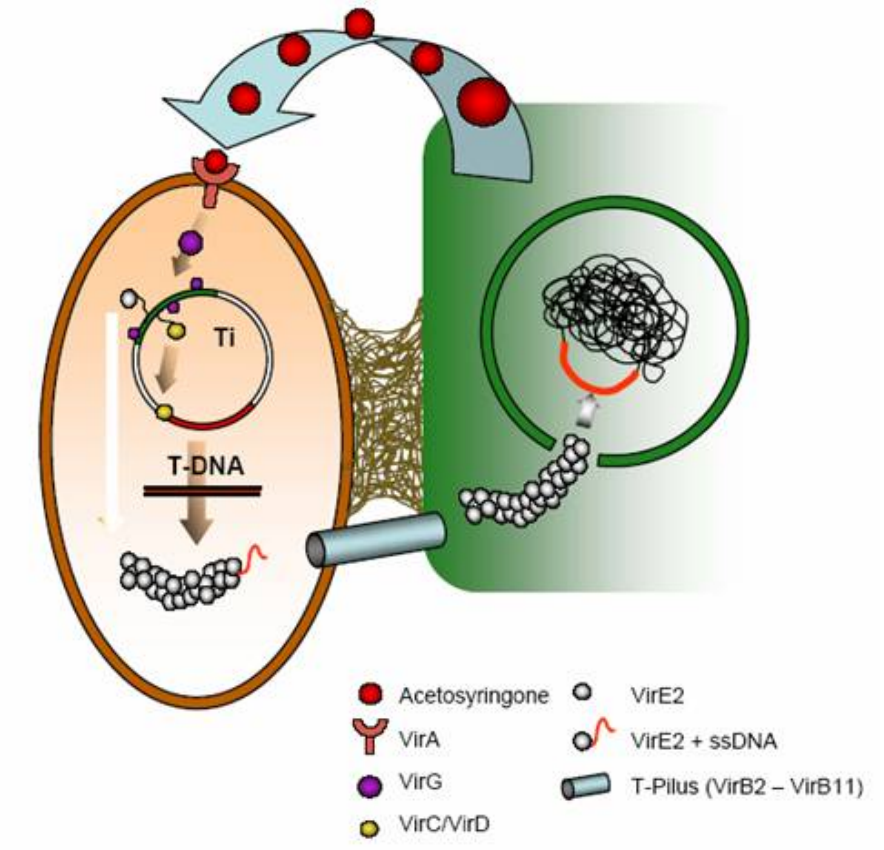


Figure 8: The natural infection cycle of *A. tumefaciens* (Frandsen et al., 2006)

2 Materials and methods

2.1 Materials

2.1.1 Instruments

Table 1: Instruments

Instrument	Manufacturer
Biometra® WT12 tumbling table	Biometra (Goettingen, Germany)
Biophotometer	Eppendorf (Hamburg, Germany)
Centrifuge Z200	Hermle (Wehingen, Germany)
Centrifuge Z300	Hermle (Wehingen, Germany)
Centrifuge Sigma-®302K	Heiz Eich (Bonn, Gemany)
DC-Cellulose-Platen 10*20cm	Roth (Karlsruhe, Germany)
Electrophoresis-System ELITE 300 Plus	Polymer (Germany)
BioRad Gen Pulser	BTX (San Diego, USA)
Electroporation cuvettes plus	BTX (San Diego, USA)
Gasprofiz	WLD TEC (Göttingen, Germany)
Gene Quant II Photometer	Pharmaci Biotech (Germany)
Ice producer mashine	Ziegra (Germany)
I Cycler iQ Multicolor Real-Time PCR Detection system	Bio-Rad (Gemany)
Hybridisation oven	Helmut Saur (Germany)
IKA MAGRCT surface heat plate	IKA Labortechnik (Staufeln, Germany)
Incubator M500	Memmert (Schwabach, Germany)
Incubator III-178	Memmert (Schwabach, Germany)
Microscope Axioplan2 HAL100	Landgraf (Langenhagen, Germany)
Microscope KL 1500 LCD	Landgraf (Langenhagen, Germany)
Microwave NN-T251	Panasonic (Gemany)
PCR Themoblock T3	Biometra (Goettingen, Germany)
pH-Meter pH211 Microprocessor	Hanna (Germany)
Refrigerator	Siemens (Germany)
Scale BP2100S	Kremer & Kreiler (Goettingen, Germany)
Scale Sartorius	Kremer & Kreiler (Goettingen, Germany)
Shake incubator UNMAX 1010	Heidolph (Gemany)
Sterile bench Kojar®	Kojart (Finland)
Thermo shake KS-10	Edmund Büher (Germany)
UVGel Image System	Intas (Germany)
VARIOKLAV® Steam sterilizer	H+P Labortechnik (Oberschleißheim, Germany)
Vortex REAX top	Heidoph (Germany)
Dishwasher	Miele (Germany)

2.1.2 Chemicals

Table 2: Chemicals

Chemical	Manufacturer
2-Mercaptoethanol	Riedel-de Häen (Seelze, Germany)
2,4-D	Duchefa (Haarlem, Netherlands)
2'-Desoxyribonucleosid-5'-triphosphate (dNTPs)	MBI-Fermentas (St. Leo-Rot , Germany)
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Roth (Karlsruhe, Germany)
Acetic acid	Roth (Karlsruhe, Germany)
Acetosyringone	Duchefa (Haarlem, Netherlands)
Plant Agar	Duchefa (Haarlem, Netherlands)
Agar-Agar	Roth (Karlsruhe, Germany)
Agarose for electrophoresis	Roth (Karlsruhe, Germany)
Ammonium nitrate	Duchefa (Haarlem, Netherlands)
Ampicillin	Duchefa (Haarlem, Netherlands)
BAP	Duchefa (Haarlem, Netherlands)
CDP- Star chemiluminescence substrate	Roche (Germany)
$CaCl_2$	Roth (Karlsruhe, Germany)
Carbenicillin	Duchefa (Haarlem, Netherlands)
Cefotaxime	Duchefa (Haarlem, Netherlands)
Cetyltrimethylammoniumbromid (CTAB)	Duchefa (Haarlem, Netherlands)
Chloroform	Roth (Karlsruhe, Germany)
Citric acid monohydrate	Roth (Karlsruhe, Germany)
D-Galactose	Duchefa (Haarlem, Netherlands)
D-Glucose	Duchefa (Haarlem, Netherlands)
EDTA	Roth (Karlsruhe, Germany)
Ethanol	Roth (Karlsruhe, Germany)
Ethidium bromide	Roth (Karlsruhe, Germany)
Ethyl acetate	Roth (Karlsruhe, Germany)
$FeSO_4$	Roth (Karlsruhe, Germany)
Formic Acid	Roth (Karlsruhe, Germany)
Gene ruler 100 bp DNA ladder	MBI Fermentas (St. Leo-Rot , Germany)
Gene ruler 1K bp DNA ladder	MBI Fermentas (St. Leo-Rot , Germany)
Gibberellic acid A3 (GA3)	Duchefa (Haarlem, Netherlands)
Glufosinate-ammonium (PPT)	Sigma-Aldrich (Deisenhofen, Germany)
Glycerin	Roth (Karlsruhe, Germany)
HCl	Roth (Kahlsruhe, Germany)
Hydrogen perocide	Roth (Karlsruhe, Germany)
IAA	Duchefa (Haarlem, Netherlands)
IBA	Duchefa (Haarlem, Netherlands)
Isoamyl alcohol	Roth (Karlsruhe, Germany)
Isopropanol	Roth (Karlsruhe, Germany)
Kanamycin	Duchefa (Haarlem, Netherlands)
KNO_3	Roth (Karlsruhe, Germany)
KOH	Roth (Karlsruhe, Germany)

Maleic acid	Roth (Karlsruhe, Germany)
Methanol	Roth (Karlsruhe, Germany)
$MgCl_2$	Roth (Karlsruhe, Germany)
$MgSO_4*7H_2O$	Roth (Karlsruhe, Germany)
MS medium	Duchefa (Haarlem, Netherlands)
MS medium micro-salt mixture	Duchefa (Haarlem, Netherlands)
MS medium vitamin mixture	Duchefa (Haarlem, Netherlands)
Myo-inositol	Duchefa (Haarlem, Netherlands)
NAA	Duchefa (Haarlem, Netherlands)
NaCl	Roth (Karlsruhe, Germany)
NaOH	Roth (Karlsruhe, Germany)
Nylon membranes positively charged	Roche (Germany)
Phenol	Roth (Karlsruhe, Germany)
Peptone	Duchefa (Haarlem, Netherlands)
Polyethylene glycol PEG 4000	Roth (Karlsruhe, Germany)
Primer	Roth (Karlsruhe, Germany)
Primer	MWG (Martinsried, Germany)
PVP40	Roth (Karlsruhe, Germany)
Rifampycin	Duchefa (Haarlem, Netherlands)
1-Sodium dodecyl sulphate (SDS)	Roth (Karlsruhe, Germany)
Sorbitol	Roth (Karlsruhe, Germany)
Sucrose	Roth (Karlsruhe, Germany)
Spectinomycin	Duchefa (Haarlem, Netherlands)
Streptomycin	Duchefa (Haarlem, Netherlands)
Tircarcillin	Duchefa (Haarlem, Netherlands)
Trishydroxylaminomethane	Roth (Kahlsruhe, Germany)
Tris-hydroxymethyl-amimomethane	Roth (Karlsruhe, Germany)
Tris-sodiumcitrate-dihydrat	Roth (Karlsruhe, Germany)
Tween	Roth (Karlsruhe, Germany)
Yeast extract	Duchefa (Haarlem, Netherlands)

2.1.3 Molecular biological kits and enzymes

Table 3: Molecular biological kits

Name of the Kits	**Manufacturer**
DIG Easy Hyb Granules Kit	Roche (Germany)
E.Z.N.A.® Gel Extraction Kit	Peqlab Biotech GmbH (Germany)
E.Z.N.A.® Plasmid Miniprep Kit I	Peqlab Biotech GmbH (Germany)
Gateway.® BP/LR clonase Enzyme Mix	Invitrogen (Germany)
QIAquick.® PCR Purification Kit	Qiagen (Hilden, Germany)
Rneasy RNA extractions kit	Qiagen (Hilden, Germany)
Revert Aid^{TM} First strand cDNA synthese kit	Fermentas
DNA- free DNase treatment & Removal Reagents	Ambion
Invisorb Spin® Plant RNA minit Kit	Invitek

Table 4: Enzymes

Enzyme	Manufacturer
BioThemTM Taq Polymerase	GeneCraft (Germany)
High fidelity BioThemTM Taq Polymerase	MBI Fermentas (St. Leon-Rot, Germany)
Restrictionsendonucleases	MBI Fermentas (St. Leon-Rot, Germany)
RNase A	Peqlab Biotech GmbH (Germany)
T4 DNA Ligase	MBI Fermentas (St. Leon-Rot, Germany)
T4 DNA Polymerase	MBI Fermentas (St. Leon-Rot, Germany)

2.1.4 Plant material

In the present work sterile *in vitro* cultures of *Malus domestica* Borkh. cv. 'Holsteiner Cox' and the hybrid 'TNR 31-35' (a descendant of *Malus sieversii* var. *sieversii* f. *niedzwetzkyana*, carrying a homozygous, dominant gene responsible for red pigmentation in all plant parts) were used. *In vitro* cultures of the cultivar 'Holsteiner Cox' were established by Prof. Szankowski, Leibniz University of Hannover, Germany (Szankowski, 2002). *In vitro* cultures of 'TNR 31-35' were kindly provided by Dr. Viola Hanke (Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, Institute of Fruit Breeding, Dresden, Germany).

2.1.5 Bacteria strains

2.1.5.1 *Escherichia coli*

In this work the *Escherichia coli* strain 'Top10' was used for propagating the plasmids.

2.1.5.2 *Agrobacterium tumefaciens*

For the *Agrobacterium tumefaciens*-mediated transfer of the *Lc* gene, the *ANS*-RNAi construct and the *FGT*- RNAi construct, the strain EHA105 was used. EHA105 is a disarmed derivative of the super-virulent *Agrobacterium tumefaciens* strain A281 and includes helper-plasmid pEHA105 (derived from the wild pTiBo542 Ti-plasmid) in a C58 chromosomal background.

2.1.6 Nucleic acids

2.1.6.1 Primers used for detection, RT- and quantitative real-time PCR

Table 5: Primers used for detection, RT- and quantitative real-time PCR.

F: forward; R: reverse

Primer		Sequence 5' to 3'	T_m °C	PCR Effici-ency %
PAL	F	AAGGGAAGTGCTGATTGAACAT	56.5	104.0
	R	CTTGGAACACCTTGTCACACTC	60.3	
CHS	F	CTAGTGACACCCACCTTGAYAG	61.2	77.5
	R	AGAAGAGYGAGTTCCAGTCYGA	60.3	
CHI	F	GGTCCGTTTGAGAAATTCATTC	56.5	126.6
	R	KGTTTTCTATCACCRYGTTTGC	57.5	
FHT	F	TCAACCAGTGGAAGGAGCTT	57.3	95.6
	R	GTCCTGCAGTTGCTGTTCCT	59.4	
FLS	F	GAAAGCAATGAGGGTACAATCC	58.4	109.0
	R	AACAGATGGAGGCCAAATCTTA	56.5	
DFR	F	AGTCCGAATCCGTTTGTGTC	57.3	99.4
	R	TTGTGGGCTTGATCACTTCA	55.3	
LAR	F	TCTTGGCCCTACTTTGACAAC	57.9	69.2
	R	AGAGTTCCCACTTCCACATCA	57.9	
ANS	F	CACCTTCATCCTCCACAACAT	57.9	113.1
	R	ATGTGCTCAGCAAAAGTTCGT	55.9	
ANR	F	CCACCTCACAGCACTACAAGAG	62.1	139.7
	R	GCAAATTTCCAAGCTGTCTTCT	56.5	
FGT	F	CCCTTCTTGTGGTCTATCAAGG	60.3	100.5
	R	TGGTAAAAACTCCATCCTCCAC	58.4	
RNAPOLY II	F	ATATGCCACCCCGTTCTCTACT	60.3	95.8
	R	CACGTTCCATTTGTCCAAACTT	56.5	
Rubisco	F	GCTTGTCCAAGAGCAAGAGAAT	58.4	73.7
	R	CTCCCTCCCCTCAATTATAACC	60.3	
NOS	F	CCC CTC GGT ATC CAA TTA GAG	63	-
	R	CGG GGG GTG GGC GAA GAA CTC		
NPTII	F	GAT TGA ACA AGA TGG ATT G	64	-
	R	CAT TTT CCA CCA TGA TAT TC		
Bar	F	GCA GGA ACC GCA GGA GTG GA	60	-
	R	AGC CCG ATG ACA GCG ACC AC		
LC	F	ATG GCG CTT TCA GCT TCC CGA	66	-
	R	TGT ACC AAG CTC AAG CAC GCC		
ANS	att B1	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCT GTG AGC TCT GAT TCA GTG A	60	-
	att B2	GGG AC CAC TTT GTA CAA GAA AGC TGG GTA CCT TGT CCA TGA GCT CGT CA		
FGT	att B1	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TCC CAT CGA AAT CGA ACC AT	60	-
	att B2	GGG ACC ACT TTG TAC AAG AAA GCT GGG TAG CTT GCC CAC GAA AAC GTA C		
Oligo dT		TTT TTT TTT TTT TTT TT (17 T)	70	-
Intron CHSA front		CCA ATT AAG ATA AAA CGT TGA ATG	-	-
intron CHSA back		CAC TTA CTT ACA CTT GCC TTG GAG	-	-

2.1.6.2 Genes for the genetic transformation

***Lc* gene**: the leaf colour (*Lc*) gene, belonging to the myc type R gene family, encodes a protein with the basic helix-loop-helix (bHLH) motif acting as a transcription factor involved in regulation of the transcription of genes of the flavonoid pathway.

***ANS* gene**: It encodes anthocyanidin synthase, which catalyzes the introduction of a double bond between C-2 and C-3 of flavan-3,4-diol (leucoanthocyanidin) and lead to a 2-flavan-3,4-diol (anthocyanidin) (Forkmann 1999).

***FGT* gene**: It encodes flavonoid 3-Ogalactosyltransferase, which catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group in flavonols and anthocyanidins. Anthocyanidins, which bear a free hydroxyl group, are unstable under physiological pH conditions, and have not been observed in nature. FGT is therefore regarded as an indispensable enzyme of the main biosynthetic pathway to anthocyanins rather than a modifying enzyme (Forkmann 1999).

2.1.6.3 Binary vectors

2.1.6.3.1 pBI121

For the transformation via *Agrobacterium tumefaciens* the binary vector pBI121 (Figure 9, Gene accession number AF485783) was used predominantly. For selection in *E. coli* and *Agrobacterium* the vector contains a kanamycin resistance gene (*npt*II).

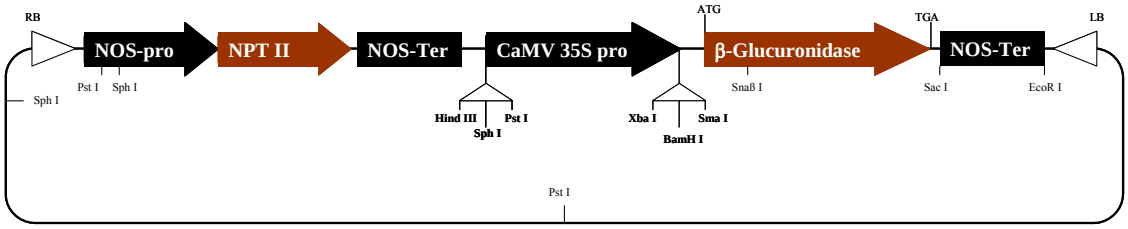


Figure 9 : pBI121 (from Clontech catalogue 1996/97). NOS-pro and NOS-ter stand as promoter and terminator of the nopalinsynthase. NPTII (neomycinphosphotransferase II) delivers a resistance against the antibiotic kanamycin. The 35S promoter originates

from the cauliflower mosaic virus (CaMV). RB and LB are the limiting border sequences of the T-DNA; furthermore for the cloning usable cutting sites are indicated.

2.1.6.3.2 pFGC5941

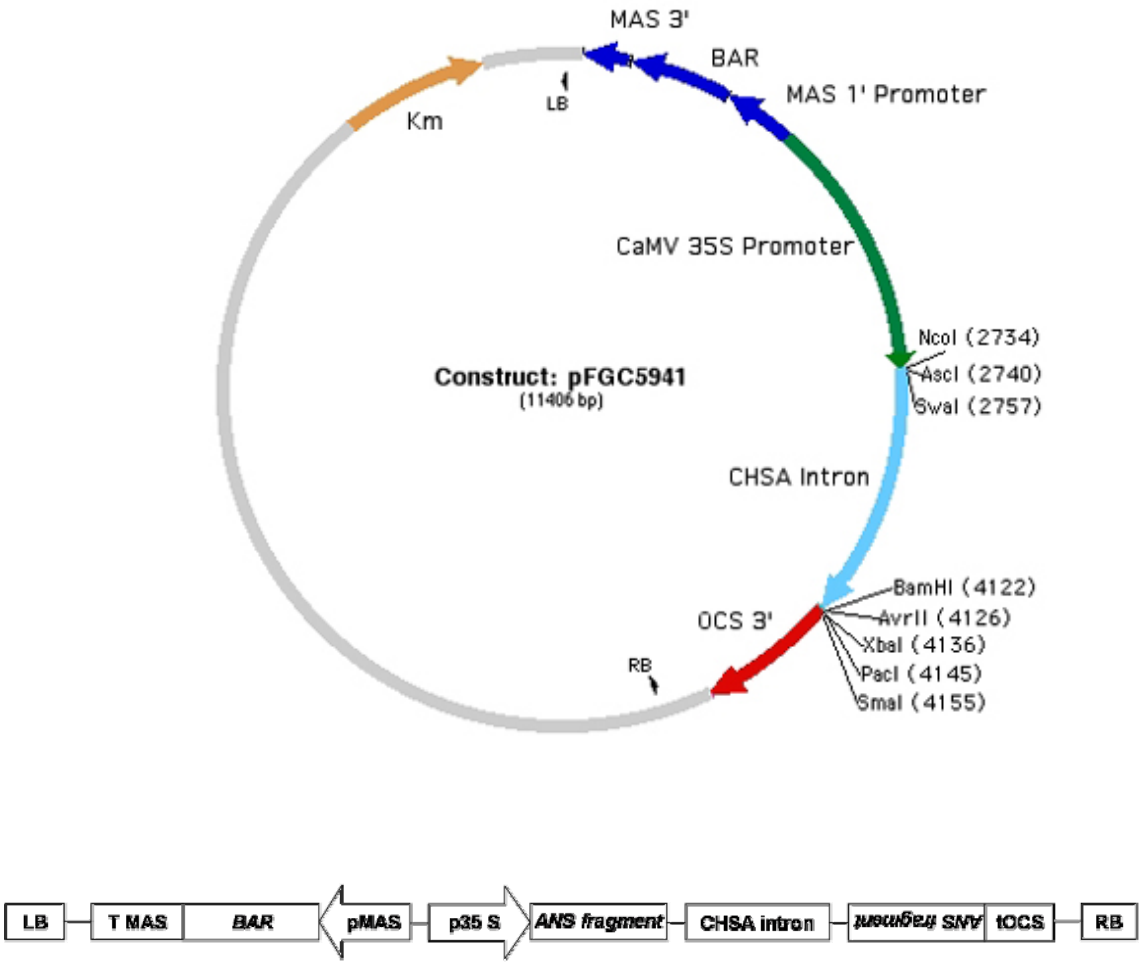


Figure 10: pFGC5941 (from chromDB)

Plasmid pFGC5941 was built from binary vector pCAMBIA1300 (ChromDB database). The CaMV 35S promoter drives dsRNA production. It has kanamycin resistance gene for the selection after transformation into bacteria and *bar* gene (confers resistance against herbicides with the active agent phosphinotricin) for the selection after transformation into plants. The vector was modified by the insertion of two Gateway (Invitrogen, Carlsbad, CA, USA) cassettes (provided by

Franziska Krajinski, University of Hannover). The Gateway technology (Invitrogen, Carlsbad, CA, USA) was used to construct binary vectors for gene silencing by RNA interference.

2.1.6.3.3 pDONR ™ 201 and pDONR ™ 207

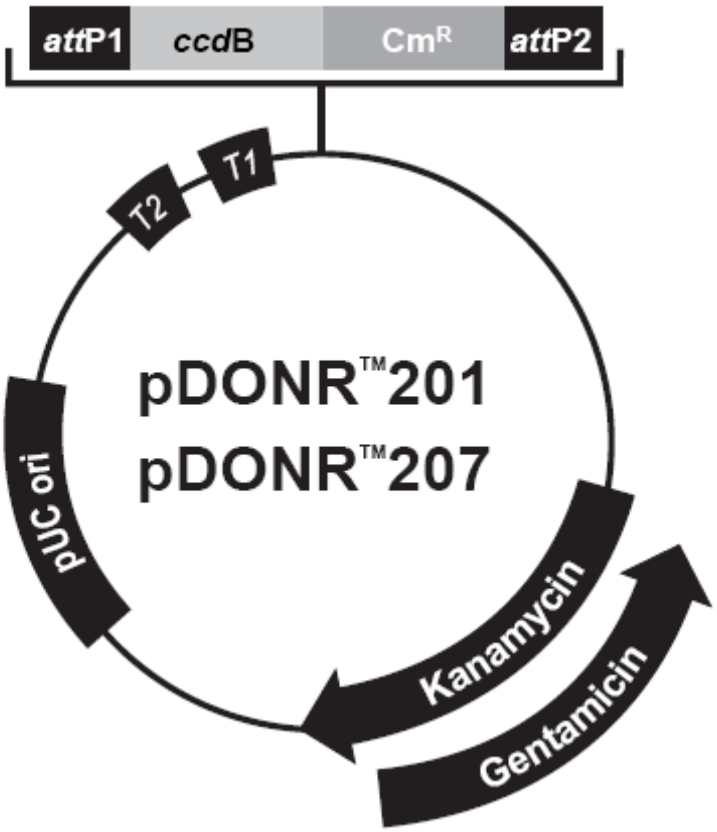


Figure 11: pDONR ™201 and pDONR ™207 (from Invitrogen, 2006)

pDONR ™201 and pDONR ™207 (Invitrogen) served for the RNAi construct building. pDONR ™201 has a kanamycin resistance gene and pDONR ™207 has a gentamycin resistance gene. Both two vectors have attP1 and attP2 sites for the BP recombination reaction.

2.1.6.3.4 pHellsgate8

pHellsgate8 (Genbank AF489904) is a high throughput vector for gene silencing in plants. It has a spectinomycin resistance gene for the selection after

recombination and transformation into *E. coli* and *Agrobacterium*. It has an *npt*II gene for the selection of transgenic plant cells. The plasmid was kindly provided by CSIRO (Australian).

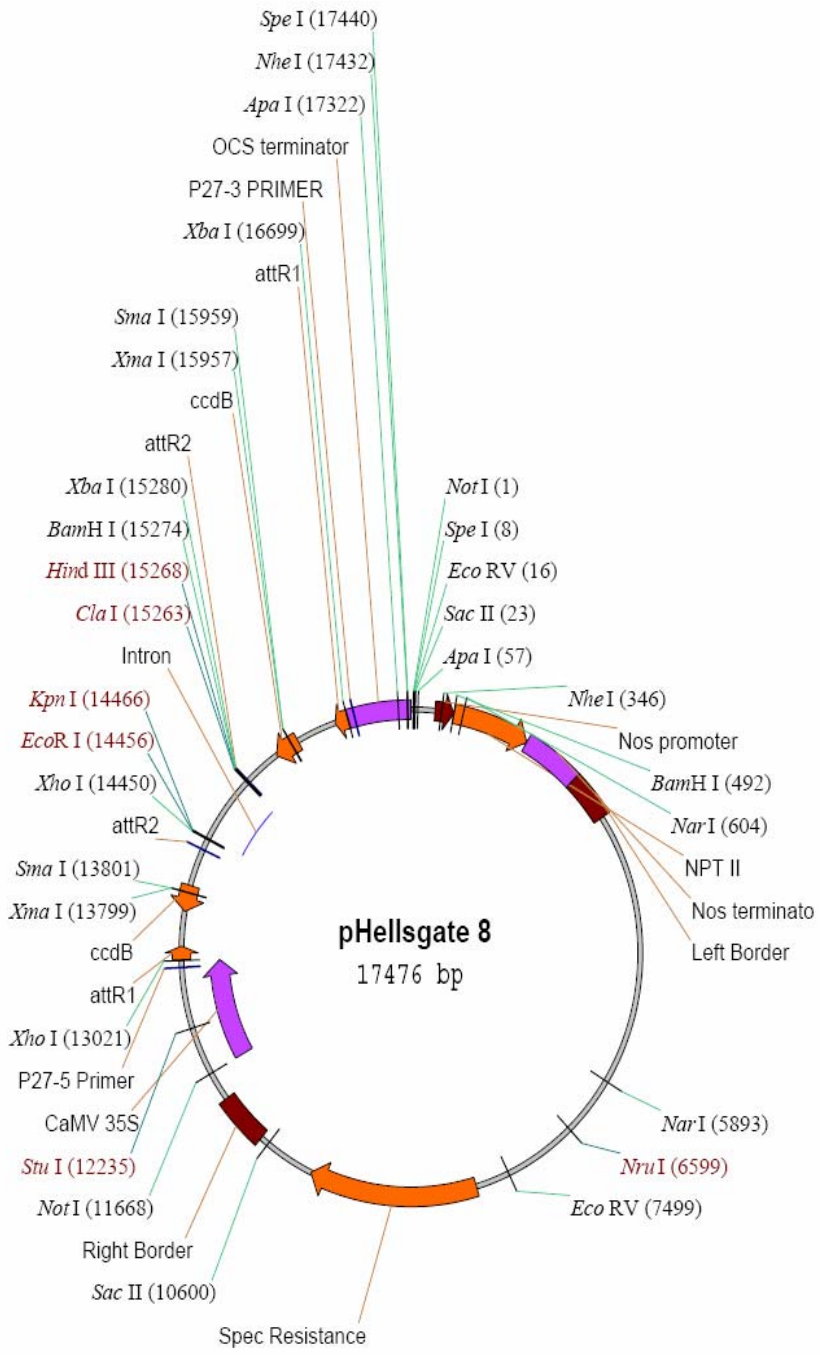


Figure 12: pHellsgate8 (from Plant Industry, CSIRO, 2002)

2.1.6.4 DNA length standard

In the molecular biology part of this work following DNA length standard were used:

-- 100bp+ (MBI Fermentas) contained following DNA fragment sizes:
3000 bp/2000 bp/1500 bp/1200 bp/1031 bp/900 bp / 800 bp/700 bp/600 bp/500 bp/400 bp / 300 bp / 200 bp and 100 bp.

-- Molecular Weight DNA markers II, DIG Labelled (Roche) contained following DNA fragment sizes:
23130 bp/9416 bp/6557 bp/4361 bp/2322 bp/2027 bp/564 bp and 125 bp.
Before using, 6 µl marker was diluted in 14 µl H_2O and 5 µl 6x sample buffer was added.

2.1.7 Media

2.1.7.1 Bacteria media

LB medium for *E. coli*:

- 10 g/l tryptone (or peptone)
- 5 g/l yeast extract
- 10 g/l NaCl

The mixture was dissolved in 1 l deionised water, for solid medium 15 g/l agar agar were added. Appropriate antibiotics were added to the medium after autoclaving. The medium was stored at 4°C in darkness.

YEP medium for *Agrobacterium:*

- 10 g/l yeast extract
- 10 g/l tryptone or peptone
- 5 g/l NaCl

The mixture was dissolved in 1 l deionised water, for solid medium 15 g/l agar were added. Appropriate antibiotics were added to the medium after autoclaving. The medium was stored at 4°C in darkness.

2.1.7.2 Basic media for plant tissue culture

2.1.7.2.1 Tissue culture media for 'Holsteiner Cox'

Table 6 : Basic tissue culture media for 'Holsteiner Cox'

Chemicals	Regeneration Medium	Elongation medium	Propagation medium	Root induction medium
MS (Murashige and Skoog, 1962) salt mixture	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l
Sucrose	-	3%	3%	3%
Sorbitol	3%	-	-	-
Myoinositol	0.1 g/l	-	0.1 g/l	0.1 g/l
IBA	1 µM	-	0.5 µM	1.5 µM
BAP	-	4.4 µM	4.4 µM	-
GA_3	-	0.28 µM	-	-
TDZ	3 µM	-	-	-
Plant Agar	-	0.8%	0.8%	0.7%
Gelrite	0.3 %	-	-	-
pH value	5.8	5.6 - 5.8	5.6 - 5.8	5.6 - 5.8

2.1.7.2.2 Tissue culture media for 'TNR 31-35'

Table 7: Basic tissue culture media for 'TNR31-35'

Chemicals	Regeneration medium	M8 Propagation medium	Root induction medium
MS (Murashige and Skoog, 1962) salt mixture	4.4 g/l	4.4 g/l	4.4 g/l
Sucrose	-	3%	3%
Sorbitol	3%	-	-
IBA	1 µM	4.9 µM	1.5 µM
BAP	-	4.4 µM	-
TDZ	3 µM	-	-
Plant Agar	-	0.8%	0.8%
Gelrite	0.3 %	-	-
pH value	5.8	5.8	5.8

2.2 Methods

2.2.1 Plant tissue culture

Shoot cultures were maintained on propagation medium (2.1.7.2) and incubated at 25°C under a 16/8-h (day/night) photoperiod. The plants were subcultured every 4 weeks. For regeneration experiments, the leaf disc method was used. Young leaves were cut into 0.5-1 cm segments. The explants were transferred with the adaxial side down on the regeneration medium and cultivated in darkness for two weeks at 25°C. Afterwards they were placed under light to encourage shoot regeneration. The regenerated adventitious shoots were cut from the explants and transferred to elongation medium. After 2 weeks elongation the shoots were transferred to propagation medium.

In order to develop, establish and optimize the regeneration of 'TNR 31-35' from leaf material, various media with different IBA and TDZ concentrations with respect to their shoots induction effectiveness were tested. After 6 weeks cultivation, following criteria were used to assess the regeneration effectiveness:

- Regeneration rate, i.e. the number of explants showing shoot regeneration. The details were given in percent.
- The number of shoots per explant. The average number was identified and only explants with shoot regeneration were taken into consideration.

2.2.1.1 *In vitro* root induction and transfer on soil

The regenerated shoots were cultivated on rooting medium under weak light to induce root formation. Rooted plantlets were transferred into the greenhouse. Before potting the plants were treated with 1.5 °/$_{00}$ Previcure (fungicide) for 1 min, in order to avoid fungal infection. The plantlets were potted on culture-plates. The plates were placed in greenhouse under high humidity, which was reduced during the further culture.

2.2.2 Plant transformation

2.2.2.1 Culture of *Agrobacterium tumefaciens*

250 µl of an *Agrobacterium tumefaciens* glycerine stock solution were inoculated in 25 ml YEP medium in an Erlenmeyer flask and appropriate antibiotics were added. The agrobacteria were cultivated overnight at 28°C and under shaking (240 rpm) in darkness. For the purification of the bacteria the total culture was centrifuged (10 min, 3600 × g). Supernatant was removed and the precipitate was resuspended in liquid MS medium. The OD_{600} value was adjusted to 0.8. This suspension was used for the transformation.

2.2.2.2 Plant transformations

Wounding seems to be essential for satisfying regeneration. Most regenerated shoots originate from the wounded leaf sides. In respect to transformation, wounding is not only beneficial for regeneration, but also enables the *Agrobacterium* to penetrate and infect the plant tissue. The epidermal cell layer of the plant acts as a protection layer and *Agrobacterium* has difficulties in penetrating this cell layer unless it is damaged. Wounding of the leaf explants with a scalpel is one method to increase both regeneration and transformation. Leaves were excised from 4 week old shoots and cut into stripes. The leaf explants were infected in the *Agrobacterium* suspension for 20 min. Afterwards the explants were put on the regeneration medium and co-cultured for 3 days in darkness at 25°C. The infected explants were washed twice with sterile water and finally in liquid MS medium supplemented with 300 mg/l tircarcillin for 15 min.

2.2.2.3 Selection and regeneration of transgenic plants

2.2.2.3.1 *Lc* and *FGT*-pHellsgate 8 transgenic plants

After washing the infected explants were cultivated on selective regeneration medium (Tab. 2.1.7.2) supplemented with 150 mg/l tircarcillin and 25 mg/l kanamycin) and continually cultivated in darkness. After 2 weeks the explants were transferred in the light and the selective pressure was increased by using 50 mg/l kanamycin. After 4 weeks cultivation explants produced callus. After further

4 weeks, explants formed new shoots; the shoots were elongated on selective elongation medium supplemented with 150 mg/l tircarcillin and 50 mg/l kanamycin. Elongated shoots were transferred to selective propagation medium.

2.2.2.3.2 *ANS*-pFGC5941 transgenic plants

Explants transformed with *ANS*-pFGC5941 were treated as described in 2.2.3.3.1 with the exception that glufosinat ammonium in concentrations from 1 mg/l up to 5 mg/l was used for selection instead of kanamycin. Propagation of regenerated shoots took place on M8 medium (M8 medium supplemented with 150 mg/l tircarcillin and 3-5 mg/l ppt).

2.2.2.4 Analysis of transgenic plants

2.2.2.4.1 Regeneration test with antibiotics

To test successful transformation, leaves were cut from regenerated shoots and cultivated on selection medium. Leaves of untransformed plants were used as a control. They were put on the selective regeneration medium (MS medium supplemented with 50 mg/l kanamycin and 100 mg/l tircacillin) to regenerate new shoots.

2.2.2.4.2 Molecular analyses

Leaves of putative transgenic shoots were frozen in liquid nitrogen. Genomic DNA and total RNA were extracted from frozen leaves following the protocols described below. Genomic DNA was used for PCR and Southern blot analysis. Total RNA was used for the synthesis of cDNA and cDNA was thereafter used for Real-Time PCR analysis.

2.2.3 Visual selection used for transformation

In some transformation experiments using the *Lc* gene, it was tried to visually select transgenic cells or tissue because of anthocyanin accumulation due to *Lc* expression. Therefore no selection agent was applied during the regeneration procedure, but red pigmented areas of the transformed explants were separated

and brought to regeneration. The process was repeated every week. After 4-6 times repeat new shoots regenerated. Afterwards the shoots were transferred to elongation medium. After 2-4 weeks elongation the shoots were subcultured on C1 propagation medium. Propagation medium was changed every 4 weeks.

2.2.4 *In vitro* suspension culture

Callus tissue was used as source material for the establishment of suspension cultures. Approximately 2 g callus tissue with 20 ml nutritive solution (after Winkelmann, 2004) for the suspension culture were cultivated in 100 ml Erlenmeyer flasks in the dark at 23-25°C while shaking (100 rpm). The cell growth needed a minimum inoculum density. If the inoculum density is too small, no more cell division will take place. In apple the cell density should not below $2x10^3$.

When the culture became high-viscosity, fresh nutritive solution was added. The propagation of the culture took place after 10 - 14 days incubation. It was distributed in two Erlenmeyer flasks and filled up with corresponding fresh nutritive solution.

For the vitality test 1 drop of the cell suspension was laid on a slide and 0.5% Evans Blue solution was added. Thereafter the solution was checked under light microscopy. Surviable cells were not dyed and dead cells appeared blue (the colouring material can pass the defective cell wall).

2.2.5 *In vitro* anthocyanidin induction

For *in vitro* anthocyanidin induction the leaf explants were used. In contrast to the normal *in vitro* culture medium, the induction medium contained no NH_4NO_3 and the macro salts of the medium were only 1/5 to ½ in comparison to the normal regeneration medium. The young leaves were cut from *in vitro* plants in 1 cm^2 explants and cultivated at 25°C under light on anthocyanidin induction medium. The medium was changed every 2 weeks.

2.2.6 Bacteria culture

2.2.6.1 *Escherichia coli*

E. coli cells of the strain Top10 (Invitrogen) were cultured at 37°C (in liquid culture under shaking: 150 U/min) in LB medium (2.1.7.1). For the selection, appropriate antibiotics were used (depending upon the vector).

2.2.6.2 *Agrobacterium tumefaciens*

Agrobacterium has an optimal growth temperature of 32-33°C. However, a temperature of 26-28°C was used, because temperatures higher than 30°C elevate the risk that the agrobacteria loose their plasmids and become avirulent. For the culture, YEP medium was used. For selection of *Agrobacterium* containing the binary vectors pBI121 or pFGC5941, 50 mg/l kanamycin was used.

2.2.7 Molecular biology methods

2.2.7.1 Isolation of nucleic acids

2.2.7.1.1 Isolation of plasmid DNA from *E. coli*

Plasmids of *E. coli* or *A. tumefaciens* were isolated either with the E.Z.N.A.® Plasmid Miniprep Kit (Peqlab) according to the manufacturer's instructions or using the following method:

The colonies of bacteria containing the plasmids of interest were incubated overnight under shaking at 37°C in 10 mL LB medium supplemented with appropriate antibiotics. Two ml were centrifuged at 10 000 rpm for 5 min. The obtained pellet was dissolved in 200 µl of buffer A and the bacteria were lysed with 400 µl buffer B, mixing and incubating at RT for 15 min. After addition of 300 µl buffer C, incubation took place on ice for 10 min. After a centrifugation step at RT for 10 min at 13000 rpm, 800 µl supernatant containing the plasmids were transferred in new tube and 600 µl isopropanol was added to elute the DNA. To pellet the precipitated DNA, the mixture was centrifuged for 10 min at 13000 rpm. The pellet was diluted in 200 µl buffer D and mixed with 400 µL ice cold

70% ethanol. After 2 min incubation, the mixture was centrifuged for 10 min at 13000 rpm. The pellet was dried for 1 hour and was dissolved in 50 µL TE buffer or distilled water with 1 mg/ml RNAse. The solution was stored at -20°C.

Plasmid isolation buffer:

Buffer A: 15 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose

Buffer B: 0.2 M NaOH, 1% SDS

Buffer C: 3 M NaOAc PH 4.8

Buffer D: 0.1 M NaOAc pH 7.0, 0.05 M Tris-HCl pH 8.0

2.2.7.1.2 Isolation of total DNA from plants

1-2 g plant material was harvested and shock frozen in liquid nitrogen. The material was grinded as fine as possible in liquid nitrogen. 5 ml CTAB solution supplemented with 0.2% mercaptoethanol were pipetted in a 50 ml tube, which contained the grinded plant material. The tube was vortexed vigorously and incubated for 30 min in a preheated thermo block at 60°C and mixed occasionally. Afterwards the same volume chloroform/isoamylalcohol (24:1) was added and the tube mixed gently and thereafter the tube was centrifuged at 4000 rpm for 10 min. Aqueous upper phase was carefully taken and pipetted in a new 50 ml tube. 2/3 (v/v) pre-chilled 100% isopropanol was added to precipitate the DNA. After a 5 min centrifugation step, the supernatant was discarded and the pellet was washed with 10 ml wash buffer. After 5-10 min drying, the pellet was dissolved in 3 ml TE buffer with 10-50 µg/ml RNAse. Incubation for 30 min at 37°C should remove RNA. The precipitation was carried out by addition of 100 µl 7.5 M NH_4-acetate and 750 µl absolute ethanol and centrifuged at 13 000 rpm for 10-20 min. The pellets were dried for 1-1.5 hours and thereafter dissolved in 500 µl Tris buffer or water.

Solvent and buffer for the DNA-Isolation

CI- mix:

- 23 ml chloroform
- 1 ml iso-amylalcohol

CTAB solvent:

- 100 mM Tris-ultra
- 1.4 M NaCl
- 20 mM EDTA
- 0.5% (w/v) PVP-40 (polyvinylpyrrolidone)

After autoclaving 3% (w/v) CTAB was added and the solution was agitated over night

- 0.2% (w/v) mercaptoethanol was added before application

DNA washing buffer:

- 76% (v/v) ethanol
- 10 mM ammoniumacetat
- double sterilized H_2O

TE buffer:

10 mM Tris-ultra (1.21 g/l)

1mM EDTA (0.37 g/l)

pH value was adjusted with HCl to 7.5 or 8.

Tris-HCl (pH 7.5):

- 121.1 g/l Tris
- 70 ml/l HCl

2.2.7.1.3 Isolation of total RNA from plants

1-2 young leaves (~ 10-20 mg) were frozen in liquid nitrogen and grinded in the mortar. RNA was isolated with the Invisorb Spin® Plant RNA mini Kit according to the manufacturer's instructions.

2.2.7.2 cDNA synthesis

For the synthesis of cDNA, reverse transcription was applied *via* the following procedure: 1 µg to 5 µg total RNA or 100 ng to 500 ng mRNA and 1 µl oligodT (or AP) primer (25 pmol) were pipetted in a 1.5 ml tube. Water was added to a volume of 16.75 µl. The mixture was incubated for 10 minutes at 70°C in order to denature secondary structures of the RNA, which can disturb the reaction. The tube was cooled down on ice and afterwards briefly centrifuged. Five µl 5-fold

reverse transcriptase buffer and 1.25 µl dNTPs (every to 10 mM) were added. The tube was preheated for 2 min at 42°C, then 1 µl reverse transcriptase (Promega) was added and the mixture was incubated for 1 h at 42°C. One to 2 µl products were applied as template for PCR.

2.2.7.3 Photometric measurement of nucleic acid concentration

RNA- and DNA-concentrations as well as the cell density of bacteria were determined directly with a photometer. Cell densities were measured via the absorption at 600 nm (OD_{600}). RNA- and DNA-concentrations were measured at a wavelength of 260 nm.

The formation of the quotient from the absorption at 260 nm and 280 nm gives information about the degree of the pollution with proteins, since proteins absorb mainly at 280 nm. A relative pure DNA-preparation gives a quotient OD_{260}/OD_{280} of approx. 1.8. All measurements were accomplished in UVette® (Eppendorf), which is light transparent in the range of 220 nm to 1600 nm.

2.2.7.4 Agarose gel electrophoresis

DNA fragment sizes were identified via gel electrophoresis. Nucleic acids can be separated in the gel because it has electronically negative phosphate groups and therefore move to the anode in an electric field. The agarose concentration of the gels was chosen depending on the size of the DNA fragments and varied between 1-3 %. Generally, gels were electrophoresed at 80 volt and 100 mA for 30 minutes to 1 hour, taking into account the agarose concentration and the purpose of electrophoresis.

DNA samples were mixed with approximately 10-20% loading buffer and loaded onto the agarose-gel (in 1x TAE), which contained ethidium bromide (0.5 µl/ml). In addition to the samples, 6 µl of the molecular weight marker 1 kbp or 100 bp marker were loaded onto the gel. The DNA was visualised under UV light (254 nm) and gel photos were taken with an UVP Video Imager.

2.2.7.5 Polymerase chain reactions (PCR)

Polymerase chain reactions (PCR) were carried out with genomic DNA, cDNA or plasmids as a template. The following standard stock solution mixture was prepared (Table 8).

Table 8: Composition of PCR

H_2O	18.3 µl
10* buffer	2.5 µl
dNTP (50 mM)	1 µl
Template (1-100 ng)	1 µl
Primer 1 (25 pM)	1 µl
Primer 2 (25 pM)	1 µl
Taq polymerase(1 µ/ml)	0.2 µl
Total	25 µl

For the PCR reaction following standard program (Table 9) was used.

Table 9: Program of PCR

Cycles	Temperature/°C	Time	Effect
1	94	3 min	Denaturation
30-40	94	1 min	Denaturation
	Depending on primer*	1 min	Annealing
	72	1-2 min	Elongation
1	72	10 min	Amplification
1	4	1:00	Prepare for storage
	Afterwards hold at 4°C		Storage

* The following annealing temperatures were used for the primers: PAL 58°C; CHS 60°C; CHI 58°C; FHT 58°C; FLS 58°C; DFR 56°C; LAR 58°C; ANS 56°C; ANR 60°C; FGT 60°C; Rubisco 60°C; NPTII 64°C; Bar 60°C; LC 66°C;ANS-attB 60°C; FGT-aTTB 60°C; oligo dT 70°C.

2.2.7.6 Cleaning of the High Fidelity PCR product

Purification and concentration of the PCR product was done using the High Pure PCR Product Purification Kit (Boehringer, Mannheim) according to the manufacturer's instructions.

2.2.7.7 Restriction digest

Restriction digest was used to confirm the presence of the correct plasmids and to cut out specific fragments from plasmids. Digestion of plasmid DNA with restriction enzymes was also employed to determine the presence and the orientation of inserted DNA fragments.

Plasmid DNA was digested either with one or two restriction enzymes. The buffers were chosen as recommended by the different enzyme suppliers.

All analytical digests were carried out in a 25-50 µl reaction volume. The reactions were incubated at 37°C in a thermo block for more than 1 hour. A general formula for the conditions of a digest reaction is given in table 10.

Table 10: Composition of DNA restriction reaction

Plasmid DNA	X µl
10x buffer	2.5 µl
Enzyme 1	1 µl
Enzyme 2	1 µl
H_2O	20.5 - x µl
Total	25 µl
37°C without shake approx. 60 min	

2.2.7.8 Concentration and purification of the plasmid

In order to concentrate the DNA and to discard disturbing substances, the DNA was EtOH-precipitated. 1/3 volume 7.5 M NH_4Ac and 2.5 volumes absolute ethanol were added to the DNA solution and they were mixed through inverting. The mixture was centrifuged for 10 min at 13.000 rpm and the supernatant was discarded. The pellet was dissolved in 50 µl TE buffer and 100µl absolute EtOH were added and the tube was inverted, afterward it was centrifuged for 10 minutes at 13.000 rpm. After discarding of the supernatant the pellet dried for about 5 min, then it was dissolved with water or TE buffer.

2.2.7.9 Ligation

Linear vector DNA and the desired DNA fragment (insert) were ligated to form a recombinant plasmid using bacteriophage T4 DNA ligase. In an optimal ligation

reaction, a 10 µl reaction was carried out using 50-100 ng of vector DNA. The concentration of the insert DNA should be greater than the concentration of vector DNA, theoretically the optimal molar ratio of insert to vector should be 3:1 according to Sambrook and Russell (2001). Because pBI121 is a low copy plasmid and due to its size (ca. 15 kp) the linearized vector was not gel-eluted after digestion. Therefore, the original insert was still present in the ligation reaction so that an about 10-fold excess of the new insert was used to minimize religation of the original insert. Table 11 presents the condition under which the ligation was performed. The ligation reaction was incubated at 14°C overnight.

Table 11: Composition of ligation reaction

pBI121 (vector DNA)	50-100 ng
Insert DNA	500 ng
Buffer 10*	2 µl
Enzyme (T4 DNA ligase)	1 µl
Sterile H_2O	After require
Total	20 µl

2.2.7.10 Heat shock method

The ligation product was transformed into chemically competent Top10 *E. coli* cells. T4 DNA ligase was inactivated for 10 minutes at 65°C. Chemical competent *E. coli* cells were slowly thawed on ice. 1-5 µl of the ligation product were added to 50 µl competent *E. coli* and carefully mixed and the mixture was incubated on ice for 30 min. The mixture was heat shocked for 1 minute at 42°C and 450 µl SOC medium were added of to the heat shocked cells. The mixture was shaken for 1 hour at 37°C on an orbital rotary shaker (200 rpm) and thereafter was plated out on solidified LB medium (with appropriate antibiotics). The bacteria were incubated overnight at 37°C.

SOC Medium:

- Tryptone 20 g/l
- Yeast extract 5 g/l
- 10 mM MgCl 580 mg/l

- 2.5 mM KCl 186.4 mg/l
- 10 mM $MgSO_4*7H_2O$ 2.465 g/l
- $MgCl_2*6H_2O$ 2.033g/l
- pH was adjusted to 7.0

20 mM glucose (filter sterilisation) freshly added to the autoclaved medium.

2.2.7.11 Production of chemical-competent *E. coli*

For the production of the competent *E. coli* strain Top10 and DH5α 5 ml LB medium (supplement with appropriate antibiotics) were inoculated with storage culture of these bacteria and cultivated over night at 37°C. 50 ml LB medium were inoculated with 1 ml over night culture and incubated 1-2 hours in an Erlenmeyer flask at 37°C until the density of the preculture has reached an OD_{600} of 0.3 to 0.4. The preculture was centrifuged for 10 min with 10.000 rpm at 4°C. The pellet was resuspended in ½ volume ice-cold 100 mM $CaCl_2$. After 20 min incubation on ice, centrifugation was repeated at 4°C and 10.000 rpm. The pellet was resuspended in 1/10 volume ice-cold 100 mM $CaCl_2$. After one hour incubation on ice, 86% glycerol was added and the glycerol vol. concentration was adjusted to 15%. The suspension was splitted in 100 µl aliquots. They were stored on ice for immediate use or frozen in liquid N_2 and stored at -80°C.

2.2.7.12 Production of electro-competent agrobacteria

From the strain EHA105 competent *Agrobacterium* were made according to following protocol. Bacteria colonies were scratched on YEP plates (supplement with appropriate antibiotics) and incubated for 2 days at 28°C in darkness. 10 ml YEP medium (with appropriate antibiotics) were inoculated with a single colony and the bacteria were grown over night at 28°C. 100 ml YEP medium were inoculated with the over night seed pre-culture and incubated 3-5 hours in a 250 ml Erlenmeyer flask at 28°C until the density of the preculture has reached an OD_{600} of 0.5 to 1.0. The medium was placed on ice and then centrifuged 10 min with 10.000 rpm at 4°C. The pellet was resuspended in 100 ml ice-cold 10% glycerol. Centrifugation was repeated at 4°C and 10.000 rpm. The pellet was

resuspended in 50 ml ice-cold 10% glycerol followed by another centrifugation step. The pellet was finally resuspended in 5 ml ice-cold 10% glycerol and splitted in 100 µl aliquots. They were stored on ice for immediate use or frozen in liquid N_2 and stored at -80°C up to 6 month.

2.2.7.13 Transformation of *A. tumefaciens* by electroporation

Electro competent bacteria were mixed with 1 µl (1µg/µl) plasmid and transferred into the pre-chilled 2 mm gene pulser cuvette. The cuvette was placed in the electroporation chamber of a Bio-Rad gene Pulser™ apparatus and the switch was pressed until peeping. Apparatus was adjusted to 1.3 kV/cm (25µF, 2.5kV, 400Ω). 500 µl YEB medium were immediately added after electroporation. The mixture was pipetted up and down; thereafter the suspension was transferred to a 1.5 ml test tube. The cell suspension was placed on ice for 15 min and thereafter incubated for 2 hrs at 28°C on a rotary shaker (300 rpm). 250µl of the suspensions were plated on solidified YEP selection medium (with appropriate antibiotics) and incubated for 2-3 days at 28°C in the darkness. Thereafter the grown colonies can be isolated.

2.2.7.14 Storage culture of *E. coli* and *A. tumefaciens*

For use at a later time, *E. coli* and *A. tumefaciens* can be stored at –80°C. The *E. coli* was cultured over night in liquid LB medium (for *Agrobacterium* in YEP medium) with selective antibiotics. 850 µl cell-suspensions were pipetted in with 150 µl autoclaved glycerol filled test tube. The tube was frozen in liquid nitrogen and conserved at –80°C.

2.2.7.15 Preparation of RNAi construct using the Gateway® technology (Invitrogen)

1. Creating an entry clone *via* the BP reaction: PCR to introduce attB recombination sites was done with appropriate primers. An approx. 300 bp PCR fragment was produced. 1-2 µl purified attB PCR product (approx 300 µg), 1 µl

pDON207 vector (300 ng), 4 µl BP reaction buffer, 9-10 µl TE buffer (pH 7.5) were added in a 200 µl tube. 4 µl BP-clonase mixture was added afterward. The mixture was incubated over night at 25°C. 2 ml Proteinase K solution were added and the mixture was incubated for 10 min at 37°C.

2. Transformation into *E. coli* Top10: 1-5 µl BP reaction product was used for the transformation of competent *E. coli* Top10 cells through heat shock method. The reaction mixture was plated on LB medium supplemented with gentamycin (40 mg/l) and cultivated over night at 37°C. Colony PCR with gene-specific primer was performed and the positive colonies were selected and cultivated in liquid LB medium supplemented with gentamycin over night at 37°C. The plasmids were isolated with a kit.

3. LR recombination into pHellsgate8 or pFGC5941: 1 µl purified RNAi vector (approx 300 µg), 1 µl pDON207 vector with insert (300 ng), 4 µl LR reaction buffer, 9-10 µl TE buffer (pH 7.5) were added in a 200 µl tube. 4 µl LR-clonase mixtures were added afterwards. The mixture was incubated over night at 25°C. 2 ml Proteinase K solution were added and the mixture was incubated for 10 min at 37°C.

4. Transformation into *E. coli* Top10: 1-5 µl LR reaction product was used for the transformation of competent *E. coli* Top10 cells through heat shock method. The reaction mixture was plated on LB medium (for pFGC5941 supplemented 50 mg/l kanamycin and for pHellsgate8 with 50 mg/l spectinomycin). The plates were cultivated over night at 37°C. Colony PCR with gene-specific primer were used for screening and the positive colonies were selected and cultivated in liquid LB-medium with corresponding antibiotics over night at 37°C. The plasmids were isolated with a kit.

5. Transformation into *Agrobacterium tumefaciens* EHA 105.

2.2.7.16 Southern Blot Analysis

2.2.7.16.1 Production of DIG labelled probes

DIG labelled probes were produced by PCR with the substances listed in Table 12. 5 µl PCR reaction mix was tested by gel electrophoresis to confirm the successful assign. Compared to the control, the probe was larger and moved

slower in gel because of the DIG assigning. The remaining probe was diluted in approx. 35 ml DIG Easy Hyb.

Table 12: Substances for the production of DIG assigned probe

	Probe	Control
H_2O	**38.1 µl**	**18.3 µl**
10* buffer	**5 µl**	**2.5 µl**
dNTPs 5 mM	**0.25 µl**	**1 µl**
Primer forward 10 µM	**2 µl**	**1 µl**
Primer reverse 10 µM	**2 µl**	**1 µl**
Template	**1 µl**	**1 µl**
DIG-mixture	**1.25 µl**	**-**
Taq polymerase	**0.4 µl**	**0.2 µl**
Total	**50 µl**	**25 µl**

2.2.7.16.2 Southern Blot

Approximately 20 µg genomic DNA was digested with appropriate restriction enzymes. The mixture was incubated overnight at 37ºC. After addition of another 20 units of enzyme, the samples were incubated for 2 hrs to complete the digestion. The precipitation was carried out by addition of 100 µl 7.5 M NH_4-acetate and 750 µl absolute ethanol and then centrifuged at 13000 rpm for 10 min. The pellets were dried and thereafter dissolved in TE buffer or water.

The samples were separated in 1% (w/v) agarose gel at 25 V overnight. The gel was washed max. 10 min with 0.25 M HCl and then 2-3 times with bi-distilled water. After twice 10 min denaturation with denaturation buffer and twice 10 min neutralisation with neutralization buffer, the DNA was transferred to nylon membranes overnight by capillarity in the presence of 20x SSC buffer. After transfer, the DNA was fixed to the membrane by incubation at 80ºC for 2 hrs.

The pre-hybridization was done by incubation at 42ºC for 2 hrs with Dig EasyHyb solution (Roche) and the hybridization on the appreciate probe at 42ºC overnight. The membrane was then washed twice for 10 min in 2 x SSC at 42ºC followed by washing with 0.5 x SSC and 0.1 x SSC for 15 min at 65°C. To avoid unspecific hybridization, the membrane was incubated for 2 hrs in a blocking solution

(Roche) and then incubated for 30 min in blocking solution containing antibodies (Anti digoxigenin-AP conjugate). After that, the membrane was washed twice for 15 min in washing buffer. To visualize the bands, the membrane was first incubated for 5 min in detection buffer and then CDP-Star was spread over the membrane. After incubation for 3 min at RT, the membranes were exposed to a chemiluminescence film for 30 min to 2 hours. The films were immersed in developing solution until the bands were visible, and then rinsed in water and immersed in fixation solution for 2 min. Afterwards the film was washed with water and dried in air.

Southern blot buffer:

Denaturation buffer:

- 0.5 M NaOH
- 1.5 M NaCl

Neutralisation buffer:

- 0.5 M Tris-HCl
- 3 M NaCl

pH value was adjusted to 7.5

Gel wash buffer:

- 1 L Maleic acid buffer
- 3 mL Tween

Maleic acid buffer:

- 0.1 M Maleic acid
- 0.15 M NaCl
- pH value was adjusted to 7.5

Blocking solution:

- 1% blocking reagent (Roche)
- Maleic acid buffer

Antibody solution:

- 150 mU/mL Anti-Digoxigenin-AP
- blocking solution

Detection buffer:

- 100 mM Tris HCl
- 100 mM NaCl

Stripping Buffer:

- 0.2 M NaOH
- 0.1% SDS

20x SSC

- 3 M NaCl
- 0.3 M Tris- sodium citratdihydrat

pH value was adjusted to 7.

For 2 x SSC, 0.5 x SSC, 0.1 x SSC 1% SDS was added.

50 x TAE buffer (Tris-acetate-EDTA electrophoresis buffer):

- 2 M Tris-ultra
- 10 M acetic acid
- 50 mM EDTA
- pH value was adjusted to 8.0 and afterward it was autoclaved.

For 1x TAE buffer 20ml 50x TAE was diluted in 980 ml H_2O.

2.2.7.17 Real time PCR

Real-time polymerase chain reaction was used to determine gene expression strength. The procedure follows the general pattern of polymerase chain reaction, but the DNA is quantified after each round of amplification.

1-2 young leaves were cut from tissue culture plants and grinded in the mortar with the help of liquid nitrogen. Afterwards, RNA was isolated using the Invisorb Spin® Plant RNA minit Kit. Specific primers for an apple gene encoding the elongation factor EF1 alpha (*EF1-α*) were used to screen for DNA contaminations. cDNA was synthesized with the Revert Aid^{TM} First strand cDNA synthesis kit and confirmed through PCR with *EF1-α* primer. The concentration of the cDNA was detected using the photometer.

The cDNA was diluted to 10ng/µl. For each PCR reaction the following combination was applied:

12.5 µl Real Time PCR Supermix

1 µl Primer forward

1 µl Primer reverse

1 µl cDNA solution

9.5 µl H_2O

Total volume was 25 µl. Four variants were prepared for every tested sample. Each variant was repeated 3 times. In the experiments ribulose-1,5-bisphosphate carboxylase/oxygenase activase (*rubisco)* and *RNA polymerase* were applied as house-keeping genes. Thereafter the samples were placed in I Cycler iQ Multicolor Real-Time PCR Detection system. The real time PCR result was analysed with the help of a specific computer program (Bio-Rad iQ5).

2.2.8 Pigment analysis

2.2.8.1 Thin layer chromatography (TLC) Analysis

In this work, thin layer chromatography analysis was kindly done by Dr. Thilo Fischer (Chair of Floriculture Crops and Horticultural Plant Breeding, TU Munich, Germany).

2.2.8.1.1 Pigment identification and thin layer chromatography

Flavones and flavonols are derivatives of γ-pyrone, present in plant material, mainly in the form of their glycosides. The extraction of pigments from plant material is usually carried out with methanol because not only flavonoids but also carotinoids and chlorophyll can be extracted by methanol in a satisfying quantity and quality. The majority of these substances fluoresce in ultraviolet light and the detection may generally be improved by the addition of some complexing agents to the mobile phase.

In order to extract flavonoids, 200 mg young leaves were placed into tubes with 300 ml methanol and the tube was laid at 4°C in the darkness for at least 24 hrs. The extract was concentrated with the Rotavapor and dissolved in a total volume of 1 ml MeOH.

To hydrolyze the extract, 200 µl of the extract were mixed with 200 µl 2N HCl in a 1.5 ml reaction tube and hydrolyzed for 15 min at 90°C, then the tube was

placed on ice for 3 to 5 min. 100 µl EtoAc were added. After vortexing and centrifugation for 2 min at 14.000 x g, the clear supernatant was spotted on cellulose plates with a glass capillary and dried with hot air.

Anthocyanins were extracted in a similar way but in this case 1% MeOH/HCl was used. To hydrolyze the extract, 200 µl extract were mixed with 200 µl 2N HCl in a 1.5 ml reaction tube and hydrolyzed for 60 min at 90°C. After cooling on ice, 80 µl IAA (Iso-amylalcohol) and, if required, some drops of water were added. Then it was vortexed and centrifuged at 14.000 x g until the formation of two phases was observed. After centrifugation for 2 min at 14.000 x g, the supernatant was spotted on cellulose plates with a glass capillary and dried with hot air.

Table 13: Thin layer chromatography solvent systems

Solvent systems for anthocyanins	Running time
BAW = Buthanol (6) : Acetic acid (1): Water (2)	approx. 8 h
HOAc-HCl= Water (82): Acetic acid (15): Hydrochloric acid (3)	approx. 3 h
Solvent systems for anthocyanidines	
Forestal= Acetic acid (30): Hydrochloric acid (3): Water(10)	approx. 12 h
BAW see above. The plate should be sprayed with 2N HCl to stabilize the anthocyanidines before application	
Formic= Formic acid (5): Hydrochloric acid (2): Water (3)	approx. 8 h
Solvent systems for other flavonoids	
CAW= Chloroform (50): Acetic acid (45): Water (5)	approx. 6 h
BAW see above	
30% HOAc= 30% Acetic acid	approx. 2-3 h

2.2.8.1.2 Pigment identification

1. Identification under normal light. The distances covered by the compounds after separation by TLC can be measured and compared with the references.
2. Identification under UV light. Ultraviolet spectrophotometry (UV) is one of the most frequently applied methods in flavonoid chemistry for both

instrumental analysis and structural investigation. Under UV light occur colour changes or invisible colour becomes visible. Comparison the colour with references is possible, too (Table 14).

Table 14: Colour properties of flavonoids in visible and ultraviolet light

[Harborne 1984]

Visible colour	Colour in UV light		Indication
	Alone	With ammonia	
Orange Red Mauve	Dull orange, red or mauve	Blue	Anthocyanidin 3-glycosides
	Fluorescent yellow ceriseor pink	Blue	Most anthocyanidin 3.5-diglycosides
Bright yellow	Dark brown or black	Dark brown or black	6-hydroxylated flavonols and flavones; some chalcone glycosides
		Dark red or bright orange	Most chalcones
	Bright yellow or yellow-green	Bright orange or red	Aurones
Very pale yellow	Dark brown	Bright yellow or yellow brown	Most flavonol glycosides
		Vivid yellow-green	Most flavone glycosides
		Dark brown	Bioflavonyls and unusually substituted flavones
None	Darke mauve	Faint brown	Most isoflavones and flavonols
	Faint blue	Intense blue	5-desoxyisoflavones and 7,8-dihydroxyflavanones
	Darker mauve	Pale yellow or yellow-green	Flavanones and flavanonol 7-glycosides

3. Anthocyanidin smoke test. After smoking with ammonia the colour of the anthocyanidin was deepened.
4. Dihydroflavonol spray test. Zinc powder was brushed on the plate and 6N HCl was sprayed on the brushed plate. The dihydroflavonols show a strong red to violet colour.
5. Flavone and flavonol spray test. 100 mg Sodium-Borhydride ($NaBH_4$) were dissolved to 1ml volume with water; 9 ml isopropanol (2- propanol) were added to the solvent. Then the solvent was sprayed on the plate. The flavones or flavonols were yellow after spraying. The plate was vaporized with 37% HCl for 2-3 minutes, and then the yellow colour was intensified and fixed.

2.2.8.2 Quantitative analysis

To research the metabolic change of flavonoid in transgenic plants, leaves from both transgenic and wild-type plants were harvested and frozen in liquid nitrogen sent to Freising (Weihenstephan, TU Munich, Germany). Polyphenolic secondary metabolites were performed by quantitative HPLC-DAD and LC-MS analysis. The analyses were kindly performed by Prof. Wilfried Schwab (Branches Biomolecular Food Technology, Research Department Nutrition and Food Sciences, TU Munich, Germany) and Prof. Dieter Treutter (Chair of Fruit Science, TU Munich, Germany).

2.2.8.3 Statistical analysis

Statistical tests were made with the help of a 1- factorial or 2-factorial variance analysis. Normal distribution with error probability of $P < 0.05$ was taking into account the calculated. For non-significant differences, the graphical representation was indicated with absolute values of the standard deviation. In the presence of significance of the absolute values were distinguished with different letters.

3 Results

3.1 Transformation of *Malus domestica* cv. 'Holsteiner Cox' with the *Lc* regulator gene

3.1.1 Cloning of the *Lc* gene into the binary vector pBI121

The *Lc* gene along with the CaMV 35S promoter and *nos* terminator was excised from pSRLC349 with the endonucleases *Hind*III and *EcoR*I. The fragment was introduced into pBI121 (Genbank accession No. AF485783) by replacing the GUS (-glucuronidase) gene by *Hind*III and *EcoR*I sites. The integration was confirmed by a restriction digest (Figure 13).

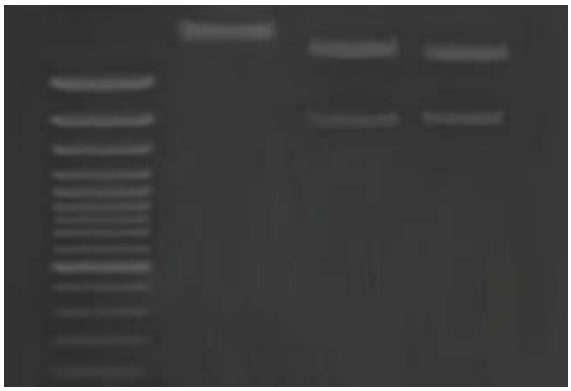


Figure 13: Restriction digest analyses of *Lc*-pBI121 vectors with restriction endonuclease *Xba*I. PK: undigested *Lc*-pBI121 Plasmid. L1, L2: colony 1 colony 2. M: 100bp+ molecular weight marker (Fermentas).

3.1.2 Transformation of the *Lc*-pBI121 constructs into cultivar 'Holsteiner Cox'

3.1.2.1 Phenotype

In order to examine the effect of the expression of the maize *Lc* regulatory gene on flavonoid biosynthesis in *M. domestica*, the gene under the control of the CaMV 35S promoter was transferred into cultivar 'Holsteiner Cox' via *Agrobacterium tumefaciens*-mediated transformation.

The shortened 2.2 kbp variant of the *Lc* gene lacking the inhibiting 200 bp of untranslated sequence was used (Lloyd et al. 1992). Explants inoculated with the agrobacteria and cultivated under kanamycin selection developed calli with red/purple groups of cells (Figure 14), later forming shoots. Five transgenic plant lines were obtained.

Figure 14: Red callus formation on leaf explants after transformation with the *Lc*-pBI121 construct and 6 weeks culture on regeneration medium (size appro. 1.5 cm).

In regenerated shoots, the pigmentation was maintained. Such pigmentation was neither observed in non-transformed control 'Holsteiner Cox' shoots nor in 'Holsteiner Cox' shoots transformed with other genes (Szankowski et al. 2003; Degenhardt et al. 2006; Rühmann et al. 2006). Transgenic shoots developed much more callus on shoot propagation medium and this callus was dark red colored. Contrarily, the callus of untransformed 'Holsteiner Cox' shoots was green. Furthermore, the growth of transgenic shoots was slower in comparison to non-transgenic shoots of the same cultivar (Figure 15).

The spatial location of the pigments within tissues of both untransformed 'Holsteiner Cox' controls and the *Lc* transgenic lines was examined. The leaves and stems were cut and observed under a microscope. Pigment accumulation in

the transgenic plants was more obvious in the nervation and mesophyll. In the stems, pigment accumulation occurs mainly in the epidermis and cambium, to a lesser extent in the phloem and in the xylem. There was plenty of pigment accumulation in the roots of the transgenic plants (Figure 16).

Figure 15: Newly propagated shoots after 4 weeks cultivation on propagation medium. Left: 'Holsteiner Cox'; Right: *Lc* transgenic plants.

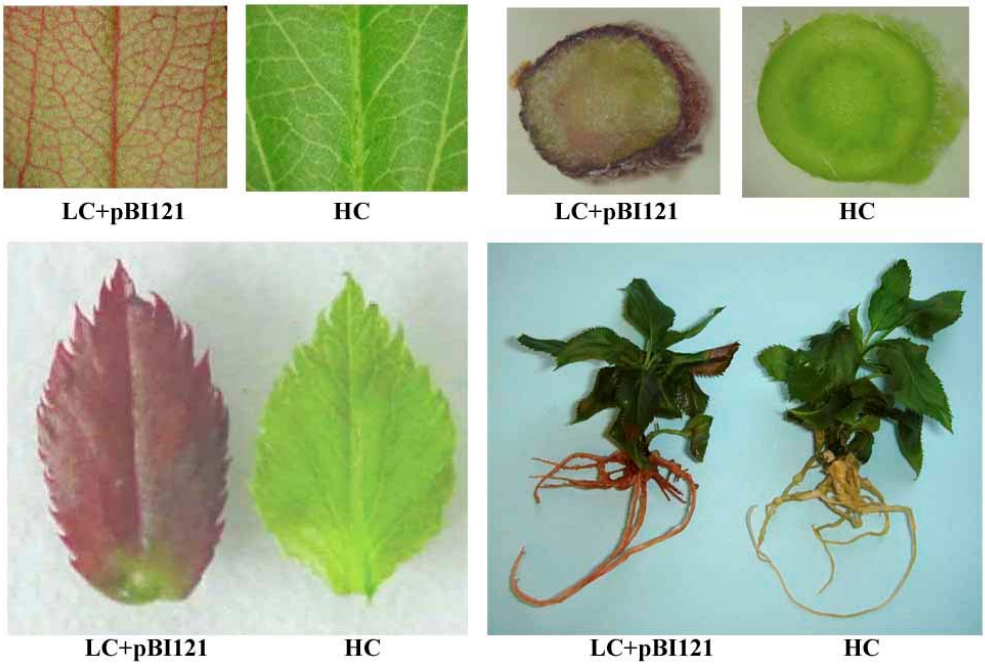


Figure 16: Pigment location in transgenic *Lc*-pBI121 plants. Left: leaf nervation; right: cut of stems; under left: leaves; under right: roots. HC: 'Holsteiner Cox' untransformed control.

3.1.2.2 Root induction and acclimatization

After shoot propagation, regenerated shoots from transgenic plants were cultivated on root induction medium supplemented with 1.5 µM IBA under light. Under these conditions, which are usually used for root induction in 'Holsteiner Cox' (Szankowski et al., 2003), none of the shoots formed roots.

In order to optimize the rooting conditions, variations of IBA concentrations in combination with or without two weeks dark treatment on rooting medium, were tested with the transgenic line 1.

In a second approach, a dark treatment of two weeks before cultivation on root induction medium was tested. About 20 shoots from treated and untreated transgenic plants were cultivated on root induction medium with 1.5, 3, 5, 7.5 and 10 µM IBA. After 4 weeks of cultivation, the root induction was rated. The results obtained showed that 2 weeks dark treatment before cultivation on medium supplemented with 3 µM IBA was the best situation for root induction in *Lc*-transgenic lines (Table 15).

Table 15: Root induction test of *Lc*-transgenic line 1 under varying conditions

	Phytohormon concentration (µM IBA)	Nr of shoots for root induction	Nr of root-forming shoots	Rooting rate (%)
Root induction without dark pre-treatment	**1.5**	**20**	**0**	**0**
	3	**20**	**2**	**10**
	5	**20**	**1**	**5**
	7.5	**20**	**0**	**0**
	10	**20**	**0**	**0**
Root induction after 2 weeks of dark pre-treatment	**1.5**	**20**	**2**	**10**
	3	**20**	**7**	**35**
	5	**20**	**3**	**15**
	7.5	**20**	**1**	**5**
	10	**20**	**0**	**0**

For the root induction of *Lc*-transgenic plants, after the 2 weeks darkness treatment, about 20 shoots from each transgenic plant line were cultivated on root induction medium (MS medium supplemented with 3 µM IBA) under weak light. After 4 weeks cultivation the roots were induced by all of the transgenic plants line (Table 16) and the roots were red (Figure 16). In order to acclimatize plants to greenhouse conditions, rooted plantlets were planted in multipot plates and

cultivated in greenhouse under high moisture. After 4 weeks cultivation, only one plant from line 1 and 2 respectively survived (Table 16).

Table 16: Results of root induction and acclimatization of *Lc*-transgenic 'Holsteiner Cox' plants

Transgenic line	Number of shoots for root induction	Root forming shoots	Root induction rate (%)	Number of plants survived in the greenhouse	Survival rate (%)
Lc1	20	9	45	1	11.11
Lc2	21	5	23,8	1	20.00
Lc3	19	6	31,6	0	0
Lc4	20	5	25	0	0
Lc6	20	9	45	0	0

3.1.2.3 Grafting of *Lc*-transgenic plants on 'Holsteiner Cox' plants

Since the root induction rate of *Lc*-transgenic plants was very low, transgenic *in vitro* shoots were grafted on non-transformed rooted acclimatized 'Holsteiner Cox' shoots.

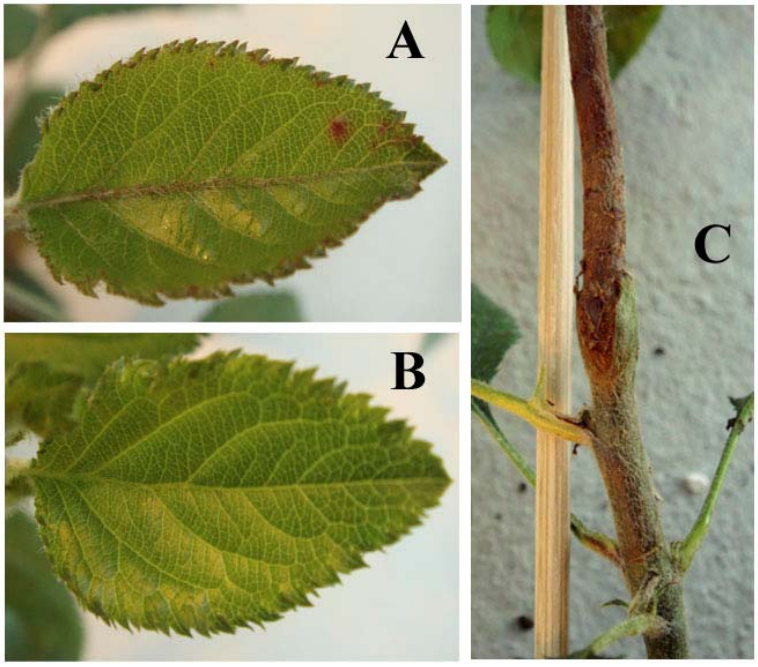


Figure 17: Phenotype of a leaf of an *Lc* transgenic grafted plant (A) grown in the greenhouse in comparison to a leaf of an untransformed control plant (B). C: *Lc* transgenic plants grafted on non-transformed 'Holsteiner Cox'

After cultivation in the greenhouse, the grafted transgenic plants grew very good. The anthocyanin accumulation decreased in comparison to the *in vitro* plants, but the plants were still distinguishable from non-transformed control plants. Anthocyanins accumulated mainly in the middle vein and at the edges of the leaves. The enhanced anthocyanin accumulation in the stem of *Lc*-transgenic plants in comparison to untransformed control plants used as the rootstock, was most obvious at the grafting side: the stem of the transgenic plant was red and stem of the untransformed 'Holsteiner Cox' was green (Figure 17).

3.1.3 Molecular analysis

3.1.3.1 Transgene integration

3.1.3.1.1 PCR analysis

Six putatively transgenic lines were regenerated on kanamycin selective medium. In order to verify the integration of the transgenes into the plant genome PCR and Southern blot analyses were performed. PCR analysis revealed the presence of both, the *Lc*-gene and the *npt*II gene, in five of six putative transgenic lines (Figure 18). The expected fragments with the sizes of 583 bp for the *Lc* gene and of 600 bp for the *npt*II gene were amplified from DNA samples of lines Lc1, Lc2, Lc3, Lc4 and Lc6. Amplification was negative when DNA of line Lc5 and of non-transformed plant material was used as template.

3.1.3.1.2 Southern Blot Analysis

In order to confirm the integration of the *Lc* gene and to investigate the copy number in the transgenic lines, the genomic DNA from transgenic plants was isolated and digested with *Hind*III. Southern blot hybridization was performed using a DIG labelled PCR amplified probe. The southern blot results showed that the *Lc* gene was successfully integrated in the genomes of the transgenic lines 'Holsteiner Cox' lines. Two gene copies were detected in line Lc6, while only one copy was found in the lines Lc1, Lc2, Lc3 and Lc4 (Figure 19).

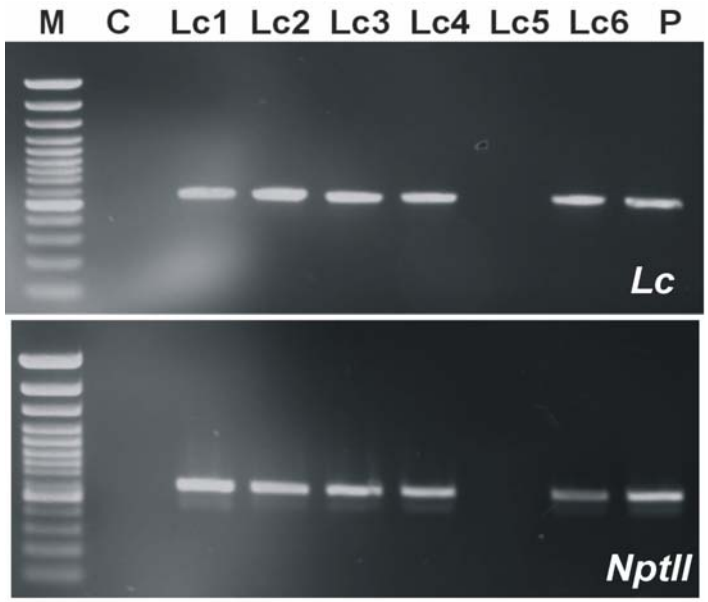


Figure 18: Electrophoresis of amplified PCR-fragments using *Lc* and *npt*II specific primer. M: 100bp+ molecular weight marker (Fermentas); C: wild type 'Holsteiner Cox' control; Lc1-Lc6: putative Lc-transgenic 'Holsteiner Cox' lines. P: plasmid *Lc*-pBI121

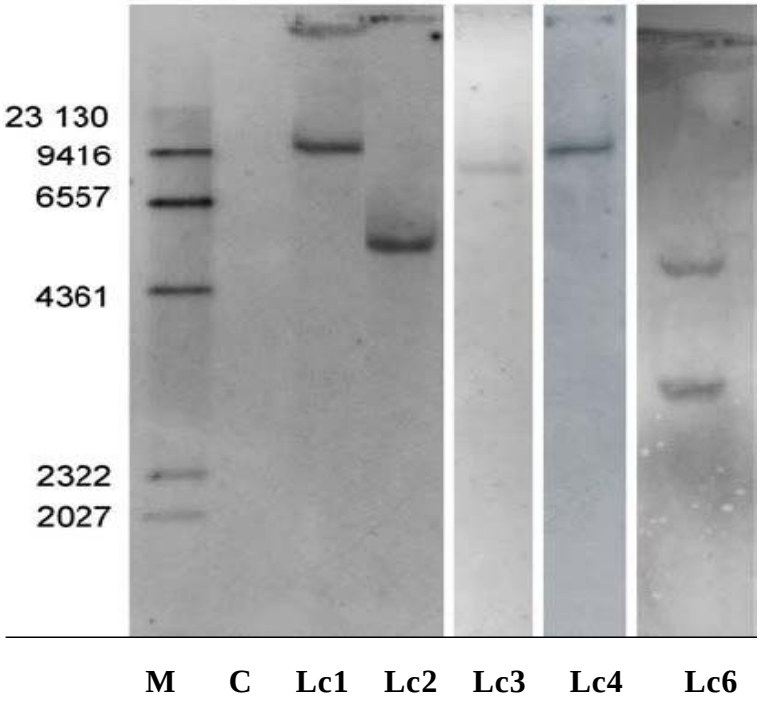


Figure 19: Southern hybridisation of *Hind*III digested DNA isolated from *Lc*-transgenic apple plants and respective control plants. Genomic DNA was

hybridised to a 583 bp *Lc*-probe. M: molecular weight markers II, C: negative control (DNA from untransformed 'Holsteiner Cox' plants); Lc1, Lc2, Lc3, Lc4, Lc6: DNA isolated from transgenic lines.

3.1.3.2 Transcription profiling

Real-time RT-PCR using gene specific primers (Table 5) was performed to obtain transcription profiles for the transgenes *npt*II and *Lc* as well as for the genes of the flavonoid biosynthetic pathway encoding PAL, CHS, CHI, FHT, FLS, DFR, LAR, ANS, ANR and FGT. Expression levels of above-mentioned genes were determined and expressed relative to the *rubisco* and the *RNA polymerase* gene. These two genes were chosen as house-keeping genes because SYBR-Green RT-PCR analysis showed that they have a high and stable mRNA expression level in apple leaves (H. Flachowsky, unpublished).

The transcripts of the transgenes *npt*II and *Lc* were detected in all transgenic lines, while neither *npt*II nor *Lc* expression was observed in non-transgenic control samples. The *Lc* gene transcription levels of the transgenic lines ranged between 137 (line Lc4) and 291 (line Lc2) (Figure 20).

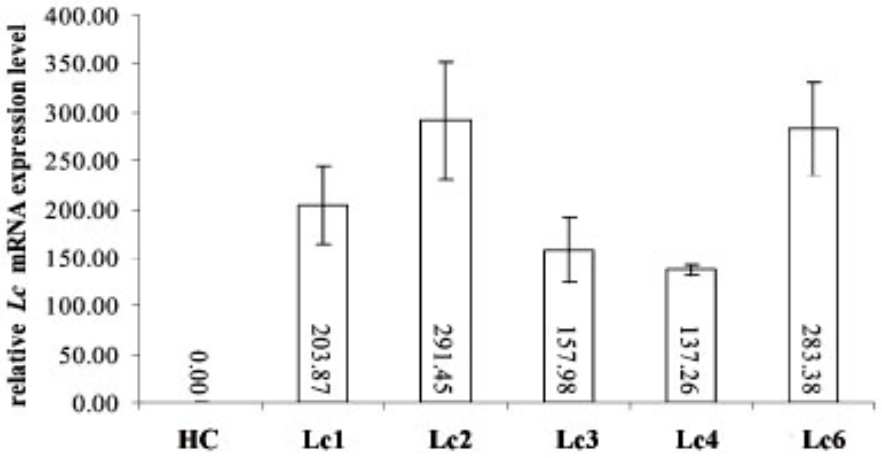


Figure 20: *Lc* gene mRNA expression levels of five *Lc* transgenic 'Holsteiner Cox' lines determined by real-time PCR. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are the mean of three replicates ±SE. HC: wild type 'Holsteiner Cox'. Lc1, Lc2, Lc3, Lc4, Lc6: transgenic plants.

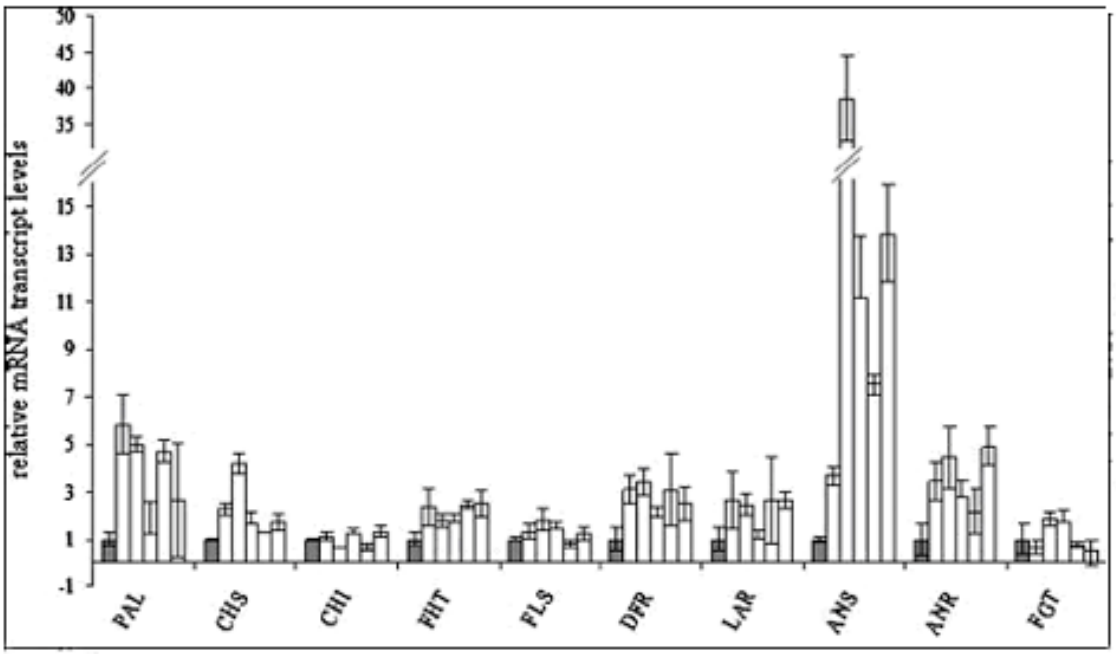


Figure 21: mRNA expression levels of genes involved in the flavonoid biosynthetic pathway in *Lc* transgenic apple lines (Lc1, Lc2, Lc3, Lc4, Lc6: white bars from left to right) determined by real-time PCR. The values are expressed relative to the level of transcripts in the untransformed 'Holsteiner Cox' (dark grey) set to be one. Values are the mean of three replicates ±SE.

The relative expression levels of the structural genes revealed that almost all *Lc* transgenic lines showed significantly increased transcription levels for *PAL, CHS, FHT, DFR, LAR, ANS and ANR* in comparison to the non-transformed wild type (Figure 21). Especially *ANS* transcription was strongly induced (10- to 50-fold) in all transgenic line related to non- transgenic control. In contrast, *FGT* was clearly induced in only some lines. Neither *CHI* nor *FLS* were induced by the *Lc* transgene.

3.1.4 Metabolic profiling

3.1.4.1 Pigment analysis by thin layer chromatography

For the identification of the flavonoids in transgenic *Lc* plants, thin layer chromatography (TLC) methods were used. The pigments of genetic transformed

plants were extracted and put on cellulose plates as described in methods. Anthocyanidin was separated in Forestal solvent. The results showed that cyanidin was not observed in non-transgenic 'Holsteiner Cox' control; in contrast all of the *Lc*-transgenic plants lines exhibit a strong accumulation of cyanidin. Flavonol was separated in CAW solvent; the TLC results showed that both quercetin and kaempferol were detected in wild-type control; in contrast to control plants, the amount of quercetin was clearly increased in all of the transgenic plants line; the increase of kaempferol was not so large in transgenic plants, but the amount of kaempferol in three transgenic lines was clearly increased. Flavanol was separated in BAW solvent and treated with DMAZA reagents; the TLC results showed that catechin and epicatechin were nearly not detectable in the wild- type control; in all of the transgenic lines the accumulation of catechin was clearly observed; the accumulation of epicatechin was very weak in transgenic plants, but it was detectable (Figure 22).

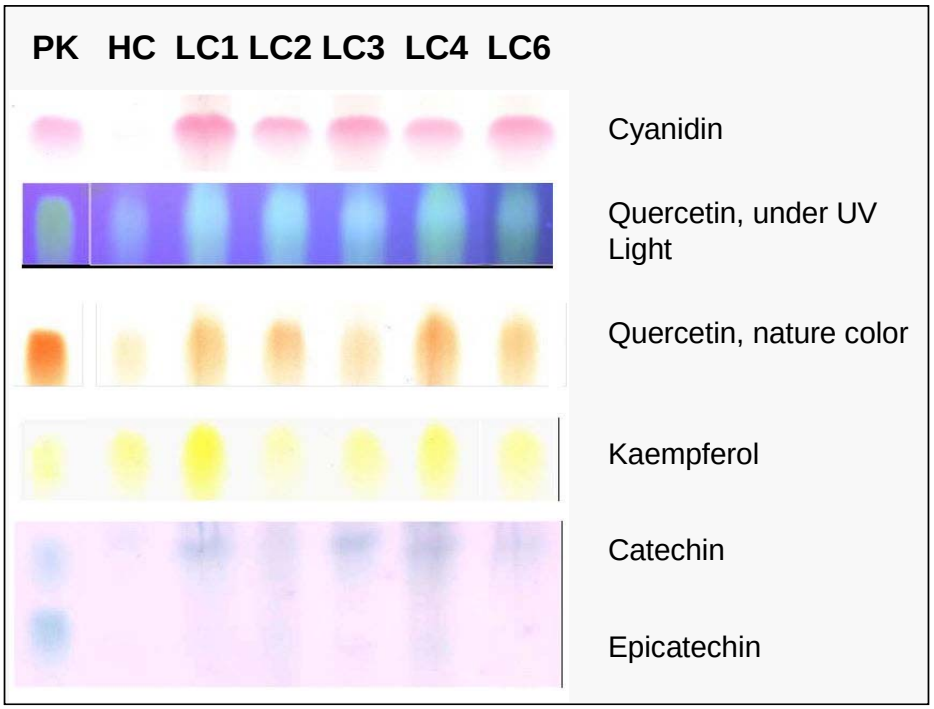


Figure 22: Results of thin layer chromatography from unhydrolyzed extracts from leaves of wildtype and transgenic 'Holsteiner Cox' plants. PK: positive control of chemical reagent; HC: 'Holsteiner Cox'; LC1. LC2, LC3, LC4, LC6: transgenic plants line. (This TLC analysis was kindly done by Thilo Fisher, TU Munich)

3.1.4.2 Quantitative analysis of flavonoid levels in the *Lc*- transgenic plants

To research the metabolic change of flavonoid accumulation in transgenic plants, leaves from both *Lc* transgenic and wildtype 'Holsteiner Cox' plants were harvested and a profile of polyphenolic secondary metabolites was performed by quantitative HPLC-DAD and LC-MS analyses. The analyses were kindly performed by Prof. Wilfried Schwab and Prof. Dieter Treutter (TU Munich, Germany).

In unhydrolyzed extracts of *Lc*-transgenic plants (Figure 23), a large increase was observed in the level of cyanidin compared with that seen in wild type extracts. In addition to cyanidin, a significant increase in the level of quercetin was detected.

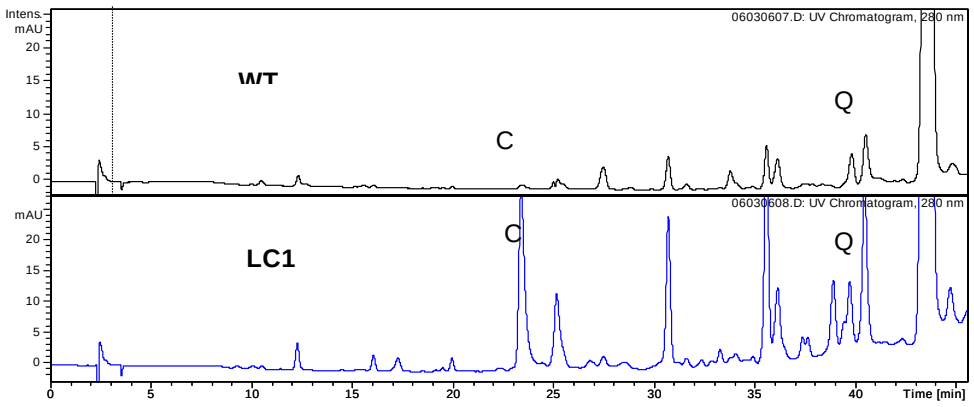


Figure 23: HPLC Results of unhydrolyzed extracts from leaves of wild- type and transgenic line 1 plants. C: cyanidin; Q: quercetin; mAU: 0.001 absorption units. Over: 'Holsteiner Cox'; under: *Lc* transgenic plant

Hydrolyzed extracts of leaves were used to quantify the levels of polyphenolic secondary metabolites in transgenic plants. A number of anthocyanins, monomeric and polymeric flavan 3-ols (catechins, proanthocyanidins), flavonols, dihydrochalcones, hydroxycinnamic acids and hydroxybenzoic acids were identified and quantified from leaves of untransformed *in vitro* plants and the five *in vitro* lines transgenic for *Lc* (Table 17, Figure 24).

Table 17: **Concentrations of phenolic compounds in leaves of untransformed ('Holsteiner Cox') and transgenic *in vitro* apple lines.** Values represent the mean of two biological replicates

Compound	HPLC (mg/g of dry weight)					
	'Holsteiner Cox'	Lc1	Lc2	Lc3	Lc4	Lc6
Anthocyanins Idaein	Below detection limit	0.99	0.52	0.20	0.35	0.46
Monomeric flavan 3- ols total	0.10	4.23	4.61	4.51	2.89	3.87
Procyanidin: Derivatives of catechin, total	0.05	0.63	0.39	0.47	0.41	0.46
Derivatives of Epicatechin, total	1.28	2.23	2.30	2.40	2.25	2.35
Flavonols, total	6.26	8.24	9.86	6.94	8.35	7.41
Dihydrochalcones, total	95.07	106.99	78.42	88.26	104.1	121.32
Cinnamic acid derivatives, total	0.43	1.72	0.84	1.41	1.05	1.54
Other phenolic compounds, total	0.54	0.67	1.27	1.19	0.51	0.70

Generally, members of almost all analysed polyphenol classes were induced by *Lc* gene. Strongest rises were observed for the anthocyanin idaein (12-fold), epicatechin (14-fold), catechin (41-fold) and proanthocyanidins. Remarkably, only two distinct proanthocyanidins, namely B2 and a yet unidentified proanthocyanidin were selectively induced by mean factors of 58 and 134, respectively (Figure 24). The concentration of hyperin (quercetin-3-O-galactoside) as the prominent *M. domestica* flavonol was only slightly increased while levels of other flavonol glycosides remained unchanged or were even reduced (Table 17). Also within the large pool of dihydrochalcones (phloretin, phloridzin and phloretin 2'-xyloglucoside) as the dominant *M. domestica* polyphenol class, the levels in the transgenic lines increased only slightly (up to threefold). Some hydroxycinnamic acids, hydroxybenzoic acids and their derivatives showed rises up to a mean factor of about 2.

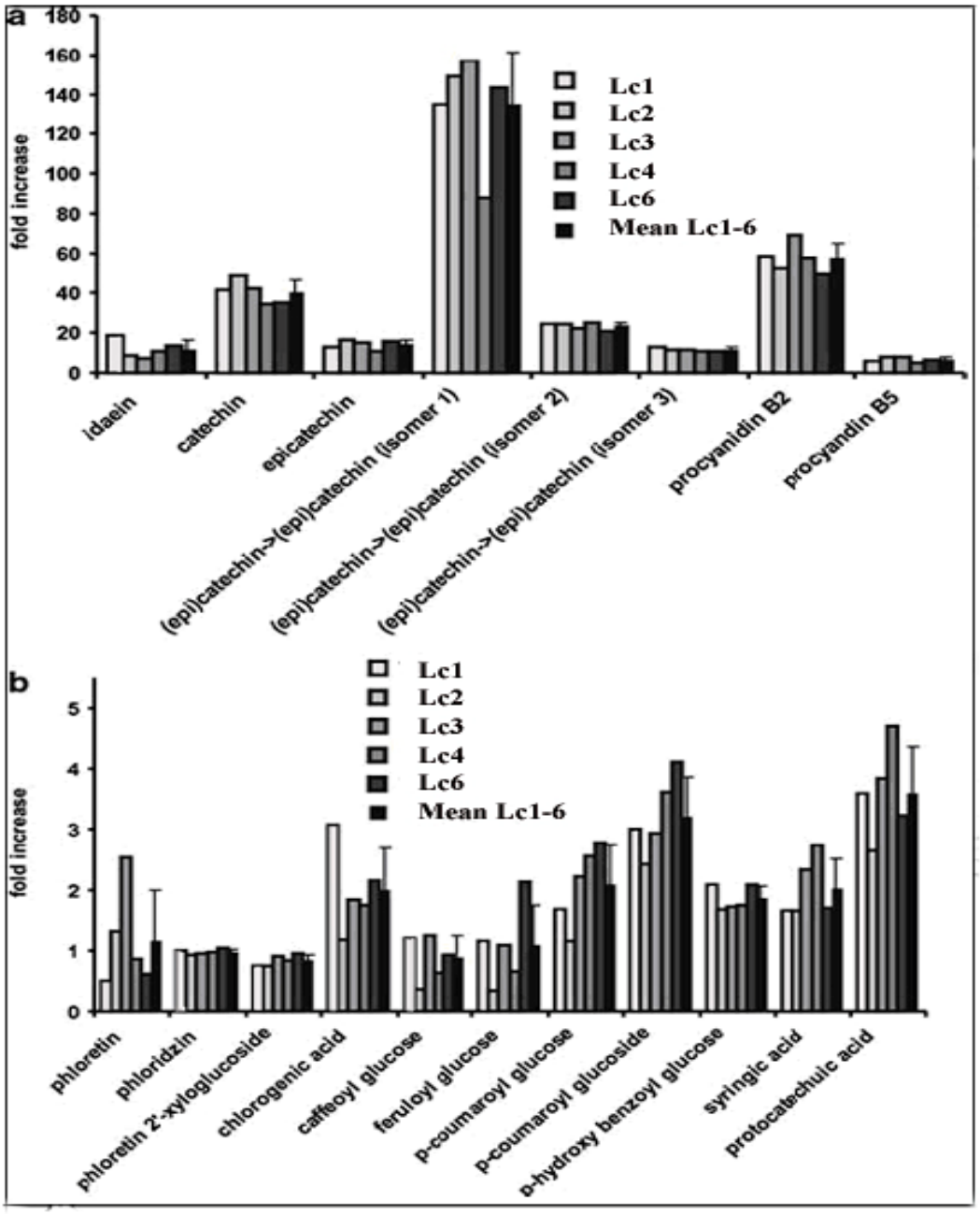


Figure 24: *Lc*-dependent increase in the levels of flavonoids (a**) and other phenolic secondary metabolites (**b**) determined by LC-MS.** Values are expressed as fold induction of metabolite levels in transgenic lines relative to metabolite levels in the untransformed controls. The *black bar* represents the means SD of values obtained from all transgenic lines (Lc1-Lc6).

3.1.5 Selection without antibiotics

Since the expression of *Lc* induced strong anthocyanin pigmentation in transformed tissue, it was examined whether this pigmentation could be used as a selectable marker for the selection of transgenic cells. The visual detection and separation of pigmented tissues could be used as an alternative to the conventional marker genes, such as herbicide or antibiotic resistance genes. In order to establish a selection method based on anthocyanin pigmentation, 200 apple leaf explants were infected with *A. tumefaciens* strand EHA105, which contains *Lc*-pBI121 construct; After 3 days coculture in darkness the infected explants were washed and cultivated on regeneration medium with 150 mg/l tircarcillin and continually cultivated in darkness. After 2 weeks culture the explants were transferred on regeneration medium with 150 mg/l tircarcillin and cultivated under light. After 2 weeks cultivation explants produced callus. In contrast to green callus of control explants, those which were inoculated with *Agrobacterium* formed red callus and green callus at the same time (Figure 25: A and B). The green area of the callus was separated and the red callus was cultivated on new medium; the process was repeated every week. After several weeks the callus regenerated new shoots; the new shoots were transferred on elongation medium and after 2 weeks on propagation medium. After propagation, shoots were large and the colours of them were red or half- red (Figure 25: C).

Young leaves of the shoots were cut and the genomic DNA was isolated and used as a template for PCR test with *Lc* primers to amplify the fragment of the *Lc* gene. In most of the samples the expected fragment of approximate 600bp was amplified (Figure 26).

The shoots were propagated on new medium and medium was changed every 4 weeks. After approximate 4 months cultivation the colour of all of the shoots changed to green. Young leaves of the shoots were cut and the genomic DNA was isolated. PCR test with *Lc* primer showed that no fragment was amplified in all of the probes. Obviously, regenerated plants were of chimeric origin.

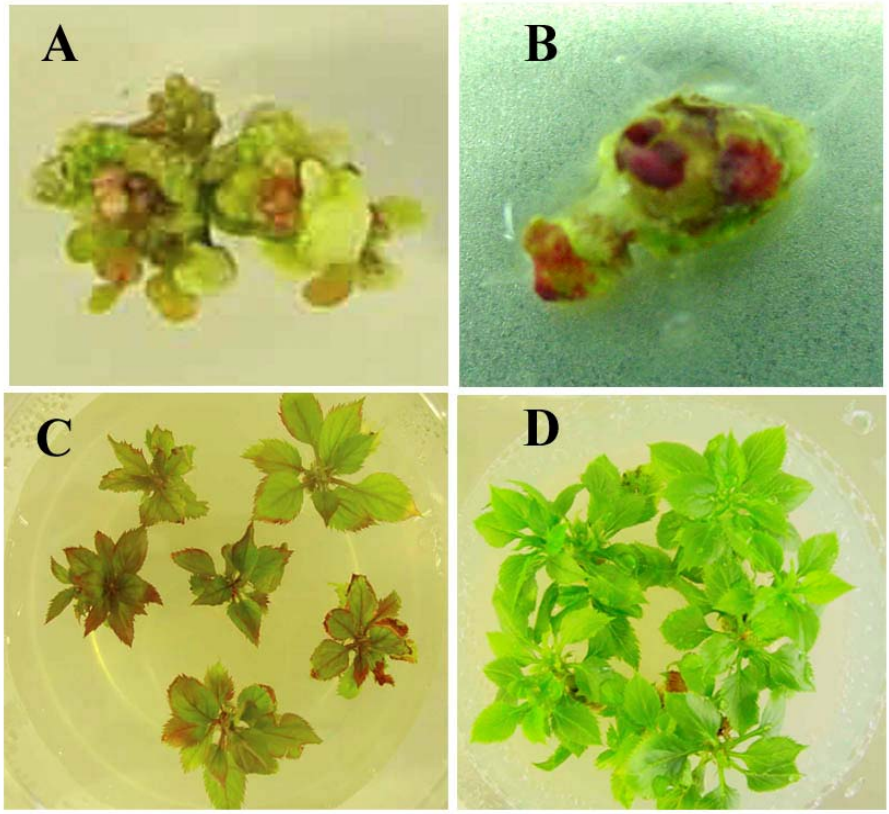


Figure 25: Selection of transgenic *Lc*-pBI121 plants by hand separation of pigmented tissue. A: callus regeneration after transformation with *Lc*-pBI121; B: red callus separated from the original explant; C: regenerated shoots. D: new propagated shoots.

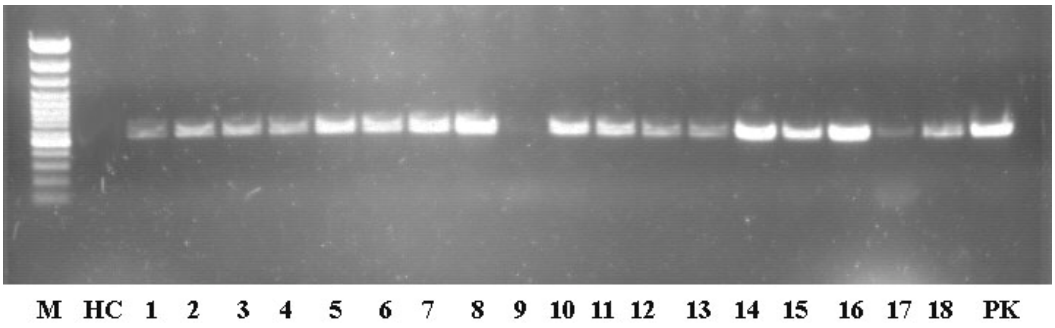


Figure 26: Electrophoresis of amplified PCR-fragments using *Lc* specific primer. M: 100bp+ molecular weight marker (Fermentas); HC: wild type 'Holsteiner Cox' control; 1-18: putative *Lc*-transgenic 'Holsteiner Cox' without antitiotics selection. PK: plasmid *Lc*-pBI121.

3.2 Metabolic engineering of flavonoid synthesis through RNA interference

There are two branching points in the anthocyanin pathway which lead to the production of (epi-) catechins and pro-anthocyanidins: one at the level of leucoanthocyanidins and one at the level of anthocyanidin aglycons. Leucoanthocyanidins can be converted either to anthocyanidins and subsequently anthocyanins through the action of ANS and FGT or reduced to catechins through the action of the enzyme leucoanthocyanidin reductase (LAR). With the aim to enhance flavanol accumulation in apple, we inhibited competitive branches by either suppression of *ANS* or *FGT*. Vectors designed to produce self-complementary hpRNA in transgenic plants were used to transform a green- (cv. 'Holsteiner Cox') and a red leaved apple cultivar ('TNR 31-35'). While regeneration procedure for 'Holsteiner Cox' was already established, the conditions for organogenesis from leaf explants had to be developed for 'TNR 31-35'.

3.2.1 Tissue culture

3.2.1.1 Regeneration of 'TNR 31-35'

As a prerequisite for the development of transgenic plants from 'TNR 31-35', a regeneration procedure based on adventitious shoot regeneration from leaf explants was established. The influence of different IBA and TDZ concentrations on regeneration rate and number of shoots per explants was tested. Results indicated that a combination of 1 µM IBA and 3 µM TDZ was most suitable for regeneration (Table 18) of 'TNR31-35' via organogenesis.

3.2.1.2 Rooting

To find the best phytohormon concentration for root induction for 'TNR 31-35', different IBA concentrations were tested. The shoots were cultivated under weak light. Evaluation was performed after 4 weeks. The results showed that the shoots on MS medium with 1 µM IBA induced a 100% rooting rate (Table 19). The hormone concentration was therefore chosen for rooting of 'TNR 31-35' shoots.

Table 18: Regeneration of 'TNR 31-35' explants on MS medium supplemented with different phytohormon concentrations and combinations.

Phytohormon concentration (µM)	Number of explants	Regeneration rate (%)	Number of regenerated shoots	Shoots per explants
1 IBA+3 TDZ	**20**	**100**	**183**	**9.15**
1 IBA+5 TDZ	**20**	**100**	**181**	**9.05**
1 IBA+10 TDZ	**20**	**100**	**155**	**7.75**
1 IBA+15 TDZ	**20**	**100**	**157**	**7.85**
1 IBA+20 TDZ	**20**	**95**	**134**	**6.70**
2 IBA+3 TDZ	**20**	**100**	**118**	**5.90**
2 IBA+5 TDZ	**20**	**95**	**145**	**7.25**
2 IBA+10 TDZ	**20**	**100**	**131**	**6.55**
2 IBA+15 TDZ	**20**	**95**	**132**	**6.60**
2 IBA+20 TDZ	**20**	**80**	**67**	**3.35**
5 IBA+3 TDZ	**20**	**85**	**71**	**3.55**
5 IBA+5 TDZ	**20**	**75**	**91**	**4.55**
5 IBA+10 TDZ	**20**	**95**	**122**	**6.10**
5 IBA+15 TDZ	**20**	**75**	**55**	**2.75**
5 IBA+20 TDZ	**20**	**60**	**77**	**3.85**
7.5 IBA+3 TDZ	**20**	**95**	**140**	**7.00**
7.5 IBA+5 TDZ	**20**	**75**	**77**	**3.85**
7.5 IBA+10 TDZ	**20**	**90**	**141**	**7.05**
7.5 IBA+15 TDZ	**20**	**65**	**40**	**2.00**
7.5 IBA+20 TDZ	**20**	**70**	**46**	**2.30**
10 IBA+3 TDZ	**20**	**60**	**44**	**2.20**
10 IBA+5 TDZ	**20**	**40**	**21**	**1.05**
10 IBA+10 TDZ	**20**	**50**	**36**	**1.80**
10 IBA+15 TDZ	**20**	**60**	**50**	**2.50**
10 IBA+20 TDZ	**20**	**65**	**90**	**4.50**

Table 19: Root formation of 'TNR 31-35' on medium supplemented with different phytohormon concentrations and combinations. The basic regeneration medium was MS medium.

Phytohormon concentration (µM)	Number of shoots used	Shoots with roots	Root induction rate (%)
1.5 IBA	26	26	100
3 IBA	30	24	80
5 IBA	31	13	41.94
7.5 IBA	31	5	16.13
10 IBA	30	5	16.13

3.2.1.3 *In vitro* flavonoid induction

To induce anthocyane formation *in vitro* and to establish a fast test system for testing successful modification of flavonoid synthesis in apple, 40 leaf explants of the apple cv. 'Holsteiner Cox' were placed on modified MS medium containing 1/5, ¼, 1/3, ½ of the normal concentration of macronutrients and no ammonium. The same number of leaf explants was put on normal regeneration medium as controls. The explants were laid in darkness to minimize the chlorophyll production. After 2 weeks of culture the explants were put under light. After one week cultivation under light, the color of the explants on low macronutrients started to change to red; the explants were transferred on new medium. After further 6 weeks cultivation, the explants on 1/5 macronutrients medium were completely changed to red and the leaves explants on ¼, 1/3 and ½ macronutrients medium appeared red color, too (Figure 27). The results of flavonoid induction showed that the anthocyane were induced on the low macronutrients concentration and without ammonium; lower macronutrient concentrations induced stronger anthocyane accumulation.

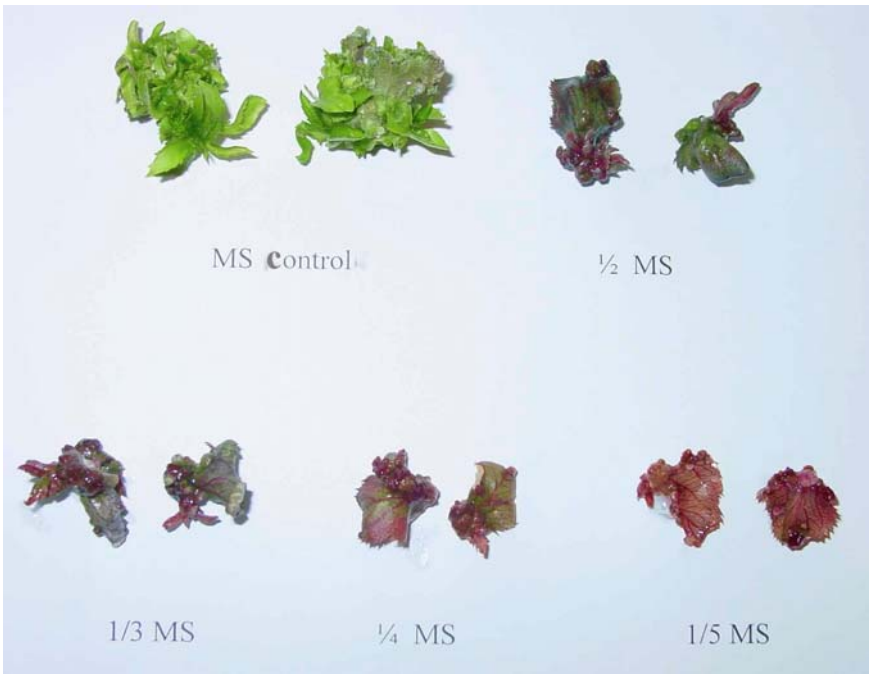


Figure 27: Leaf explants of 'Holsteiner Cox' after 8 weeks of *in vitro* anthocyane induction medium.

3.2.2 RNA interference vectors

In order to induce post-transcriptional gene silencing in apple, the binary vectors pHellsgate8 and pFGC5941 were used. The vectors allow cloning of cDNA fragments using the recombination based Gateway® cloning technology. The fragments are arranged as an inverted repeat downstream the CaMV35S promoter, resulting in a transgene that produces self-complementary hpRNA in transgenic plants. A 292 bp *ANS* fragment and a 289 bp *FGT* fragment were amplified with primers through PCR, which also introduce attB-sites for subsequent recombination reactions. The orientation of the *ANS* and *FGT* fragments in the expression vector was examined by PCR and restriction analysis. *Xba*I and *Xho*I were chosen as vector specific restriction enzymes for pHellsgate8. Two restriction sites for *Xba*I were located at the both side of the first fragment and two restriction sites for *Xho*I were located at the both side of the second fragment (Figure 28). Therefore, using the enzymes *Xba*I or *Xho*I, it was expected that right orientation of the fragments should lead to approximate 400 bp restriction fragments. For the PCR examination, only one primer was used for the amplification: attB1 primer for the pHellsgate8 vector (it should lead to an approximate 1500 bp fragment) and attB2 primer for pFGC5941 constructs (it should lead to an approximate 1500 bp fragment) (Figure 28).

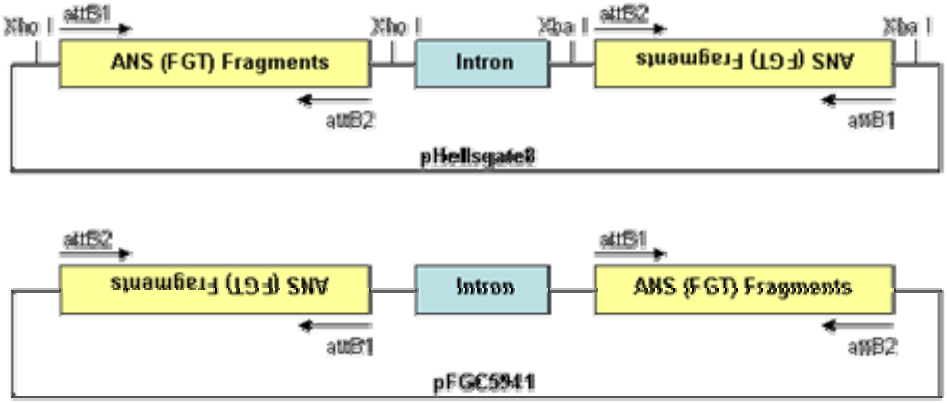


Figure 28: Orientation of the *ANS* and *FGT* fragments in the plasmids pHellsgate8 and pFGC5941.

The positive colonoes were selected and cultivated in liquid medium and afterwards the plasmids were isolated and tested by PCR and restriction digest. The digest and PCR analyses showed that: for *ANS*-pHellsgate8 plasmids of locus 4 and locus 5 had matching bands (Figure 29: B and C); for *FGT*-pHellsgate8 plasmids of colony 1 and colony 3 had matching bands (Figure 29: D, E and F); for *ANS*-pFGC5941 plasmids of colony 1, 2, 3 and 4 had matching bands (Figure 30: A and B). For *FGT*-pFGC5941 constructs plasmids of colony 1, 5 and 6 were positive (Figure 30: C and D).

The clones of the four constructs were sent for sequencing and the sequence data confirmed the successful cloning. The four constructs were transferred in *A. tumefaciens* strain EHA105 through electroporation as described in methods. After cultivation and selection with appropriate antibiotics the agrobacteria were applied for the following plant transformation.

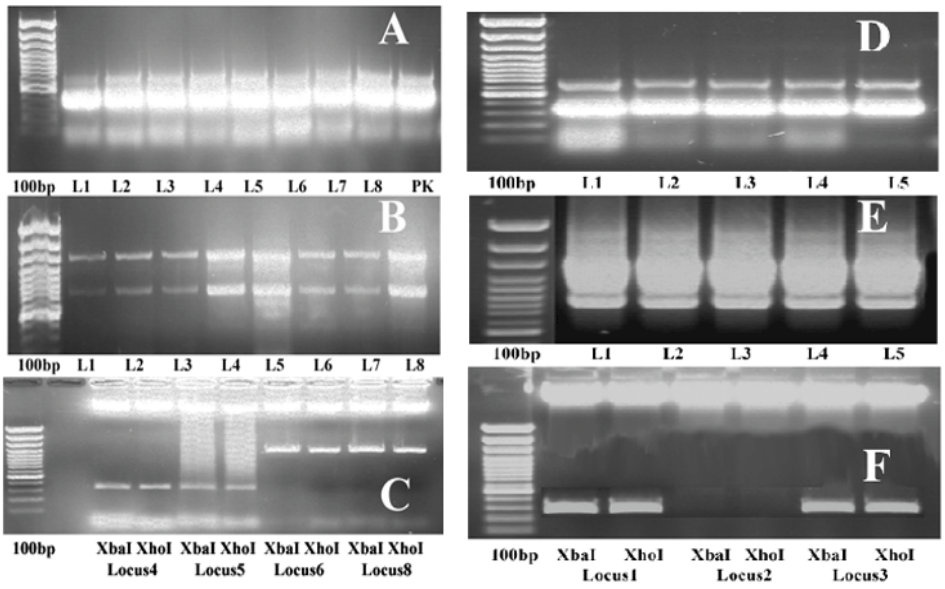


Figure 29: PCR and restriction digest analyses of *ANS*- and *FGT*-pHellsgate vectors. A: colony PCR of *ANS*-pHellsgate8; B: PCR of *ANS*-pHellsgate8 with ANS attB1 Primer; C: digest analysis of *ANS*-pHellsgate8 with restrictions endonucleases *Xba*I and *Xho*I; D: colony PCR of *FGT*-pHellsgate8; E: PCR of *FGT*-pHellsgate8 with FGT attB1 Primer; F: digest analysis of *FGT*-pHellsgate8 with restrictions endonucleases *Xba*I and *Xho*I. L: colony line; PK: positive control.

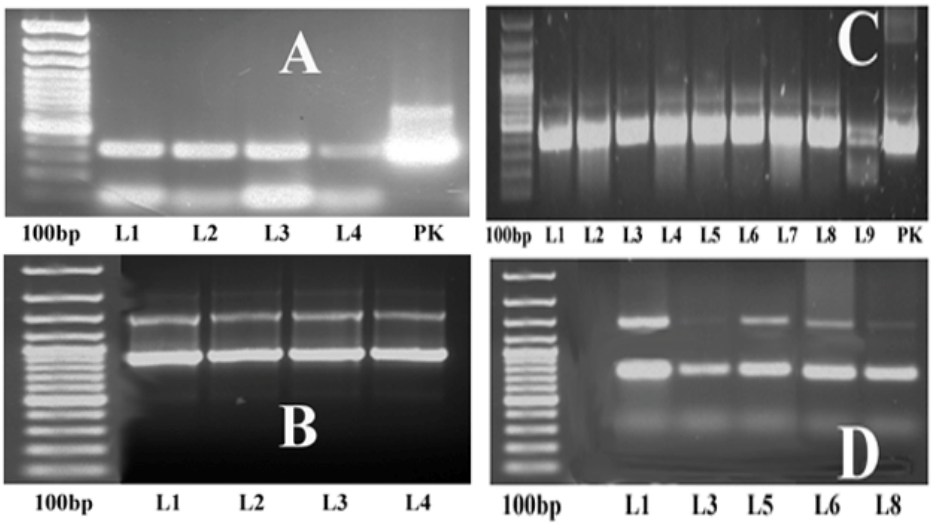


Figure 30: PCR and digest analyses of transformation results of *ANS*- and *FGT*-pFGC5941 constructs. A: colony PCR of *ANS*-pFGC5941; B: PCR with ANS attB2 Primer; C: colony PCR of *FGT*-pFGC5941; D: PCR with FGT attB2 Primer. L: colony line; PK: positive control.

3.2.3 Transformation of *ANS*-pFGC5941 into 'TNR 31-35' and 'Holsteiner Cox'

In order to proof the principle of the effect of RNAi-silencing of *ANS* in apple the red leaved cultivar 'TNR 31-35' and green leaved cultivar 'Holsteiner Cox' were used for transformation. The red shoot colour of 'TNR 31-35' should be caused from large amount of anthocyane accumulation in the plant tissue. When the ANS transcription is blocked, the anthocyane accumulation should be inhibited and the shoots should no more be red. The colour change was easy to be visually observed and the change of anthocyane accumulation was easy to be detected with HPLC and TLC analysis.

Plants successfully regenerated on selective medium were tested by PCR and Southern blot analyses for integration of the selectable marker gene (*bar* gene). Influence of RNAi on expression of the *ANS* gene as well as on metabolite accumulation was tested with Real time PCR and HPLC analyses respectively.

3.2.3.1 Transformation and regeneration

The transformation of the apple cultivars 'TNR 31-35' and 'Holsteiner Cox'was performed as previously described. After coculture, transformation and selection, four putative transgenic lines of 'TNR 31-35' and five putative transgenic lines of 'Holsteiner Cox' were regenerated on selective medium. In case of 'TNR 31-35', the color of regenerated shoots and leaves was changed compared to untransformed wild type plants: some were green and some were both green and red under lower selection stress; and the shoots were completely green under higher selection stress (Figure 31). No phenotypic changes were observed for 'Holsteiner Cox' (Figure 32).

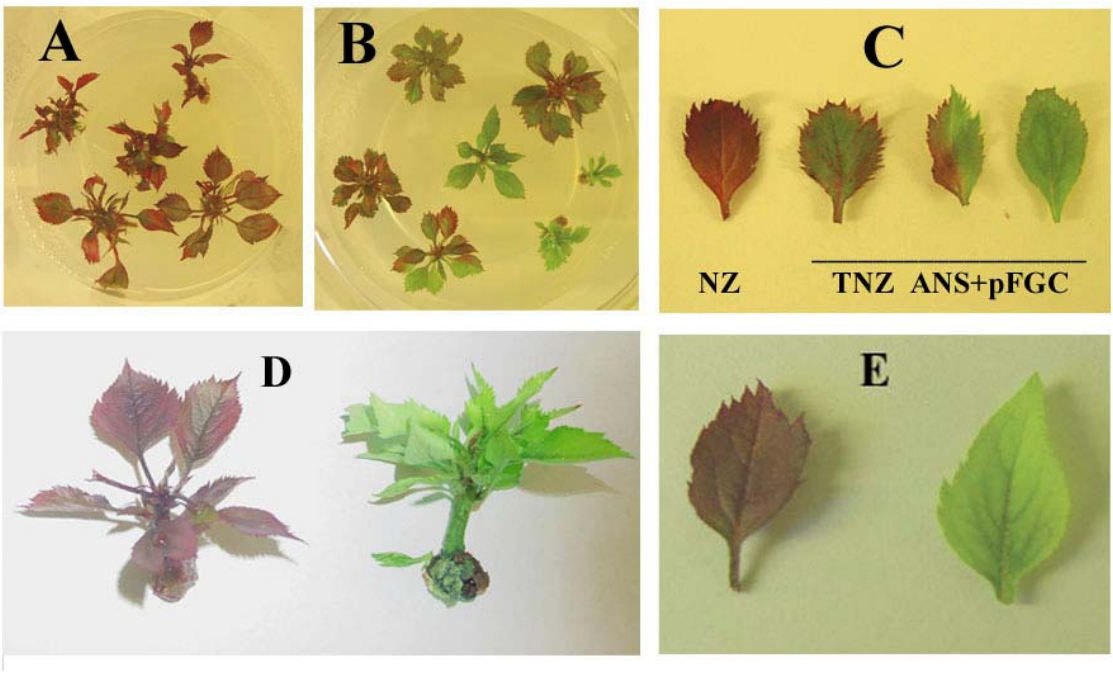


Figure 31: Shoots and leaves of transgenic 'TNR 31-35' plants transformed with the *ANS*-pFGC5941 construct. A. Untransformed control shoots of 'TNR 31-35'. B: Shoots of transgenic 'TNR 31-35' after ppt selection (1mg/l). C: Leaves from transgenic 'TNR 31-35' shoots after selection with 1 mg/l ppt; NZ: 'TNR 31-35' untransformed control. TNZ: transgenic plants. D: Shoots of transgenic 'TNR 31-35' plants after selection with 5 mg/l ppt; left: 'TNR 31-35' control; right: transgenic plants. E: Leaves from transgenic 'TNR 31-35' shoots after selection with 5 mg/l ppt; left: 'TNR 31-35' control; right: transgenic plants.

A B C D

Figure 32: Leaves of transgenic 'Holsteiner Cox' transformed with the *ANS*-pFGC5941 construct. A. Young leaf of an untransformed 'Holsteiner Cox' control plant. B: Old leaf of an untransformed 'Holsteiner Cox' control plant. C: Young leaf from transgenic 'Holsteiner Cox' shoots. D: Old leaf from transgenic 'Holsteiner Cox' shoots.

3.2.3.2 Root induction

The shoots of transgenic 'TNR 31-35' and 'Holsteiner Cox' plants were cultivated on root induction medium to induce root development. Transgenic 'TNR31-35' shoots failed to develop roots while transgenic 'Holsteiner Cox' exhibited normal root development. Thereafter the rooted transgenic 'Holsteiner Cox' shoots were cultivated in the greenhouse. Table 20 showes root induction results of transgenic 'Holsteiner Cox' and the survival rate in greenhouse.

Table 20: Results of root induction experiments and acclimatization of *ANS*-pFGC5941 transgenic 'Holsteiner Cox' plants to greenhouse conditions.

ANS-pFGC5941 transgenic line	Nr of shoots	Root forming shoots	Rooting rate (%)	Acclimatized to the greenhouse	Survival rate (%)
Line1	22	16	72,72	4	25.00
Line1B	22	14	63,63	3	21.43
Line 3	20	11	55.00	6	54.55
Line 4	23	14	60,87	7	50.00
Line 5	21	8	38.09	2	25.00
'Holsteiner Cox' control	20	18	90.00	13	72.22

3.2.3.3 Flavonoid induction test

To observe the influence of the silencing of the ANS in transgenic 'Holsteiner Cox' 20 leaves explants of transgenic plants and wild type control plants were placed on MS medium containing 1/5 of the normal concentration of macronutrients and without ammonium, which was previously proven to induce anthocyanin accumulation in apple leaf explants. The explants were cultivated in darkness for 2 weeks and thereafter under a 16/8 hrs photoperiod. After 2 week culture under light, the color of the wild type control explants was completely change to red; whereas the colour of leaves explants from transgenic plants was green or fewer red (Figure 33). This demonstrates that the suppression of ANS leading to down regulation of anthocyanin synthesis.

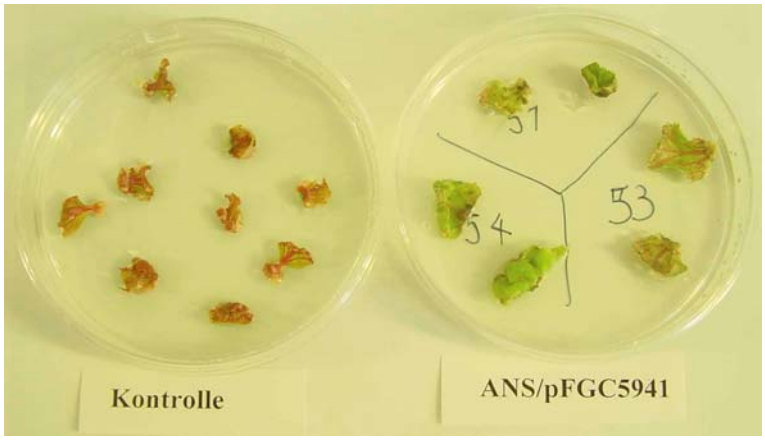


Figure 33: Color change of transgenic plants after 4 weeks anthocyane induction. Left: 'Holsteiner Cox'; right: transgenic plant with *ANS*-pFGC5941 constructs.

3.2.3.4 PCR analysis

In order to test the integration of the transferred T-DNA into the apple genome, PCR analyses were performed to confirm the presence of the selectable marker gene *bar*. The expected fragment of the size of 264 bp was found in all tested lines of 'TNR 31-35' and 'Holsteiner Cox' (Figure 34 and 35). No fragment was observed for the untransformed control plants.

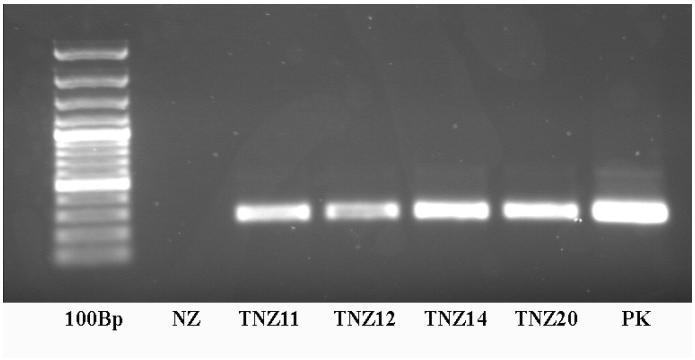


Figure 34: PCR analysis of transgenic 'TNR 31-35'. The Fragments were amplified by the *bar* primers. 100Bp: 100bp+ molecular weight markers; NZ: wild type 'TNR 31-35' control; TNZ11, TNZ12, TNZ14, TNZ20: Transgenic 'TNR 31-35'; PK: *ANS*-pFGC5941 plasmid

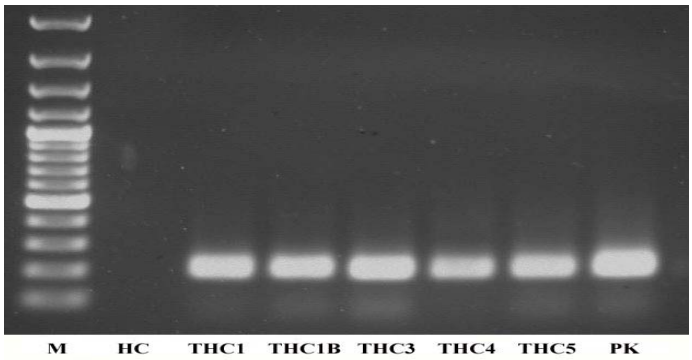


Figure 35: PCR analysis of transgenic 'Holsteiner Cox'. The Fragments were amplified by the *bar* primers. M: 100bp+ molecular weight marker; HC: Wild type 'Holsteiner Cox' control; THC1, THC1B, THC3, THC4, THC5: transgenic 'Holsteiner Cox'; PK: *ANS*-pFGC5941 plasmid

3.2.3.5 Southern blot

To confirm the successful integration of the transgenes into genomic DNA of transgenic 'TNR 31-35' and 'Holsteiner Cox' and to investigate the number of copies of the transgenes, Southern Blot analyses were performed. The results indicated the integration of one copy of the *bar* gene in all of the transgenic lines of 'TNR 31-35' (Figure 36). In the transgenic 'Holsteiner Cox' lines one integration was found for lines 1, 1B and 3 while two integrations were found in lines 4 and 5 (Figure 37).

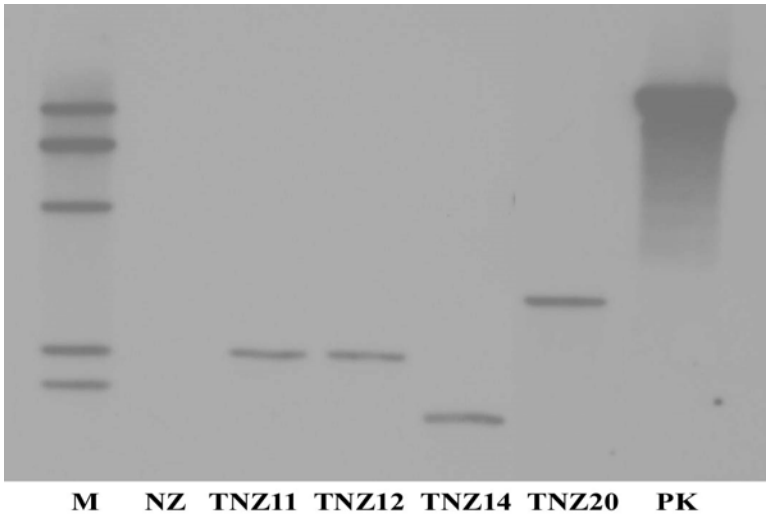


Figure 36: Southern hybridisation of *EcoR*I digested DNA isolated from *ANS* RNAi-transgenic apple plants and respective control plants. Genomic DNA was hybridised to a 300 bp *bar*- probe. Lanes: M – molecular weight markers II, NZ: negative control (DNA from wild plants of 'TNR 31-35'); TNZ11, TNZ12, TNZ14, TNZ20: DNA isolated from transgenic plants; PK: plasmid *ANS*-pFGC5941.

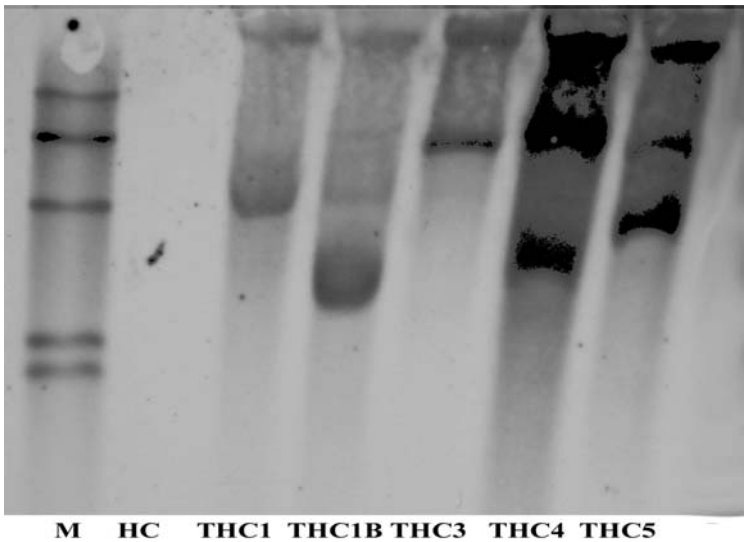


Figure 37: Southern hybridisation of *Bam*HI digested DNA isolated from *ANS* RNAi-transgenic apple plants and respective control plants. Genomic DNA was hybridized to a 300 bp *bar* probe. M: molecular weight markers II, HC: wild type 'Holsteiner Cox'); THC1, THC1B, THC3, THC4, THC5: transgenic plants

3.2.3.6 Effect of *ANS* dsRNA on the expression level of the *ANS* gene

The effect of the introduced *ANS* dsRNA on the expression level of the flavonoid synthesis pathway gene in transgenic 'TNR 31-35' and 'Holsteiner Cox' lines was analyzed by quantitative Real time PCR. Young leaves of transgenic *in vitro* shoots were used for RNA isolation. Expression levels of *ANS, FGT, FLS, DFR, LAR, ANR, CHI, FHT, CHS* and *PAL* gene were determined and expressed relative to the *rubisco* and the *RNA polymerase* gene.

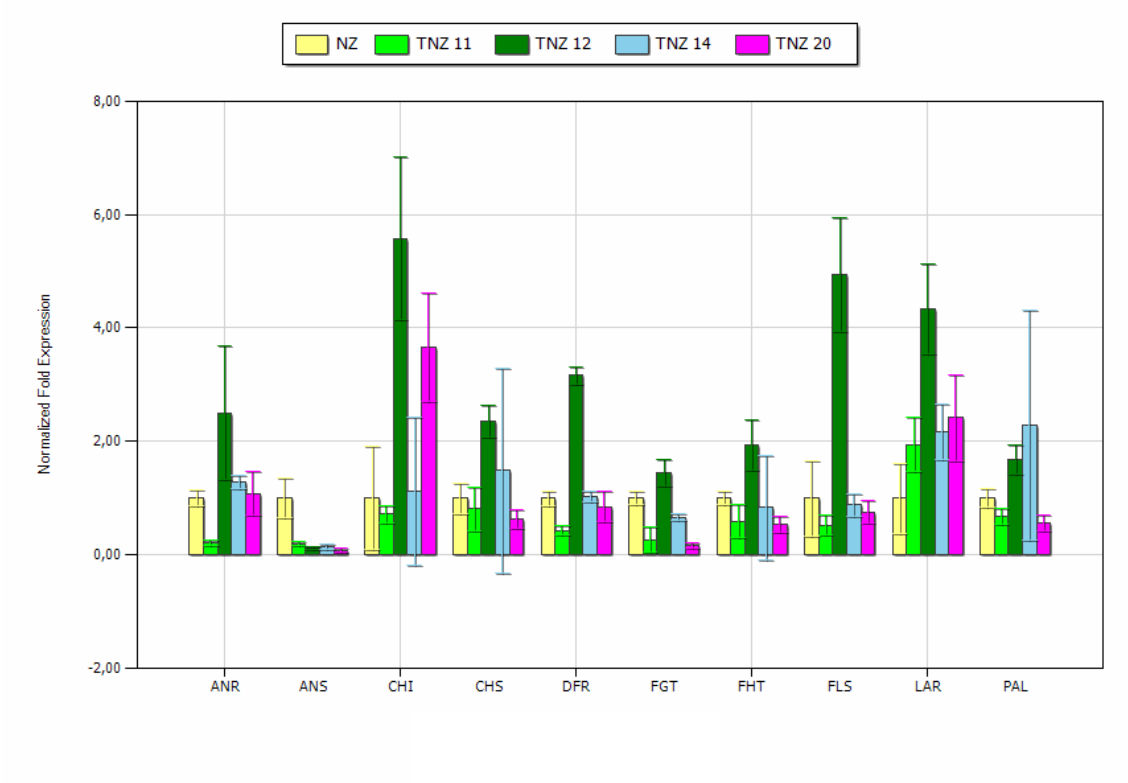


Figure 38: Real time analysis of flavonoid synthesis gene expression in *ANS* RNAi- transgenic 'TNR 31-35' plants and respective control plants. NZ: non-transgenic control (wild plants of 'TNR 31-35'); TNZ line 11, TNZ line 12, TNZ line 14, TNZ line 20: transgenic line 11, 12, 14, 20. The value of control plants was 1.

The Real time PCR results for transgenic 'TNR 31-35' showed that the transcript levels of the *ANS* gene were dramatically decreased in all transgenic

lines. In contrast to the *ANS* gene, the expression of *LAR* gene was slightly increased. There was not significant change in the expression levels of *FLS, DFR, ANR, CHI, FHT, CHS, FGT* and *PAL* gene (Figure 38).

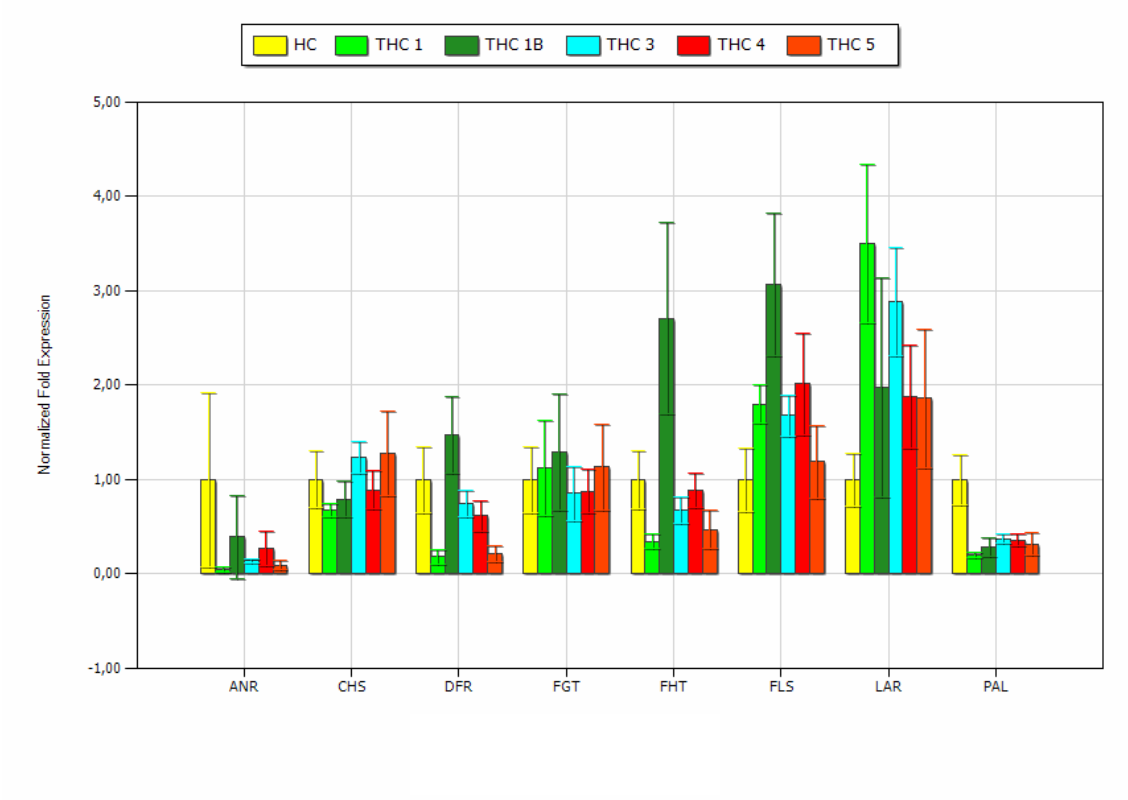


Figure 39: Real time analysis of flavonoid synthesis gene expression in *ANS* RNAi- transgenic 'Holsteiner Cox' plants and respective control plants. HC: non-transgenic control (wild plants of 'Holsteiner Cox'); THC Line1, THC Line 1B, THC Line 3, THC Linie4, THC Line 5: transgenic line 1, 1B, 3, 4, 5. The value of control plants was 1.

The Real time PCR results of samples taken from transgenic 'Holsteiner Cox' lines showed that the transcript levels of the *ANS* and *PAL*gene were dramatically decreased in all transgenic plants in contrast to wild type control. The transcript levels of the *FLS* and *LAR* genes were slightely increased. The transcript levels of the *ANR, CHS, DFR, FGT, FLS* genes were not significant changed (Figure 39).

3.2.3.7 Analyses of flavonoid levels in the transgenic plants

The influence of the decreased expression of *ANS* in transgenic 'TNR 31-35' and 'Holsteiner Cox' plants on accumulation of polyphenolic secondary metabolites was determined *via* quantitative HPLC-DAD and LC-MS. The analyses were kindly performed by Prof. Wilfried Schwab and Prof. Dieter Treutter (TU Munich). Hydrolyzed extracts of leaves from *in vitro* grown transgenic and respective untransformed control shoots were used to quantify the levels of polyphenolic secondary metabolites in transgenic plants. A number of catechin and putative catechin derivates, epicatechin, phloridzin, phloretin, flavonols, p-coumaric acid derivate and caffeic acid derivate were identified and quantified from leaves of untransformed *in vitro* plants and the *in vitro* lines transgenic for *ANS*-pFGC5941.

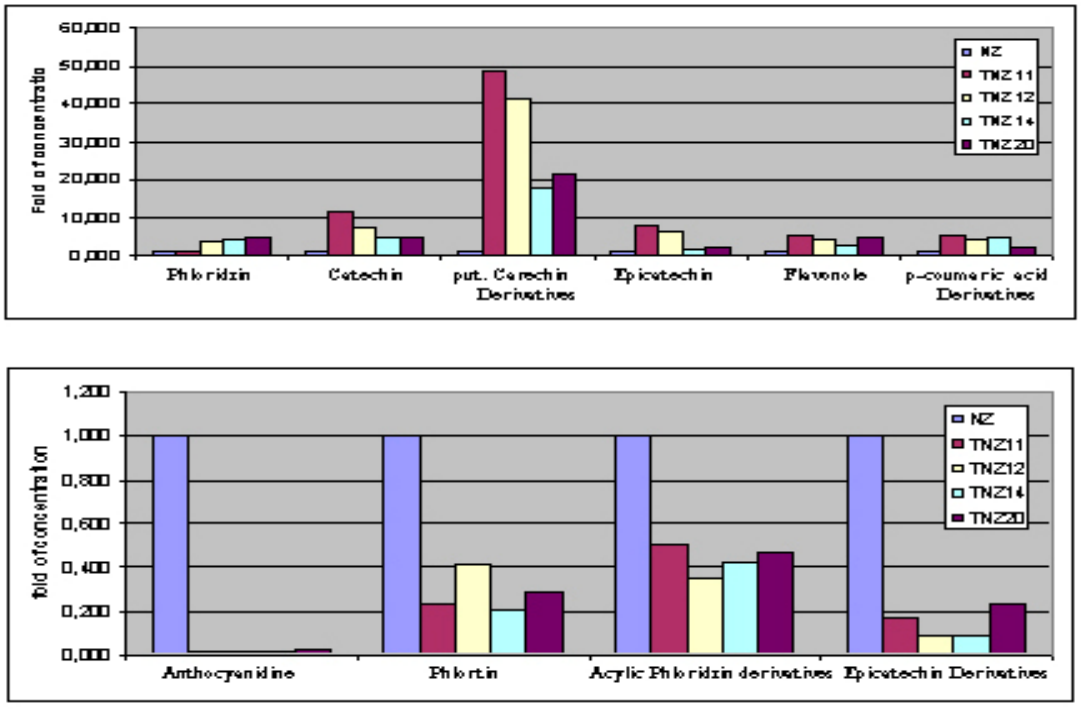


Figure 40: *ANS*-RNAi dependent modification in the levels of flavonoids and other phenolic secondary metabolites determined by LC-MS. Values are expressed as fold induction of metabolite levels in transgenic lines relative to metabolite levels in the untransformed controls. NZ: non-transgenic control (wild plants of 'TNR 31-35'); TNZ11, TNZ12, TNZ14, TNZ20: transgenic lines 11, 12, 14, 20.

Table 21: Quantification of polyphenolic secondary metabolites in hydrolyzed extracts of leaves from plants transformed with *ANS*-pFGC5941 (mg/g of dry weight). NZ: non-transgenic control (wild plants of 'TNR 31-35'); TNZ11, TNZ12, TNZ 14, TNZ20: transgenic line 11, 12, 14, 20.

Polyphenolic Compound	NZ	TNZ11	TNZ12	TNZ14	TNZ20
Anthocyanidine	1,896	0,034	0,026	0,031	0,052
Phloridzin	23,474	21,854	86,494	96,636	108,423
Phloretin	5,372	1,279	2,222	1,093	1,560
Acylic phloridzin derivate	2,717	1,363	0,963	1,145	1,262
Catechin	0,126	1,446	0,930	0,601	0,574
putative Catechin Derivate	0,092	4,505	3,824	1,637	1,970
Epicatechin	1,039	8,564	6,412	1,360	2,295
Epicatechin derivate	2,094	0,347	0,194	0,187	0,483
Flavonols	6,861	34,856	27,233	20,401	31,338
p-Coumaric acid derivate	0,550	2,926	2,400	2,481	1,266
caffeic acid derivate	0,681	1,182	0,709	0,512	0,991

Table 22: Quantification of polyphenolic secondary metabolites in hydrolyzed extracts of leaves from plants transformed with *ANS*-pFGC5941 constructs (mg/g of dry weight). HC: non-transgenic control (wild plants of 'Holsteiner Cox'); THC1, THC1B, THC3, THC4, THC5: transgenic line 1, 1B, 3, 4, and 5.

Polyphenolic Compound	HC	THC1	THC1B	THC3	THC4	THC5
Anthocyanidine	0	0	0	0	0	0
Phloridzin	18,315	5,432	56,491	95,515	49,389	65,761
Phloretin	2,477	7,597	1,159	6,917	3,918	5,463
Acylic phloridzin derivate	1,232	0,919	1,247	1,773	1,357	1,292
Catechin	0,090	0,083	1,515	0,171	0,364	0,349
put Catechin Derivate	0,024	0,044	8,863	0,064	0,090	0,097
Epicatechin	0,058	0,166	10,327	0,590	0,820	1,204
Epicatechin derivate	0,789	0,622	0,452	1,149	0,667	0,848
Flavonols	2,983	6,645	15,491	8,462	16,523	11,554
p-Coumaric acid derivate	0,664	0,546	1,305	0,495	0,618	0,579
Caffeic acid derivate	0,203	0,142	3,350	0,282	0,335	0,889

The HPLC analyses of transgenic 'TNR 31-35' plants indicated a significant decrease in the accumulation of anthocyanidines in transgenic plants compared with wild type plants. In addition to anthocyanidines, a slight decrease in the levels of phloretin, acylic phloridzin derivate and epicatechin derivate was detected. In contrast to the decrease of these substrates, the concentration of catechin and putative catechin derivates was significantly increased in transgenic plants, while a slight increase of phloridzin, p-coumaric acid derivate, epicatechin and flavonols was detected. The concentration of caffeic acid derivate was not significantly changed (Table 21, Figure 40).

In transgenic 'Holsteiner Cox' plants, no anthocyanidin was detected in all of the tested plants. In transgenic plants, a large increase was observed in the level of flavonols compared with that seen in wild type extracts. In addition to flavonols, a significant increase in the level of epicatechin and putative catechin derivates was detected in all transgenic lines. There was slightly increase of phloridzin, phloretin and catechin concentration in some transgenic line. No significant change was observed for the concentration of acylic phloridzin derivate, epicatechin derivate, p-Coumaric acid derivate and caffeic acid derivate (Table 22).

3.2.4 Transformation of *FGT*-Hellsgate8 into 'Holsteiner Cox'

In order to observe the effect of down regulation of *FGT* expression, an RNAi approach was performed. A gene construct leading to production of ds *FGT* RNA was used to transform *Malus domestica* Borkh. cv. 'Holsteiner Cox' *via* the *Agrobacterium*-mediated transformation. Afterward the successfully transformation was confirmed by regeneration test, PCR analysis and Southern blot; the effect of the transformation on gene expression levels and flavonoid accumulation was tested with Real time PCR and HPLC analysis.

3.2.4.1 Transformation and regeneration

The transformation of *FGT*-pHellsgate8 construct into apple cultivar 'Holsteiner Cox' was performed as previously described. About 200 explants were cut from *in vitro* 'Holsteiner Cox' leaves. Three transgenic lines survived on selective medium and were micropropagated.

3.2.4.2 Root induction and transfer to the greenhouse

After successful transformation, the shoots of transgenic plants were excised and transferred on root induction medium with 100 mg/l tircacillin to induce root development. The materials were placed under weak light conditions. After 4 weeks on root induction medium, the shoots were planted in multipot plates and cultivated in greenhouse under high moisture. Table 23 shows the results of root induction and survival rate of the plants in the greenhouse.

Table 23: Results of root induction and transfer to the greenhouse of *FGT*-pHellsgate8 transgenic plants

FGT-pHellsgate8 transgenic line	Nr of tested explants	Nr of shoots forming roots	Root induction rate	Nr of plants survived in greenhouse	Survival rate (%)
Line 12	22	15	68,18	6	40.0
Line 13	23	20	86,90	11	22.0
Line 14	24	13	54,20	7	53.8
'Holsteiner Cox' control	20	18	90.00	13	72.22

3.2.4.3 Flavonoid induction test

Figure 41: Color change of leaf explants after 4 weeks on anthocyane induction medium. Left: leaf explants of transgenic plants with *FGT*-pHellsgate8 constructs; right: leaf explants of an untransformed 'Holsteiner Cox' control plant.

To observe the modification effect of anthocyane synthesis in transgenic apple plants, 20 leaf explants of transgenic and untransformed 'Holsteiner Cox' plants were placed on MS medium containing 1/5 of the normal concentration of macronutrients and no ammonium. The explants were laid in darkness for 2 weeks

and thereafter cultivated under light. After 2 week culture under light, the color of the wild type control explants was completely change to red; whereas the leaves explants from transgenic plants had fewer red colour (Figure 41).

3.2.4.4 PCR and Southern blot analyses

PCR amplification with the *npt*II primers showed an about 300 bp fragment for the transgenic plants and for the plasmid *FGT*-pHellsgate8. No fragment was observed for the untransformed control plant. The PCR analysis results showed that the *npt*II gene was present in the transformed plant (Figure 42).

To confirm successfull integration of the *npt*II gene into genomic DNA and investigate the number of copies of the *npt*II gene present in the genome of these cultivars, the genomic DNA from transgenic plants was isolated and thereafter digested with the restriction endonuclease *BamH*I. Southern blot hybridization was performed using a *npt*II specific dig labelled probe.

The Southern blot results showed that the *npt*II gene was successfully integrated in the plant genomes. Two copies were found in the Line 12, 1 copy in line 13 and 5 copies in the line 14 (Figure 43).

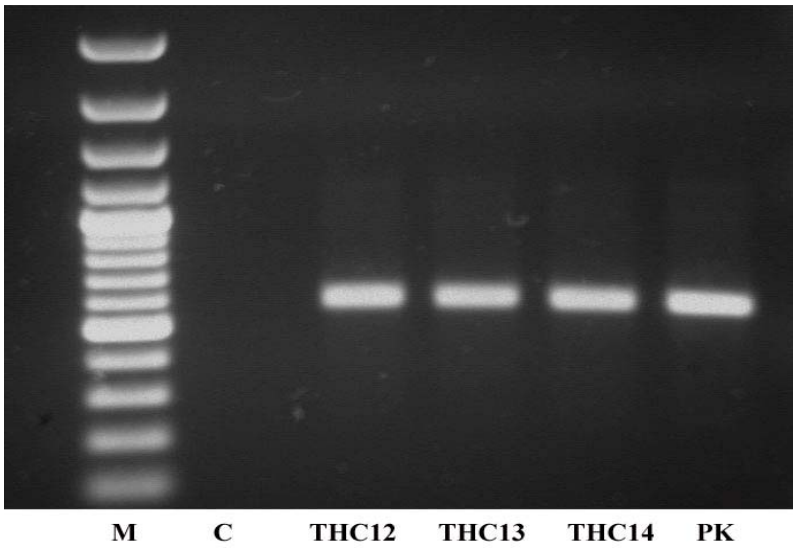


Figure 42: Fragments of the *npt*II gene amplified by PCR. M: 100bp+ molecular weight marker; C: Wild type 'Holsteiner Cox' control; THC12, THC13, THC14: transgenic 'Holsteiner Cox' line 12, 13, 14; PK: Plasmid *FGT*-pHellsgate8

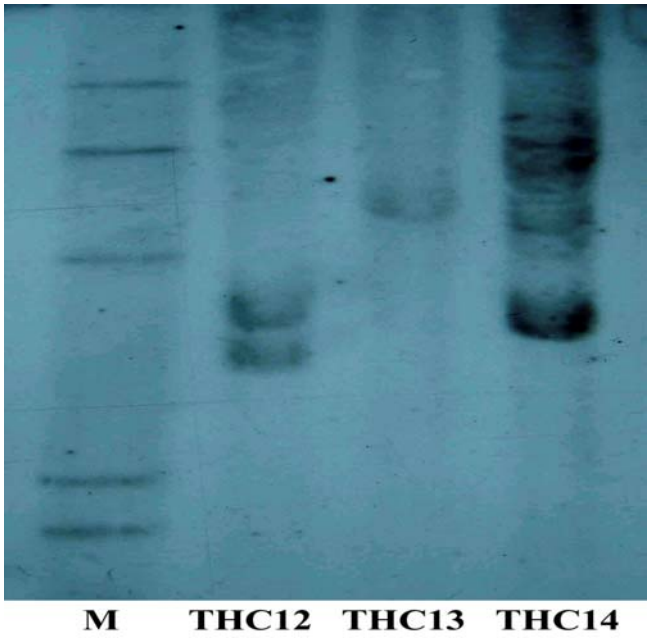


Figure 43: Southern hybridisation of *BamH*I digested DNA isolated from *FGT* RNAi transgenic apple plants and respective control plants. Genomic DNA was hybridised to a 300 bp *npt*II-probe. Lanes: M: molecular weight markers II; THC12, THC13, THC14: transgenic line12, 13, 14.

3.2.4.5 The effect of *FGT* RNAi on expression of the genes of the flavonoid biosynthetic pathway

The influence of the expression of *FGT* dsRNA was analyzed by real-time quantitative reverse transcriptase mediated PCR (Real Time PCR). Young leaves were harvested, and total RNA was isolated from each tissue as described in methods. Expression levels for UDP- glucose flavonoid glucosyltransferase (FGT) were determined and expressed relative to the expression of *rubisco* and the *RNA polymerase* gene. The Real time PCR results showed that the transcript levels of the *FGT* gene was dramatically decreased in transgenic probes in contrast to wild type control. Transcript levels of *ANS, FLS, DFR, LAR, ANR, CHI, FHT* and *PAL* were slightly decreased in transgenic 'Holsteiner Cox', plants compared to the wild type control plant (Figure 44).

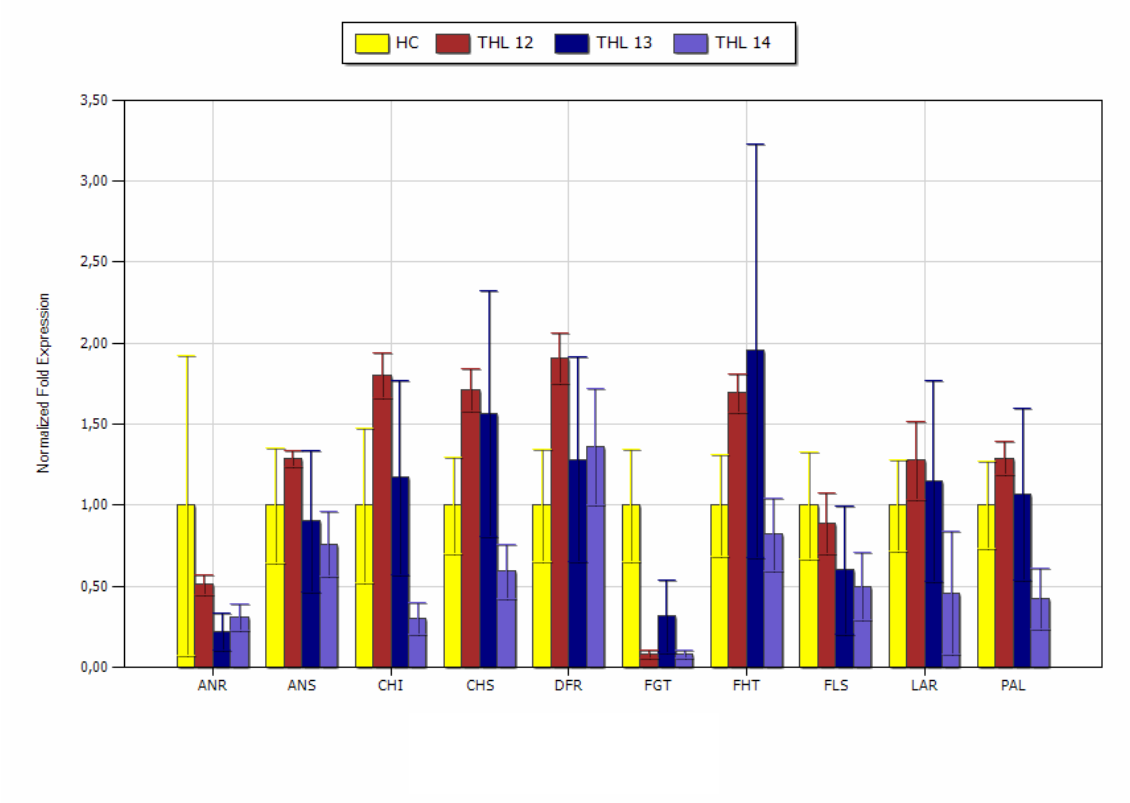


Figure 44: Real time analysis of the flavonoid synthesis gene expression in *FGT* RNAi transgenic plants and respective control plants. HC: non-transgenic control (wild type 'Holsteiner Cox'); THC12, THC13, THC14: transgenic line 12, 13, 14. The value of control plants was 1.

3.2.4.6 Analysis of flavonoid levels in the transgenic plants

To determine the metabolic change of flavonoids in transgenic plants leaves from both *FGT* RNAi transgenic and wild type 'Holsteiner Cox' plants were harvested and polyphenolic secondary metabolites were analysed by quantitative HPLC-DAD and LC-MS analysis. The results showed that no anthocyanidin was detected in all of the tested plants; phloridzin concentration was clearly increased in transgenic plants. Other researched compounds, such as phloretin, acylic phloridzin derivate, catechin, putative catechin derivate, epicatechin epicatechin derivate, p-coumaric acid derivate and caffeic acid derivate, were not clearly changed through genetic transformation with the *FGT*-pFGC5941 constructs (Table 24).

Table 24: Quantification of polyphenolic secondary metabolites in hydrolyzed extracts of leaves from plants transformed with *FGT*-pHellsgate8 constructs (mg/g of dry weight). HC: non-transgenic control (wild plants of 'Holsteiner Cox'); THC12, THC13, THC14: transgenic line 12, 13, 14.

Polyphenolic compound	**HC**	**THC12**	**THC13**	**THC14**
Anthocyanidine	0	0	0	0
Phloridzin	18,315	**83,030**	**30,601**	**128,009**
Phloretin	2,477	6,583	1,659	0,158
Acylic phloridzin derivatives	1,232	2,442	1,946	0,354
Catechin	0,090	0,024	0,025	0,018
putative Catechin Derivatives	0,024	0,000	0,043	0,000
Epicatechin	0,058	0,044	0,063	0,030
Epicatechin derivatives	0,789	1,216	1,169	0,537
Flavonols	2,983	2,890	10,500	4,599
p-Coumaric acid derivatives	0,664	0,583	0,579	0,405
caffeic acid derivatives	0,203	0,190	0,096	0,034

4 Discussion

4.1 Stimulation of flavonoid biosynthesis in apple through heterologous *Lc* gene expression

4.1.1 Phenotype change

A number of *M. domestica* cv. 'Holsteiner Cox' transformants expressing high levels of a full length *Lc* cDNA under the control of the CaMV35S promoter were successfully generated. The recombinant expression of the maize *Lc* regulatory gene enhances flavonoid biosynthesis in transgenic *M. domestica*. *Lc* transgenic 'Holsteiner Cox' shoots were phenotypically distinguishable from non-transgenic plantlets by strong anthocyanin accumulation in leaves and stems. *Lc* transgenic shoots also grew slower in comparison to non-transformed shoots. Similar growth inhibiting effects due to *Lc* expression were observed in a Petunia hybrid (Bradley et al. 1998), in *L. esculentum* and in Arabidopsis transgenic lines, where calli failed to give rise to shoots or the shoots failed to root (Quattrocchio et al. 1993). Since flavonoids can negatively regulate polar auxin transport *in vivo* (Taylor and Grotewold 2005), disturbances in transport of endogenous auxins might be the reason.

4.1.2 Induction of structural gene expression

Phenotypic colour change is in accordance with transcription analyses which confirmed an accompanied increase in transcription levels of most anthocyanin structural genes (Figure 21). Especially expression of anthocyanidin synthase (ANS), the enzyme converting leucoanthocyanidins into anthocyanidins, dramatically increased in *Lc*-transgenic lines. Similar expression pattern changes, especially the unaffected *CHI* expression, were also found in *Lc*/*C1* transgenic *L. esculentum* (Bovy et al. 2002). Gene expression of the diverging pathways to epicatechin (*ANR*) or catechin (*LAR*) was induced in *Lc* overexpressing *M. domestica* plants, too. But the *FLS* expression responsible for the divergent pathway to the flavonols was not induced. The coordinate transcriptional regulation of *ANR* and *LAR*1 by a bHLH transcription factor was also found in

transgenic *Lotus corniculatus* expressing the maize bHLH *Sn* gene (Paolocci et al. 2007).

4.1.3 Metabolites

The LC protein caused pronounced enhancement of accumulation of anthocyanins, of monomeric flavan 3-ols (catechin, epicatechin) and of some polymeric flavan 3-ols (proanthocyanidins) (Table 17). Generally, this corresponds well with the observed changes in transcription levels of structural genes responsible for anthocyanin and flavan 3-ol biosynthesis (Figure 21). However, the reason for the strongly increased levels of distinct proanthocyanidins (B2 and another unidentified dimer), and catechin as one of their supposed precursors remains unknown. While the level of procyanidin B2 (epicatechin-(4β-8)-epicatechin) was 58- fold increased in the *Lc* transgenic lines the concentration of B5 (epicatechin-(4β-6)-epicatechin) was only sevenfold higher. From the current data, no obvious correlation of transcript levels with either the stereochemistry of the respective monomers or the types of inter-flavonoid linkage can be seen. The most likely explanation seems the existence of specific condensing enzymes that are differentially regulated and, thus, differentially influenced by the transgenic *Lc* factor. However, only recently, the ANS catalysed dimerization of catechin monomers has been described (Wellmann et al. 2006). Thus, it is also conceivable that *ANS*, which is highly expressed in the *Lc* transgenic lines, plays an important role in the formation of selected proanthocyanidins.

The concentration of hyperin (quercetin 3-O-galactoside) is increased in the transgenic lines, but other quercetin glycosides are reduced and therefore the total amount of flavonols is unchanged. UDP-glycosyl: flavonoid 3-O-glycosyltransferases often show broad substrate specificity for anthocyanidins as well as for flavonols (Forkmann. 1999). Therefore, the induced UDP-galactosyl: flavonoid 3-Ogalactosyltransferase from anthocyanin (idaein) biosynthesis may cause this shift towards galactosylated quercetin at the cost of the other quercetin

glycosides. Furthermore, the levels of some hydroxycinnamic acids and some hydroxybenzoic acids are slightly increased by *Lc*.

The enhanced *PAL* expression, but also the rise in some hydroxycinnamic acids, argues for a rise in p-coumaroyl-CoA substrate supply for flavonoid biosynthesis. The alternative explanation, a drain of substrates from the major pool of dihydrochalcones, can hardly be tested, as the levels of dihydrochalcones in the *Lc* lines are almost unaffected. With respect to the enormous concentrations of the dihydrochalcones, their biological variation would hide what can be expected for a substrate drain explaining the observed rises in anthocyanins and flavan 3-ols. Nevertheless, this substrate drain may also take place. It appears that dihydrochalcones synthesizing enzymes are not induced by *Lc*; this could indicate a generally different regulation for them in comparison to the regulation of flavonoid structural genes. The closely related pear (*Pyrus communis* L.), which is also a member of the *Maloidae,* does not contain dihydrochalcones (Challice and Williams 1968; Andreotti et al. 2006). This hints to a phylogenetically young pathway in *M. domestica*.

4.1.4 Mode of transcription factor action

In maize and many other plants, the presence of both myb and bHLH (myc) type transcription factors is required for anthocyanin expression (Heim et al. 2003; Grotewold, 2005). In 'Holsteiner Cox' apple, constitutive *Lc* expression was sufficient to strongly enhance anthocyanin, catechins and specific proanthocyanidin accumulation, making it likely that interacting myb-type transcription factors are expressed in the cultivar under the described experimental conditions. Recently, the light induced transcription factors *MdMYB1* and *MdMYB10*, determining apple fruit colour, were described (Espley et al. 2006; Takos et al. 2006). In *MdMYB10* a signature motif for the interaction with bHLH proteins was identified (Espley et al. 2006). It may be expected that the LC protein interacts with or even induces myb-type transcription factors, which leads to the observed induction of anthocyanins and other phenylpropanoids. Further work is required to confirm this.

4.1.5 Rooting of *Lc*-transgenic plants

In contrast to wild type shoots, shoots obtained from the transformation process did not show good root formation on selective root induction medium, on which the wild type 'Holsteiner Cox' exhibited a very good root regeneration rate (Table 16). When the transgenic shoots were pretreated under weak light for two weeks and thereafter cultivated on higher IBA concentrations under dim light, the shoots showed root formation. It might be caused from the effects due to *Lc* expression. The previous results have shown that the flavonoids accumulation was enhanced through *Lc* expression. Flavonoids act as negative regulators of auxin transport in Arabidopsis (Brown et al. 2001). This might also be the reason for the slow growth of *Lc* transgenic plants. When the shoots were pretreated under weak light for two weeks, the shoots were not as red as before. It might be the signal of weaker flavonoid accumulation. The combination of weak light treatment and the increased concentration of 3 µM IBA induced root formation. These results confirmed that the flavonoids act as negative regulators of auxin effect.

4.1.6 Perspective

In this study we have shown that heterologous expression of maize *Lc* enhanced flavonoid biosynthesis in leaf tissues of apple. Whether the effect will also be true for the fruit appears to be strongly influenced by and depending on expression patterns of other transcription factors and can only be determined when plants have fruited.

Flavonoids have become targets for genetic plant modification to modify flower colour and to improve forage traits, crop resistance and healthiness of the human diet. Especially flavan-3-ols and their derivatives have significant health promoting effects and show even pharmacological activities. The role of flavonoids in pathogen resistance is well documented (Jambunathan et al. 1990; Byrne et al. 1996; Römmelt et al. 1999, 2003b; Halbwirth et al. 2003). Studies on apple provided evidence for the significance of flavonoids in resistance to apple scab caused by the fungus *Venturia inaequalis*. Flavan 3-ols accumulate at the infection site (Treutter and Feucht, 1990) and their concentration is associated

with field resistance (Mayr et al. 1997). Inhibition of *PAL*, catalysing the first step of the phenylpropanoid pathway and supplying the precursors for flavonoid biosynthesis, led to severe apple scab symptoms on leaves of a resistant cultivar (Mayr et al. 1997), whilst vice versa enhanced biosynthesis improved resistance (Römmelt et al. 1999; Bazzi et al. 2003; Leser and Treutter 2005). Flavonoids also play a major role in post-harvest resistance of fruits and vegetables (Lattanzio et al. 1994; Lattanzio 2003). Based on these studies it can be assumed, that *Lc*-transgenic apple plants may exhibit increased pathogen resistance.

4.2 Transformation of *Lc* gene into 'Holsteiner Cox' without antibiotics selection

In the present study it was tried to establish a selection system based on anthocyanin pigmentation. For this reason, after inoculation of leaf explants with agrobacteria, the red callus, which partly developed from the explants, was separated from the original explant and cultivated on new medium. After several times of subcultivation with visual selection, red pigmented shoots were obtained and the PCR analyses revealed the amplification of a specific *Lc* fragment. But after further cultivation of the shoots, the strong anthocyanin pigmentation disappeared and PCR test with *Lc* specific primer showed that no fragment was amplified in all of the probes. It is obvious that the visual selected tissue was chimeric leading to the regeneration of non transgenic shoots.

By the selection with visual detection, the separated pigmented tissues contained transgenic and non-transgenic plant cells. Previous work have showed that the non-transgenic cells had a growth advantage because they do not accumulate high levels of flavonoids, which act as negative regulators of auxin transport. In the new regenerated shoots, non-transgenic cells grown faster than transgenic cells. So the amount of non-transgenic cells increased faster than transgenic cells and the colour of the shoots changed to green.

In order to avoid the chimera formation by the transformation without antibiotic selection, a cell suspension culture system of 'Holsteiner Cox' was established (data not shown). The shoot regeneration system from suspension culture would be tested. Suspension culture of cyclamen formed somatic embryos regeneration with various phytohormon concentrations (Winkelmann et al. 2002,

Winkelmann 2004). When the shoot regeneration system is established, the transformed suspension culture would be selected without antibiotics, but visual detection. The selection would be done under microscopy. Single transgenic cells with deeper colour would be selected und further cultivated, thereafter regenerated new shoots. Because the shoots come from single cells or at least from a cluster of few cells, the likelihood of chimerism is reduced. When all of previous description works, a new selection system without antibiotic selection might be established.

4.3 Modification of flavonoid biosynthesis through dsRNA-mediated gene silencing

In this study, our goal was the enrichment of flavanol synthesis through RNA interference. Silencing of the expression of the structural genes of the flavonoid pathway anthocyanidin synthase (ANS) and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (FGT) was performed to induce a shift towards flavanols, which play important roles in phytopathology.

Anthocyanins are responsible for red pigmentation in the shoots and the flavonols acted as resistance substance against plant disease (Treutter and Feucht, 1990; Mayr et al. 1997).

The results of this study show that dsRNA-mediated genetic transformation can operate in *M. domestica* cultivar 'Holsteiner Cox' and 'TNR31-35' to efficiently induce sequence-specific inhibition of gene function. *Agrobacterium*-mediated transformation provides a convenient and efficient method to introduce dsRNA-expressing constructs into the plant genome. In this study, two kinds of dsRNA-expressing constructs, *ANS*-pFGC5941 and *FGT*-pHellsgate8, were used to investigate RNAi effects.

4.3.1 Phenotype change

The shoots of *ANS*-pFGC5941 transgenic 'TNR31-35' were phenotypically distinguishable from non-transgenic plantlets by strong colour change: the leaves

and stems of original plants were red and in transgenic plants they were green (Figure 32) since 'Holsteiner Cox' is a green leaved cultivar. No phenotypical changes were observed in *ANS*-pFGC5941 and *FGT*-pHellsgate8 transgenic shoots of 'Holsteiner Cox'. A clear difference was observed when the leaves of these two type transformants were cut and cultivated on anthocyanidin induction medium: the leaf colour of non-transgenic plantlets was complete red; the leaf colour of transgenic plantlets with *ANS*-pFGC5941 constructs remained almost complete green and the colour of transgenic plantlets with *FGT*-pHellsgate 8 constructs was significance weaker (Figure 35). *ANS* and *FGT* are important pathway genes for the anthocyanidin synthesis (Figure 4). When the expression of these genes was down regulated, the synthesis of anthocyanidin is obviously blocked. Because the anthocyanidin was the reason of the red colour formation, when the anthocyanidin synthesis was blocked, the red colour formation would be blocked, too. Similar colour changing effects due to *RNAi* expression were observed in a petunia hybrid (Metzlaff et al. 1997), where Chalcone synthase A was silenced and the red colour formation in flower petals was blocked.

4.3.2 Change of structural gene expression

Phenotypic colour change is in accordance with transcription analyses which confirmed an accompanied change in transcription levels of several anthocyanin structural genes. In the transgenic cultivar 'TNR31-35' and 'Holsteiner Cox' with *ANS*-pFGC5941 constructs, the expression of anthocyanidin synthase (ANS), the enzyme converting leucoanthocyanidins into anthocyanidins, dramatically decreased in transgenic lines. The expression decrease of *ANS* gene confirmed the effect of RNA interference. In contrast to *ANS*. the expression of *LAR* was slightly increased (Figure 39). This might because of the feedback effect: when the anthocyanidin synthesis was blocked, there might be accumulation of leucoanthocyanidins and other upstream substance, therefore the expression of *LAR* was promoted. By the transgenic cultivar 'Holsteiner Cox' with *FGT*-pHellsgate8 constructs, the expression of glucose-flavonoid glucosyltransferase (FGT), the enzyme modified anthocyanidine by glycosylation (Forkmann et al.

1999), dramatically decreased in transgenic lines (Figure 44). The decrease of *FGT* expression showed the effect of RNA interference. By the anthocyanidin induction test of transgenic *FGT*-pHellsgate8 on anthocyanidin induction medium, the red colour of leaves of transgenic plants was weaker than that of non-transformants plants, possibly because of the non-glycosied anthocyanidine was not stable.

4.3.3 Metabolite changes

By the *M. domestica* cultivar 'TNR31-35', the *ANS* RNA interference caused a decrease of accumulation of anthocyanidines. In contrast to this, enhancement of accumulation of flavonols, of catechin and of epicatechin was observed. Generally, the metabolite change of anthocyanidine and catechin corresponds well with the observed changes in transcription levels of structural genes responsible for anthocyanindine and catechin (Figure 39). There was a decrease of accumulation of anthocyanidines because the transcription levels of *ANS*, which encodes the enzyme converting leucoanthocyanidins into anthocyanidins (Forkmann et al. 1999), was strong reduced in transgenic plants. In contrast to *ANS*, transcription level of *LAR*, which encoded the enzyme converting leucoanthocyanidins into catechins, was strong induced in transgenic plants. However, the reason for the strongly increased levels of flavonols and epicatechin was remains unknown. The increase of epicatechin levels purposed to be because the conversion between catechin and epicatechin. The most likely explanation of the increased levels of flavonols seems to be feed back effect: when the anthocyanidin synthesis was blocked, as the alternative metabolic substance, the accumulation of flavonols was enhanced.

In the *M. domestica* cultivar 'Holsteiner Cox', the *ANS* RNA interference caused similar effect of metabolic change of catechin, of epicatechin and of flavonols: the accumulation of catechin, epicatechin and flavonols was enhanced. No anthocyanidine was detected either in non-transformed or in transgenic plants.

The RNAi-mediated downregulation of *FGT* caused no significantly metabolic change in the *M. domestica* cultivar 'Holsteiner Cox'. No anthocyanidins were

detected in non transgenic plants. The *FGT* gene, which induced UDP-galactosyl of flavonoid 3-Ogalactosyltransferase from anthocyaninidine shift towards galactosylated anthocyane, would have no effect of metabolic change because of the lack of original substance.

4.3.4 Colour change of 'TNR31-35' by transformation phase

By the transformation of the red leaved 'TNR31-35' with *ANS*-pFGC5941, the colour of transformants at end phase was different from selection phase. During the selection phase, the colour of many survived shoots was a mixture of green and red, many leaves even are just half red and half green (Figure 31). After selection, the transgenic shoots were complete green. These might be the reason of chimera formation by the selection phase. The untransformed cells escape from selection under low antibiotic concentration and formed red colour. The untransformed cells were killed after further selection with higher antibiotic concentration.

4.3.5 Root induction of transgenic plants

The root induction of wild type 'TNR31-35' was very good using IBA. In contrast to this, shoots obtained from the transformation process did not show favourable root formation on selective roots induction medium. It may be caused from the pattern change of flavonoid, which can negatively regulate polar auxin transport in vivo (Taylor and Grotewold 2005). To optimize root induction of transgenic shoots, different phytohormon combination should be tested.

4.3.6 Perspective

In this study, the results have shown that anthocyanidin synthesis was blocked in the *ANS*-RNAi transgenic 'TNR31-35' shoots. Whether the effect will also be true for the fruit appears to be strongly influenced by and depending on

expression patterns of other transcription factors and can only be determined when plants have fruited. When the anthocyane accumulation was also blocked in the fruit, RNAi can be used for the production of new sort.

In contrast to the inhibition of anthocyanidin accumulation, the accumulation of catechin and epicatechin, which belong to flavan-3-ols, was enhanced. Studies on apple provided evidence for the significance of flavonoids in resistance to apple scab *(V. inaequalis)*. Flavan 3-ols accumulate at the infection site (Treutter and Feucht, 1990) and their concentration is associated with field resistance (Mayr et al. 1997). Based on these studies it can be assumed, that *ANS* RNAi transgenic apple plants may exhibit increased pathogen resistance.

4.4 Transformation efficiency and selection

Transformed plant cells of apple containing *npt*II can theoretically survived under selection of 100 µg/ml kanamycin (Szankoeski, 2002). 50 µg/ml kanamycin had been applied for the selection by the transformation at first. After several times transformation, there was always only callus formation, but no shoots regenerated under this selection stress. Thereafter 25 µg/ml kanamycin was applied for the selection. Transgenic shoots regenerated and the shoots survived under selection of 50 µg/ml kanamycin.

By the transformation of *ANS*-RNAi construct into 'Holsteiner Cox' and 'TNR31-35', the transgenic plants with *bar* gene can survive under selection of 10 µg/ml ppt (Szankowski, 2002). 5 µg/ml ppt had been applied for the selection by the transformation at first. After several times transformation, there was always only callus formation, but none shoots regenerated under this selection stress, too. Thereafter 1 µg/ml ppt was applied for the selection. Transgenic shoots regenerated and the shoots were gradually selected with 2, 5 and 10 µg/ml ppt. The transgenic shoots can survive under selection of 10 µg/ml ppt.

The reason might be in the transgenic cells, the synthesis of flavonoid, which acted as auxin transport regulators, was modified. The growth of the transgenic cells was not good under higher selection stress. The advantage of gradual selection with raised antibiotic stress can improve the transformation efficiency,

because the transgenic cells were easily survived under lower selection stress. Disadvantage of this method was that the non-transgenic cells had more chance to escape from the selection under lower selection stress and formed chimeras. More times were needed for further selection under higher selection stress.

4.5 Anthocyane induction through low concentration nutrient

Anthocyanin accumulation is very weak in 'Holsteiner Cox' during the vegetative phase. The stimulation and inhibition effect of anthocyanidin by transgenic plant need long time to be observed. A quick method should be established to test the transgenic effect. By the transformation of *ANS* RNAi construct into apple cv 'Holsteiner Cox', the metabolic blocking effect of anthocyanidin biosynthesis was difficult to be detected because anthocyanidin was not detectable in the young plants tissue. When the anthocyane synthesis was induced through changed culture medium, the metabolic blocking effect would easy to be detected. The induction of anthocyane synthesis had been induced through adjust of nitrogen and sugar concentration in torenia (Aida et al. 1998; Li, 2003).

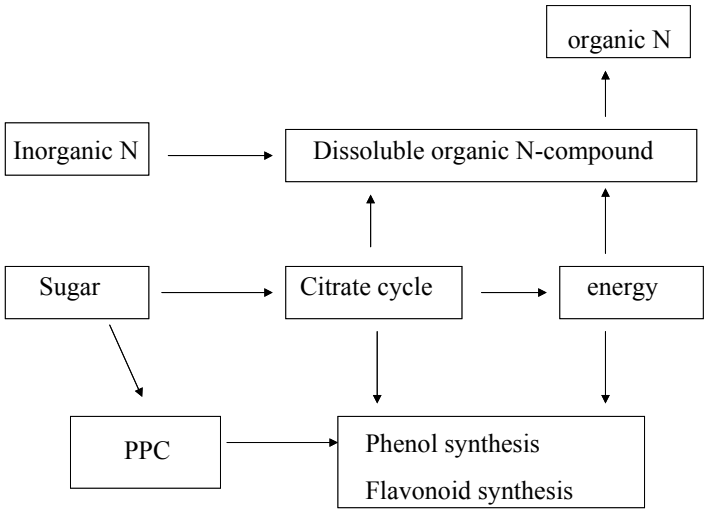


Figure 45: Effect of Nitrogen and Sugar in the plant metabolism

[Treutter, 2002. Personal communication]

In the experiment performed in this work, leaf explants were used as starting material. The explants were placed on MS medium containing 1/5, ¼, 1/3, ½ of the normal concentration of macronutrients and without ammonium. The explants were cultivated under darkness to reduce the chlorophyll production. After 2 weeks culture in darkness and one week cultivation under light, the color of the explants growing on low macronutrients started to change to red. Anthocyans were induced on the low macronutrients concentration and without ammonium; lower macronutrients concentration induced more anthocyans (Figure 27).

The observed colour-change in the shoots can be explained as the result of the lower of nitrogen concentration in culture medium. The lower nitrate concentrations can promote the anthocyanin accumulation (BaoDo et al. 1991). Figure 45 illustrates that when the nitrogen is deficient in plants, the biosynthesis of flavonoids is stimulated. Through it is not clear, if the induced anthocyane was the same pattern as it in fruit, but it can be used for a fast evaluation of genetic modification of flavonoid biosynthesis, because the transgenic effect can already be tested in the leaves phase.

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet habe.
Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

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