Metabolic Engineering in Apple: Overexpression of Apple Transcription Factors Involved in the Regulation of the Flavonoid Pathway for Increased Disease Resistance

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Dedicated with much love and affection to my beloved parents,

my wife, my children (Hanin and Hamza),

my sisters, my brothers

and my friends

ABSTRACT

Metabolic engineering in apple: Overexpression of apple transcription factors involved in the regulation of the flavonoid pathway for increased disease resistance

Khaled Al Rihani

Apple (*Malus domestica* Borkh) is one of the most important fruit trees rich in flavonoids. the defensive role of flavonoids in apple has been studied before, but, there is a need to improve our knowledge of this role.

The aim of the present study was to modify the flavonoid pathway in apple by overexpressing transcription factors involved in this pathway in order to analyse the hypothesis whether or not there will be an effect on plant disease resistance. The *MdMyb9, MdMyb10* and *MdMyb11* transcription factor genes were used; these genes belong to a family of similar plant transcription factors and have been found to upregulate several genes of the phenylpropanoid pathway. Therefore, the binary vector pJan harbouring those transcription factors individually was used for transformation experiments, with two apple cultivars 'Holsteiner Cox' and 'Gala' respectively. The pJan vector contains the *npt* II gene as a selectable marker gene that, however, might cause some regulatory problems.

Thus, in order to improve the selection system, the *MdMyb10, MdMyb11* transcription factors were cloned into the binary vector pGIIMH35S, which contains the *bar* gene as a selectable marker gene, and the new constructs were used in new transformation experiments with only the 'HC' cultivar.

Leaf discs from 4-5 week old *in vitro* apple plants CVs.'HC' and 'Gala' were used as explants for *Agrobacterium*–mediated transformation. several shoots were regenerated after transformation experiments, which were healthily growing on media supplemented with 50mg/l kanamycin when the pJan binary vector was used, and supplemented with PPT concentrations up to 10mg/l, when the pGIIMH35S binary vector was used. Shoots were rooted and transferred into pots, then transfeered to the greenhouse. the time course for each transformation experiment from explant to transfer the plants to the greenhouse was 3-4 months. The transformation efficiencies ranged between 0.5 % and 1.2 %, with an average of 0.4% for the whole 'HC' transformation experiments. When eliminating the experiments, which did not render any transgenic shoots, the efficiency became 0.64%. The transformation efficiency obtained for the whole 'Gala' transformation experiments ranged between 0.45% and 1.32%, with an average of 0.6%, but when eliminating the experiments, which did not render any transgenic shoots, the efficiency became 0.84 %.

Detection of transgenes was made by PCR using different primer combinations for *MdMyb9, MdMyb10, MdMyb11*, *npt* II and *bar* genes, respectively. The results clearly indicated and confirmed the successful integration of T-DNA into genomic DNA of 'HC' and 'Gala'. Copy numbers and integration patterns were investigated using southern blot analysis with different probes (*MdMyb9, MdMyb10* and *MdMyb11*). One copy was detected in all plants analysed (non-transgenic and transgenic) representing the homologous endogenus gene. In addition, the presence of an additional copy in most of transgenic plants testedwere observed, while two or four copies were also found in some transgenic plants.

Leaf paint analysis showed positive results in the tested 'HC' transgenic plants transformed using the constructs pGIIMH35S-MdMyb10, pGIIMH35S-MdMyb11, indicating a positive *bar* gene expression by BASTA® herbicide detoxification.

RT-PCR was performed to confirm transcription of the transgenes using different primer combinations for *MdMyb9, MdMyb10,* and *MdMyb11*.

Real time PCR analysis was made to see mRNA expression levels in both non-transgenic and transgenic plants. The transcript was detected in both transgenic and non-transgenic plants, with dramatically increases up to 1261 and 847-fold, for *MdMyb10* 'HC' and 'Gala' transgenic plants, respectively. Also dramatically increases up to 47 and 1451-fold were found in the case of *MdMyb9* 'HC' and 'Gala' transgenic plants, respectively. Moderate increases up to 6 and 9.6- fold were observed in the case of *MdMyb11* 'HC' and 'Gala' transgenic plants, respectively.

HPLC analysis was carried out to detect the levels of different flavonoid compounds in both non-transgenic and transgenic plants. Some of the compounds analysed were induced and others were reduced, with an observed increase in the level of Cyanidin 3-O-galactoside in the case of *MdMyb10* 'HC' transgenic plants, and an increase of total contents of flavon-3-ols and hydroxycinnamic acids in the case of *MdMyb9, MdMyb11* transgenic plants from both cultivars used in this study.

Keywords: *Agrobacterium*, Apple, flavonoids, transcription factors, Myb, overexpression

ZUSAMMENFASSUNG

Metabolic engineering im Apfel : Überexpression von Apfel Transkriptionsfaktoren, die an der Regulation des Flavonoid Stoffwechselweges für verbesserte Krankheitsresistenz beteiligt sind.

Apfel (*Malus domestica* Borkh.) gehört zu den fruchtragenden Bäumen, die reich an Flavonoiden sind und deren defensive Rolle am besten studiert worden ist, aber es gibt bezüglich der Regulation weiteren Erkenntnisbedarf.

Das Ziel der vorliegenden Untersuchung war, herauszufinden ob durch Überexpression von Transcriptionsfaktoren der dadurch geänderte Flavonoid- Stoffwechsel im Apfel einen Einfluß auf die Krankheitsresistenz hat. Bei den Transkriptionsfaktoren handelt es sich um *MdMyb9, MdMyb10* und *MdMyb11*. Diese Faktoren gehören zu einer Genfamilie zunächst die Gene im Phenylpropanoidstoffwechsel stark regulieren. Als binärere Vektor wurde pJan benutzt, in den die Transkriptionsfaktoren einkloniert und mittels *Agrobacterium tumefaciens*-vermittelten Gentransfer in die Apfelsorten '*Holsteiner Cox*' und '*Gala*' transformiert wurden. Der pJan Vektor enthält das *npt-II* Gen als Selektionsmarker, das jedoch regulatorische Probleme verursachen kann. Um das Selektionssystem zu verbessern, wurden *MdMyb10* und *MdMyb11* in den binären Vektor pGIIMH35S einkloniert, der das *bar* Gen als Selektionsmarker enthält und anschließend in die Apfelsorte *Holsteiner Cox* transformiert.

Blattstücke vom 4-5 Wochen alten in-vitro Apfel Pflanzen 'HC' und 'Gala' wurden als Explantate für *Agrobacterium* -vermittelten Gentransfer benutzt. Diese Explantate wurden auf Medium mit entweder 50mg/l Kanamycin für binäre Vektoren pJan und 10 mg/l PPT für pGIIMH35S selektioniert und neue Triebe regeneriert. Verschiedene Triebe wurden bewurzelt und anschließend im Gewächshaus eingetopft. Die Zeitspanne für die Transformation betrug 3-4 Monate. Die Transformationseffizienz lag zwischen 0.5%-1.2%, mit einem Durchschnitt von 0.4% für die gesamten `HC' Transformationen und zwischen 0,45%-1,32% mit einem Durchschnitt von 0,6% für die gesamten 'Gala' Transformationen. Unter Nichtbeachtung der Transformationen ohne jede Bildung von transgenen Trieben ergaben sich Effizienzen von 0,64% für 'HC' und 0,84% für 'Gala'.

Leaf paint analysis zeigte positive Ergebnisse in den getesteten mit pGIIMH35S-MdMyb10 und pGIIMH35S-MdMyb11 transformierten 'HC' Pflanzen. Dies deutet auf eine positive BASTA® herbicide detoxification durch *bar* Genexpression hin.

Der Nachweis positiver transgener Pflanzen erfolgte mittels PCR mit spescifischen Primerpaaren für *MdMyb9, MdMyb10, MdMyb11, npt* II und *bar.* Die Resultate zeigen klar

die erfolgreiche Integration von T-DNA in das Genome von‘HC’ und ‘Gala’
Kopienanzahl und Integrationsmuster wurden mittels *Southern blot* Analyse und verschiedenen Proben (*MdMyb9, MdMyb10* und *MdMyb11*) untersucht. Eine Kopie des homologen endogenen Gens wurde in allen Pflanzen (transgene und Nicht-transgene) und zusätzliche Kopien (2-4) für die meisten transgenen Pflanzen gefunden.
Die Bestätigung von Transcripten für *MdMyb9, MdMyb10,* und *MdMyb11* erfolgte mittels RT-PCR und specifischen Primerpaaren.
Veränderungen in der mRNA Expression konnte mit Real Time PCR in Nicht-transgenen und transgenen Pflanzen gezeigt werden. Für *MdMyb10* mRNA in ‘HC’ und ‘Gala’ konnte ein sehr hoher Anstieg von 847-1261-fach gegenüber Nicht-transgenen Pflanzen beobachtet werden. Für *MdMyb9* waren es 47-1451-fach und etwas moderater für *MdMyb11* 6-9,6- fach.
Die durchgeführte HPLC Analyse für verschiedene flavonoide Stoffwechselprodukte in Nicht-transgenen und Transgenen Pflanzen ergab einen Anstieg der Cyanidin 3-O-galaktoside in transformierten *MdMyb10 'HC'* Pflanzen und eine Zunahme der totalen Menge von *Flavon-3-ols* und Hydroxycinnamic acids in transformierten *MdMyb9* 'HC' als auch transformierten *MdMyb11* ‘Gala’ Pflanzen. Daneben konnten einige weitere Komponenten identifiziert werden, die entweder induziert oder reduziert worden sind.

Stichworte: *Agrobacterium*, Apfel , Flavonoide, Transkriptionsfaktoren, Myb, Überexpression

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ABBREVIATIONS AND TERMS

ANS	Anthocyanidin synthase
ANR	Anthocyanidin reductase
BHLH	Basic helix-loop-helix domain
BAP	6-Benzyl-amino-purine
bar	Bialaphos resistance gene
bp	Base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementare DNA
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	CoenzymA
CTAB	Cetyl Tri-methyl ammonium bromide
C1	Color 1
C4H	Cinnamate 4-hydroxylase
ºC	Celsius
DAFB	Days after full bloom
DFR	Dihydroflavonol 4-reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphat
DHK	Dihydrokaempferol
dsDNA	Double stranded DNA
dry wt	Dry weight
EDTA	Ethylene-diaminetetraacetic acid
EtOH	Ethanol
EtBr	Ethidium bromide
FAO	Food and Agriculture Organization
FGT	UDP-Glucose flavonoid 3-O-glucosyltransferase
FHT	Flavanon 3 beta-hydroxylase
FLS	Flavonol synthase
F3′H	Flavonoid 3′-hydroxylase
F3′5′H	Flavonoid 3′,5′-hydroxylase
F3GTs	Flavonoid 3-O-glucosyltransferases
FLS	Flavonol synthase
GA	Gibberellic acid
g/l	Gram per litre
GUS	β-glucuronidase
gDNA	genomic DNA
GS	Glutamine Synthetase
HPLC	High performance liquid chromatography
HC	Holsteiner Cox
h	hour
IBA	Indole-3-butyric acid
IFS	Isoflavone synthase
kb	Kilo base pair
kV	kilovolt
LB	Left border
LAR	Leucoanthocyanidin reductase
LC	Maize leaf colour

mg/l	Milligram per litre
MYB	DNA-binding domain
LC-MS	Liquid chromatography/mass spectroscopy
LB medium	Luria Bertani medium
mM	Milli mole
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
min	minute
NAA	1-naphthyl-acetic acid
nptII	Neomycin-phospho-transferase (gene)
nm	nonometer
ng	nanograme
OD	Optimal density
Nod factor	Nodulation
PAP1(2)	Production of anthocyanin pigment 1 (2)
PAL	Phenylalanin ammonia-lyase
PAT	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
pMole	Pico mole
PPT	Phosphinothricin
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RB	Right border
RNAi	RNA interference
rpm	rounds per minute
RT-PCR	Reverse transcription polymerase chain reaction
RT	Room temperature
Sec	second
T-DNA	Transferred DNA
TDZ	Thidiazuron
Ti-Plasmid	Tumour-inducing plasmid
TIA	Terpenoid indole alkaloids
TT8(2)	Transparent Testa8(2)
Tris	Trishydroxylaminomethane
Tm	melting temperature of the PCR product
UV	Ultra Violet
U/µl	Unite per microliter
YEP	Yeast extracts pepton
vir	Virulence
V/V	Volume to volume
µl	Micro litre
µM	Micro mole
µg	microgram
µFd	microfarad
WT	Wild type

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1 INTRODUCTION

Apple is one of the most important sources of flavonoids in human nutrition (approx: 1-5 mg/100 g weight), after onion and tea (Hertog *et al.*, 1993). The major flavonoid classes occurring in apple fruit are flavonols such as quercetin 3-glycosides, monomeric and oligomeric flavan-3-ols such as catechin, epicatechin and procyanidins, dihydrochalcones such as phloridzin, and in red-coloured cultivars, anthocyanins such as cyanidin 3- glycosides. Because of the high content of flavonoids in apple, research suggests that apples may reduce the risk of colon cancer, prostate cancer and lung cancer. Compared to many other fruits and vegetables, apples contain relatively low amounts of vitamin C as well as several of other antioxidant compounds. The fiber content, while less than in most other fruits helps to regulate bowel movements and may thus reduce the risk of colon cancer. They may also help with heart disease, weight loss and controlling cholesterol, as apples do not have any cholesterol, but fiber, which reduces cholesterol by preventing re-absorption. Apples are bulky for their caloric content like most fruits and vegetables (http://en.wikipedia.org/wiki/Apple_Tree).

The defensive role of flavonoids in apple has been studied before, but there is a need to improve our knowledge of this role.

Treutter (2006) reported that the presence of flavonoids in apple trees might account for efficient defence against insects and pathogens attack.

With all of the beneficial effects of flavonoids in protecting plants from pests and pathogens, one may create the idea to stimulate their biosynthesis and accumulation in order to support the plant's defence mechanisms at the time and site of attack. Simmonds (2003) stated that at least "in theory we could be creating a world of plants richer in flavonoids." It is often described that pathogens induce the biosynthesis of resistance-related metabolites but also non-pathogenic strains are capable to elicit secondary metabolism (Yamamoto *et al.*, 2000). Metabolic engineering is the tool that may improve our understanding the role of flavonoids in plant resistance and may be helpful for use of their beneficial effects.

The flavonoids biosynthetic pathway was the first target for metabolic engineering since the early 1990s, as the pathway was well known and the results could easily be observed by changes in flower colour (Dixon and Steele, 1999; Forkmann and Martens, 2001). The first gene isolated from the flavonoid biosynthetic pathway was a *CHS* gene from parsley (*Petroselinum hortense*) (Kreuzaler *et al.*, 1983). Transcriptional control of the representive structural genes has been most intensively studied in relation to the biosynthesis of

anthocyanins.

Two classes of genes can be distinguished within the flavonoid pathway: (I) the structural genes encoding enzymes that directly participate in the formation of flavonoids, and (II) regulatory genes (transcription factors) that control the expression of the structural genes. The precursors of the synthesis of most flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derived from the carbohydrate metabolism and phenylpropanoid pathways, respectively.

This present research proposes to use metabolic engineering as a tool to study the transcription factors involved in the flavonoid biosynthesis pathway in order to improve plant disease resistance by overexpressing three of transcription factors genes involved in this pathway (*MdMyb9*, *MdMyb10*, and *MdMyb11*). The effects of this engineered overexpression on phenotype, mRNA expression level, and on the content levels of some analysed flavonoids in this pathway, will be the parameters to better understand the role of flavonoids in plant defence.

2 LITERATURE REVIEW

2.1 Importance of Apple *Malus domestica*, Origin and Taxonomy

The apple tree, which originally came from southwestern of Asia, has spread to most temperate regions of the world. Over the centuries, many hybrids and cultivars have been developed, giving us the 7,500 varieties in the market today, were the major production come from the temperate zone of the Northern and Southern hemispheres (O´Rourke, 1994). According to the FAO (2009): http://faostat.fao.org nearly 71.7 million tonnes of apples were produced in almost 5 million hectares in 2009; where China, USA, Turkey, Poland, and Italy were the major producers worldwide.

Table.1. Main Apple producing countries in 2009

Countries	**tonnes**
Argentina	1300000
Austria	485609
Belarus	431573
Brazil	1220499
Canada	413096
China	31204163
Egypt	550000
France	2050000
Germany	965100
Hungary	575368
Italy	2176200
Morocco	400000
Poland	2626273
Romania	517491
Russian Federation	1596000
South Africa	702284
Spain	552900
Syrian Arab Republic	360978
Turkey	2782365
Ukraine	853400
United States of America	4514880
Uzbekistan	635000

http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor

Table. 2. World apple yield and production in comparison to other fruits in 2009

Item	Area harvested	Production (tonnes)	Yield(Kg/Ha)
Apples	4957192	71736938	144712
Apricots	520455	3831823	73624
Avocados	438325	3585156	81792
Bananas	4843595	95595965	197365
Berries Nes	123315	903676	73281
Blueberries	72554	306383	42228
Carobs	93253	180249	19329
Cashewapple	681001	1851005	27180
Cherries	369766	2150107	58147
Citrus fruit, nes	1102360	7778197	70559
Cranberries	22591	409523	181277
Currants	114617	631108	55062
Dates	1256953	7226947	57495
Figs	448474	1184884	26420
Fruit Fresh Nes	4095419	27627853	67460
Fruit, tropical fresh nes	2431926	17673114	72671
Gooseberries	26083	120996	46388
Grapefruit (inc. pomelos)	250953	4496868	179191
Grapes	7437141	66935199	90001
Kiwi fruit	85983	1285553	149512
Lemons and limes	1014635	13949600	137483
Mangoes, mangosteens, guavas	4745782	35035641	73824
Oranges	4192351	67601635	161249
Papayas	413227	10213069	247153
Peaches and nectarines	1655285	18579393	112242
Pears	1739632	21907395	125931
Persimmons	784252	3807843	48553
Pineapples	852109	18448674	216506
Plantains	5383032	34316133	63748
Plums and sloes	2525048	10679206	42293
Pome fruit, nes	12500	81400	65120
Quinces	68420	497237	72674
Raspberries	91103	483620	53084
Sour cherries	259605	1358326	52322
Stone fruit, nes	84557	479630	56722
Strawberries	253900	4132352	162755
Tangerines, mandarins, clem.	2159170	30587778	141664

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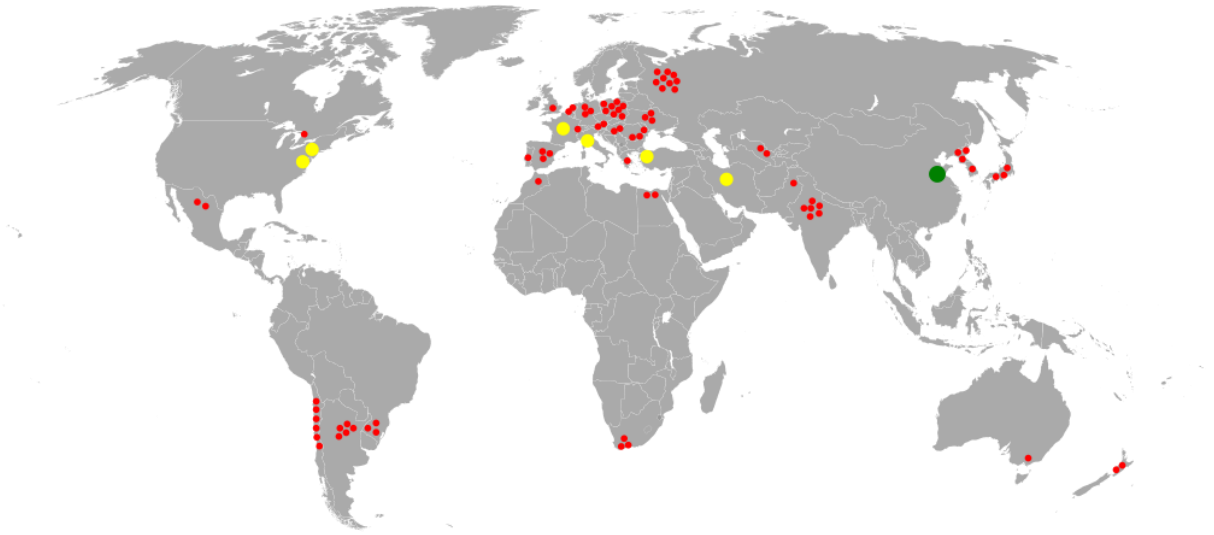


Fig.1. World map showing areas inclusive of apple production (colors referred to the top producers as a percentage, green: 100; yellow: 10; red: 1)
http://upload.wikimedia.org/wikipedia/commons/thumb/1/1b/2005apple.svg/1000px-2005apple.svg.png

Apple belongs to the *Pomoideae* family, subfamily *Rosaceae*, along with other important fruit crops such as pear (*Pyrus communis* L.), prune (*Prunus domestica* L.) and cherry (*Prunus avium* L.). Domesticated apple probably originated from the area around of the Himalaya Mountains on the border of Western China, in the former USSR and in Central Asia, and it is putatively a complex inter-specific hybrid, designated *Malus domestica* Borkh. (Korban and Skirvin, 1984; Phipps *et al*., 1990).

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 number of chromosomes is X=17. It has been possible to generate haploids by sexual crossing (Lespinasse *et al*. 1983), diploid and triploid forms are known.

Most of the cultivated apple lines are diploids (*2n* = 2x = 34), self-incompatible, open-pollinated, and display a juvenile period that ranges from 6 to 10 years or more (Korban and Chen, 1992).

2.2 Flavonoids

Flavonoids are a class of low-molecular-weight phenolic compounds that are widely distributed in the plant kingdom. More than 7,000 diverse flavonoids have been identified in different plant species (Ververidis *et al.,* 2007). Flavonoids are found in most plant tissues. The most important sources for human nutrition are fruits, tea and soybean. Green and black tea contains about 25% percent flavonoids. Other important sources of flavonoids are apple, citrus fruits (Awad *et al*., 2000).

These compounds frequently serve as pigments in plants, but are also involved in many biological interactions. Flavonoids are built upon a C6-C3-C6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclised with oxygen. Based on the degree of unsaturation and oxidation of the three-carbon segment, flavonoids are divided in several structural classes like flavanones, isoflavones, flavones, flavanols and anthocyanins. Most flavonoids reported in the literature are glycosides of a relatively small number of flavonoids aglycons, which are generally water-soluble and accumulate in the vacuoles of plant cells (Bohm, 1998; Seigler, 1998).

2.2.1 Flavonoid biosynthetic pathway

Flavonoids are synthesized via the phenylpropanoid pathway. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. PAL also shows activity with converting tyrosine to *p*-coumarate, albeit to a lower efficiency. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate and 4-coumarate: CoA ligase (4CL) converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3- hydroxylase (F3H) catalyzes the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT) which stabilizes the anthocyanidins by 3-*O*-glucosylation (Harborne, 1994; Bohm, 1998).

The flavonoid pathway is presented in Fig.2, there is evidence that the enzymes involved in flavonoid metabolism might be acting as membrane-associated multienzyme complexes, which has implications on the overall efficiency, specificity, and regulation of the pathway (Stafford, 1991; Winkel-Shirley, 1999, 2001). Studies of the flavonoid pathway range from classical genetic analysis of flower color inheritance patterns by Mendel, through the establishment of their chemical structures, to efforts to understand the factors involved in their biochemical synthesis (Bohm, 1998).

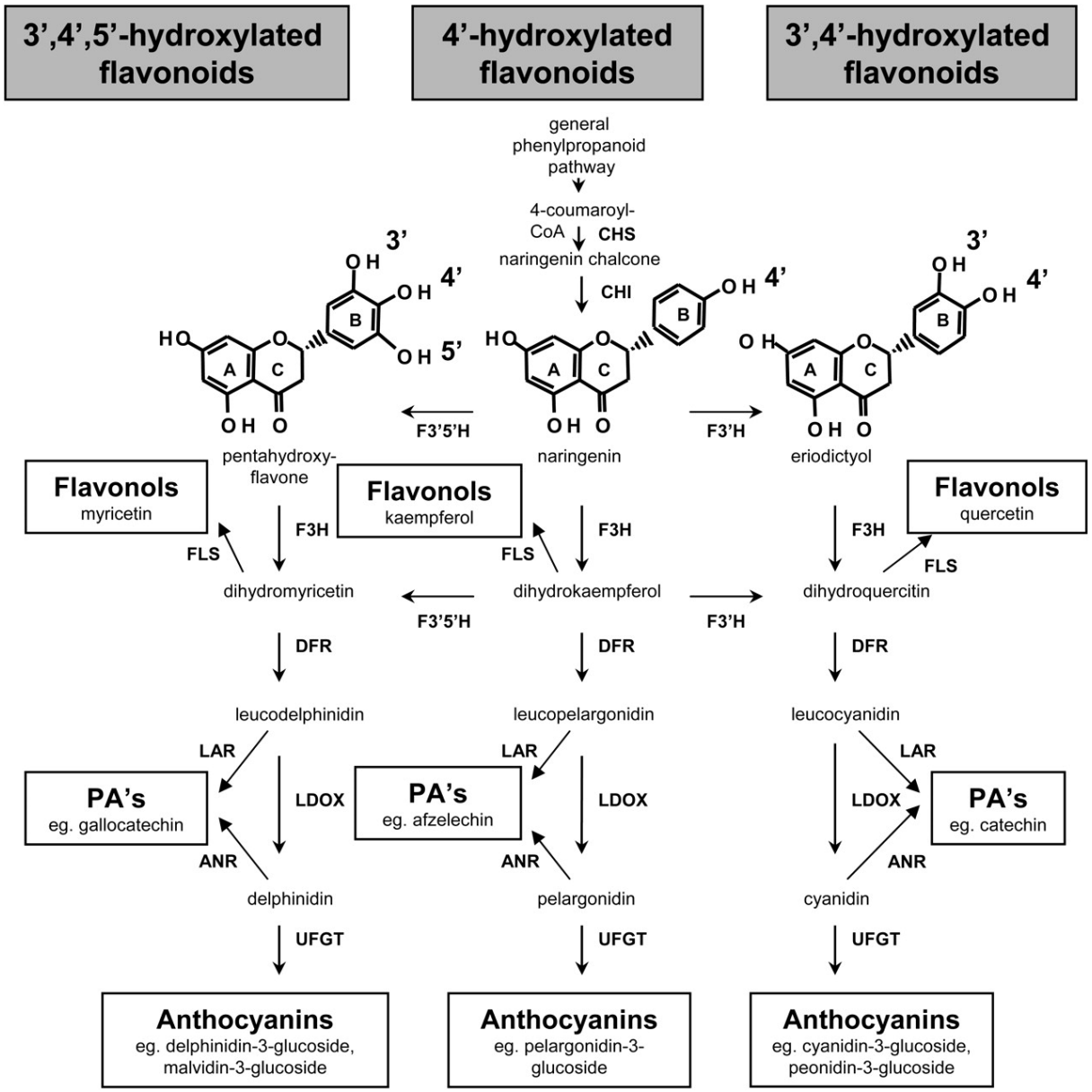


Fig.2. Schematic representation of the flavonoid biosynthetic pathway. Enzymes involved in the pathway shown are CHS, chalcone synthase; CHI, chalcone isomerase; F3'H; F3'5'H; F3H, flavanone-3β-hydroxylase; DFR; LDOX; FLS, flavonol synthase; LAR; ANR; and UFGT. Examples for the different hydroxylation patterns of the flavonoid B-ring are given for naringenin (4'-hydroxylated), eriodictyol (3', 4'-hydroxylated), and pentahydroxyflavone (3', 4', 5'-hydroxylated). (Bogs *et al.*, 2006)

2.2.2 Flavonoids biosynthetic pathway in apple

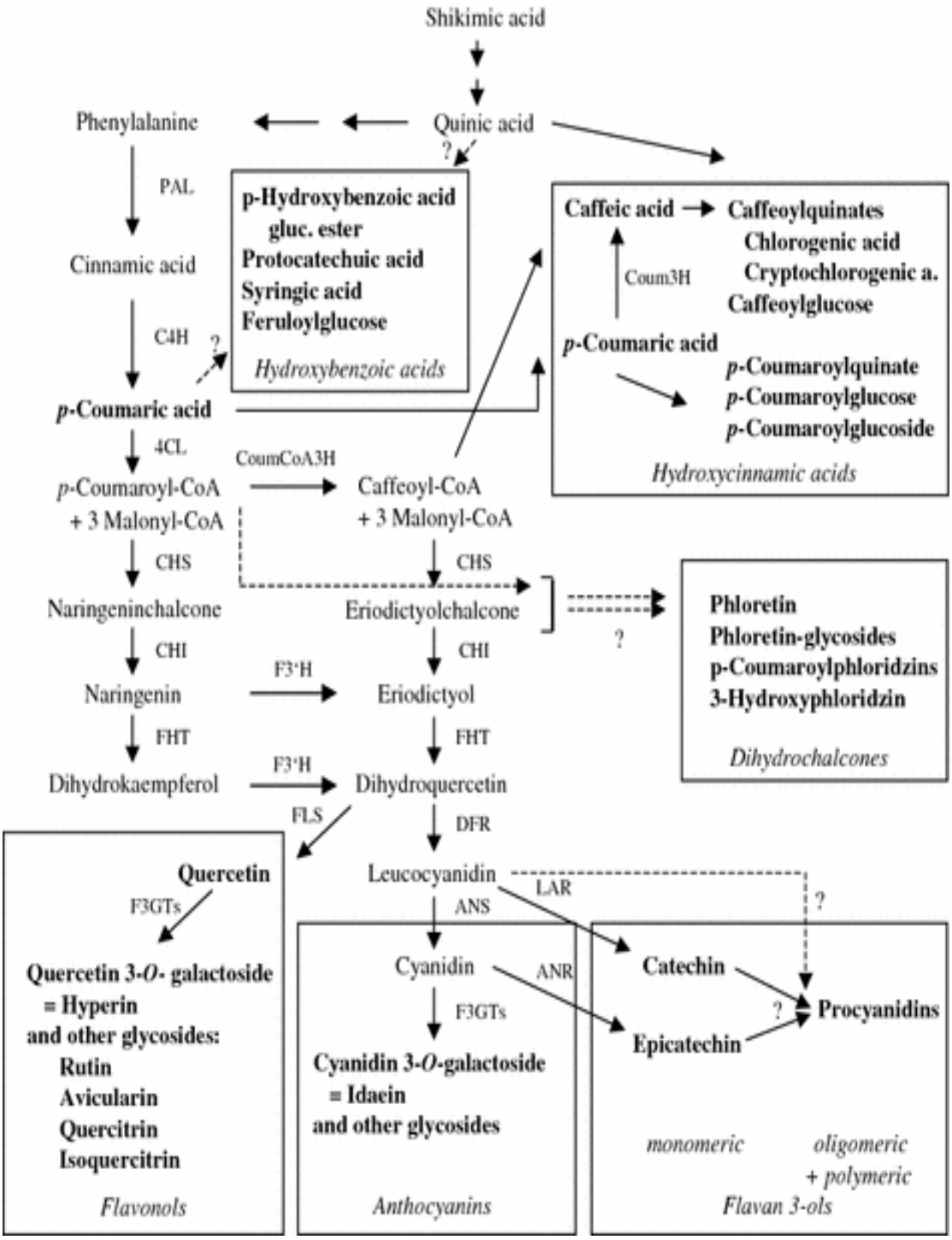


Fig.3. Flavonoids biosynthetic pathway in apple (Li *et al.*, 2007)

2.2.3 Defensive role of flavonoids in plants

The defensive role of flavonoids in plants was an important target since the twenties of the last century. The first demonstrated example of phenolics providing disease resistance was the case of onion scales accumulating sufficient quantities of catechol and protocatechuic acid to prevent onion smudge disease, *Colletotrichum circinans*. (Link *et al*., 1929). The coloured outer onion scales of resistant onion varieties contain enough of these two phenols to reduce spore germination of *C. circinans* to below 2%, while susceptible varieties lack these compounds and the spore germination rate is over 90% (Link *et al*., 1929).

Treutter (2005) divided the defence-related flavonoids into two groups: "preformed" and "induced" compounds. Plants in response to physical injury, infection, or stress synthesize the "induced" compounds. They may also be constitutively synthesized but, additionally, their biosynthesis is often enhanced under the influence of several types of stress. They may also occur only after infection or following several types of stress, as the so-called phytoalexins.

The "preformed" flavonoids are innate compounds, which are synthesised during the normal development of plants. Schlösser (1994) reviewed the involvement of preformed flavonoids in several host-pathogen interactions. These preformed compounds are often stored at strategically important sites where they may play a signalling and/or a direct role in defence. The relation between the localised accumulation of flavonoids and their activity is plausible with regard to the accumulation of UV-absorbing flavonoids in epidermal tissues. The site of accumulation seems to clearly indicate a beneficial function in other cases. Proof, however, is much more difficult to find. The occurrence of flavanols in the nuclei of several tree species is still highly debated (Feucht *et al.*, 2004, 2005).

2.2.3.1 Plant-microbe interactions

Because of their *in vitro* antimicrobial activity, specific classes of flavonoid and isoflavonoid compounds have long been thought to play a role in plant–microbe interactions as part of the host plant's defensive arsenal (Dixon, 1999; Dixon and Harrison, 1990). Several flavonoids are exuded from plant roots and act as signals that induce the transcription of bacterial genes, where protein products are required for the infection process (Hungria and Stacey, 1997; Broughton *et al*., 2003; Cooper, 2004); this is a significant action of flavonoids in improving plant growth and fitness. The expression of a symbiosis-related gene is also induced in a cyanobacterium by naringin (Cohen and Yamaseki, 2000). In root cells of *Medicago trunculata* colonised by *Glomus versiforme* elevated levels of PAL and CHS transcripts were detected (Harrison and Dixon, 1994). Ironically, flavonoids can also act as attractant

molecules for pathogenic micro-organisms such as *Agrobacterium tumefaciens* or *Pseudomonas syringae* and as inducers of their virulence genes (Mo *et al.*, 1995).

Several studies have also revealed new roles for isoflavonoids as signal molecules in plant-microorganism interactions. For example, the isoflavonoid pterocarpans, maackiain and pisatin act as classical phytoalexins in the interaction between garden pea (*Pisum sativum*) and the fungal pathogen *Nectria haematococca,* and maackiain and pisatin detoxifying enzymes are fungal virulence factors (Enkerli *et al*., 1998; Wasmann and Van Etten, 1996). The flavonol rutin is assumed to be one of the signalling molecules in *Eucalyptus globulus* ssp. *Bicostata*, promoting the hyphal growth of the ectomycorrhizal fungus *Pisolithus* sp. (Lagrange *et al*., 2001).

Scervino *et al*. (2005) studied the effect of flavonoids on pre-symbiotic growth, such as spore germination, hyphal length, hyphal branching and the formation of auxiliary cells and secondary spores, of the arbuscular mycorrhizal fungi *Gigaspora rosea*, *G. margarita*, *Glomus mosseae* and *G. intraradices* was studied. According to the effect on each fungal growth parameter, the tested compounds could be classified to be genus and/or species specific or specific only for a certain developmental stage of pre-symbiotic growth.

2.2.3.2 Plant-pathogen interactions

The possibility that phenolic oxidation products could have an antifungal action by polymerizing and forming a protective seal on cell walls has been proposed by Beckman *et al.* (1974) who showed that artificial membranes of calcium oxalate-pectin, that had been infused with the oxidation products of 3-hydroxy-tryptamine, were resistant to degradation by *Fusarium oxysporum* f. sp. *cubense*.

Grayer and Harborne (1994) in their survey of antifungal compounds from higher plants found that distinction is made between constitutive antifungal agents and phytoalexins, which are specifically formed in response to fungal inoculation.

Skadhauge *et al*. (1997) in their study of barley mutants showed that proanthocyanidins and even small amounts of dihydroquercetin are involved in the defence against *Fusarium* species. They explained the mechanisms and the role of barley flavonoids in resistance against *Fusarium* might be related to the crosslinking of microbial enzymes, inhibition of microbial cellulases, xylanases, pectinases, and– chelation of metals necessary for enzyme activity. Formation of a hard, almost crystalline structure as a physical barrier is the result of response against pathogen attack.

Padmavati *et al*. (1997) reported that the fungal blast pathogen *Pyricularia oryzae* shows a

differential sensitivity to growth inhibition by naringenin, kaempferol, quercetin and dihydroquercetin, in decreasing order. Naringenin also inhibited the spore germination of the bacterial blight pathogen *Xanthomonas oryzae* pv. *Oryzae*.

Beckman (2000) reviewed the possible role of preformed phenolics in periderm formation in wilt disease resistance. He described some examples where phenolic compounds, among them flavonoids, are stored in specialised cells from where they can be infused into attacked tissue, such as xylem vessels. Such leaching is probably involved in the hypersensitive response and programmed cell death as common mechanisms of pathogen defence.

On the other hand, if pre-existing antifungal phenolics are not sufficient to stop the development of the infectious process, plant cells usually respond by increasing the level of pre-existing antifungal phenols at the infection site, after an elicited increased activity of the key enzymes (PAL and chalcone synthase) of the biosynthetic pathway (Yedidia *et al.*,2003).

Quercetin and its derivatives inhibited the growth of not only *Arabidopsis thaliana* but also of the fungus *Neurospora crassa* (Tomita-Yokotani *et al*. 2003; Parvez *et al*., 2004).

Reported research using an alfalfa split root system provides evidence for systemic suppression of *nod* gene-inducing flavonoid compounds after initial nodulation as a means of inhibiting new infections (Catford *et al.*, 2006)

2.2.3.3 Plant-insect interactions

The role of flavonoids in plant-insect interactions is widely accepted. Moreover, a popular concept assumes that plant secondary metabolites, among them flavonoids, evolved for defence against herbivores (Feeny, 1976).

The biochemical basis for nematode resistance of banana was found to depend on flavan-3, 4-diols and condensed tannins (Collingborn *et al.*, 2000).

In order to analyze the effect of flavonoids on *Tenebio molitor* larvae growth, (Sosa *et al*., 2000) have investigated 20 flavonoids isolated from Argentina native plants *and* others, commercially purchased. During the study, each flavone was incorporated into the feed at a concentration of 6.0 μmol/g. Although all flavones slightly diminished the larval weight after 60 days of treatment, results indicated that quercetin was the most effective growth inhibitor for *T. molitor* larvae.

An inheritance study with groundnut provided good evidence that the flavonols quercetin and its glycoside rutin are related to larval mortality of the tobacco armyworm *Spodoptera liture* (Mallikarjuna *et al*., 2004). The species *Arachis hypogaea* is a non-resistant host, whereas the wild species *A. kempff-mercadoi* is resistant due to its flavonols. Feeding experiments with

interspecific hybrids revealed a positive correlation between the flavonol content of the plants and larval mortality.

In order to determine how flavonoids can confer resistance to crops against insect attack, Lattanzio *et al*. (2000) found a positive relationship between resistance/susceptibility characteristics against aphids and flavonoid glycoside content of cowpea lines. The resistant lines showed a flavonoid content higher than that of susceptible ones. In vitro bioassays proved that, among endogenous flavonoids, quercetin and iso-rhamnetin possess a good inhibitory aphid reproduction effect.

However, it is not a simple equation as to which of the partners may gain most benefit (Simmonds, 2003). There are insects, which can sequester plant flavonoids in their body cuticle as a defence against predators, or into their wings to attract mates (Burghardt *et al.*, 2000).

Flavonoids can also have negative effects on non-adopted insects or may reduce the nutritive value of the food. They may behave as feeding deterrents, as digestibility reducers and as toxins. Several insects are sensitive to flavonoids or are deterred by flavonoids in feeding tests (Brignolas *et al*., 1998; Thoison *et al*., 2004; Chen *et al*., 2004).

However, flavonoids do not simply act as broad spectrum defensive mechanisms (Forkner *et al*., 2004; Nykänen and Koricheva, 2004).

Sosa *et al*. (2004) described a physiological mechanism which flavonoid exudates could elicit an avoidance reaction in herbivores. The exudates of *Cistus ladanifer* L. contains apigenin and 3, 7-di-O-methylkaempferol, which inhibit the skeletal muscle sarcoplasmic reticulum (Ca^{2+}-ATPase), thus impairing mouth skeletal relaxation.

Thirty-seven flavonoid compounds (9 flavones, 18 flavonols, 8 flavanones, and 2 flavanonols) were investigated for their effect on feeding choice with Bertha Armyworm (*Mamestra configurata* Walker; BAW). Feeding choice was dependent upon subtle differences in biochemical structure. Unsubstituted flavone and flavanone were the strongest feeding deterrents in the choice bioassay, while 7, 40-dihydroxyflavone and dihydroquercetin stimulated BAW to feed. The constitutive flavonoids of *Brassica napus*, isorhamnetin-3-sophoroside-7-glucoside and kaempferol-3,7-diglucoside, were effective deterrents when supplemented at concentrations higher than endogenous levels. In a no-choice bioassay, flavone reduced both larval weight as well as larval and pupal development time (Onyilagha *et al.*, 2004).

Misra *et al*. (2010) have expressed an Arabidopsis (*Arabidopsis thaliana*) transcription factor, *AtMyb12*, in tobacco (*Nicotiana tabacum*), which resulted in enhanced expression of genes

involved in the phenylpropanoid pathway, leading to severalfold higher accumulation of flavonols. Global gene expression and limited metabolite profiling of leaves in the transgenic lines of tobacco revealed that *AtMyb12* regulated a number of pathways, leading to flux availability for the phenylpropanoid pathway in general and flavonol biosynthesis in particular. The tobacco transgenic lines developed resistance against the insect pests *Spodoptera litura* and *Helicoverpa armigera* due to enhanced accumulation of rutin.

2.2.3.4 Plant-plant interactions

Antimicrobial effects of flavonoids have been described to participate in allelopathic plant-plant interactions (Chou, 1999; Inderjit and Gross, 2000). Their roles and mode of action are not yet fully understood.

Bais *et al.* (2003) identified catechin as a phytotoxic allelochemical, they found that Centaurea maculosa exudes catechin from its roots and inhibits growth and seed germination of Centaurea diffusa and Arabidopsis thaliana. The flavanol triggers a wave of reactive oxygen species, which leads to a Ca^{2+} signalling cascade and to the death of the root system.

Deng *et al.* (2004) found that the growth reduction found in some gramoineous plants (maize, rice, and *Echinochloa oryzicola*) caused by the flavanone naringenin is attributed to the inhibition of 4-coumarate CoA ligase and therefore of lignification.

Subramanian *et al.* (2007) showed that flavonoids can play multiple roles at different stages of nodulation. Flavonoids are thought to serve as signal molecules in the rhizosphere to concentrate compatible rhizobia and induce Nod signal biosynthesis.

Zhang *et al.* (2009) silenced different flavonoid-biosynthesis enzymes to generate transgenic *Medicago truncatula* roots with different flavonoid profiles. Silencing of chalcone synthase, the key entry-point enzyme for flavonoid biosynthesis led to flavonoid-deficient roots. Silencing of isoflavone synthase and flavone synthase led to roots deficient for a subset of flavonoids, isoflavonoids (formononetin and biochanin A) and flavones (7, 4′-dihydroxyflavone), respectively.

Supplementation of roots with the flavonol kaempferol (an inhibitor of auxin transport), in combination with the use of flavone pre-treated *Sinorhizobium meliloti* cells, completely restored nodulation in flavonoid-deficient roots. In addition, *Sinorhizobium meliloti* cells constitutively producing Nod factors were able to nodulate flavone-deficient roots, but not flavonoid-deficient roots. These observations indicated that flavones might act as internal inducers of rhizobial nod genes, and that flavonols might act as auxin transport regulators during nodulation. Both these roles of flavonoids appear critical for symbiosis in *M.*

truncatula (Zhang *et al.,* 2009).

2.2.4. Defensive role of flavonoids in apple *(M. domestica)*

The presence of preformed flavanols in apple leaves may account for efficient defence against the fungus *Venturia inaequalis* (Picinelli *et al*., 1995; Treutter and Feucht, 1990). Resistant apple cultivars have a higher content of hydroxycinnamic acids and flavanols (Picinelli *et al*, 1995).

However, it is not yet clear if 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). 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 metabolized through an unusual pathway towards the 3-deoxycatechins (Römmelt *et al*., 1999, 2003; Halbwirth *et al*., 2003).

Lattanzio *et al* (2001) have shown that in cold stored 'Golden Delicious' apples, when rot caused by *Phlyctaena vagabunda* appears in infected tissues surrounding the rotten zone, a general increase in phenolic levels was observed, as compared to a healthy tissue of the same fruit.

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). 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).

Hamauzu (2006) reported that phloridzin also plays a role in host resistance to pathogens. He suggested that phloridzin can be hydrolysed *in vivo* by various fungi such as *V. inaequalis* to give phloretin, which in turn, is degraded to phloroglucinol, phloretic acid and *p*-hydroxybenzoic acid, which inhibits the development of the fungus.

Phenolic compounds were measured in leaf tissues of apple cvs 'Jonagold' and 'Golden Delicious'. Healthy leaves and leaves infected by *Venturia inaequalis* were analyzed by high performance liquid chromatography. Infection by *V. inequalis* caused an accumulation of phenolic compounds in infected leaves with a 1.4 to 6.2- fold increase of flavonols, a 2 to 6-fold increase of chlorogenic acid (Mikulic-Petkovšek *et al.,* 2008).

The apple leaves and fruits infected with the *Venturia inaequalis* fungus enhanced the metabolism of phenolics at the infection sites, especially in the boundary tissue. The infected tissue showed in comparison to the healthy tissue up to 7.6 times more hydroxycinnamic acids, up to 2.6 times more flavan-3-ols and up to 2.9 times higher values of flavanols. The content level of total phenolics in the infected tissue was 1.3–2.4 times higher than in the healthy leaves and fruit (Mikulic-Petkovšek *et al*., 2009).

Flachowsky *et al.* (2010) reported that transgenic apple plants cv. 'Holsteiner Cox' overexpressing the *Leaf Colour* (*Lc*) gene from maize (*Zea mays*) exhibit strongly increased production of anthocyanins and flavan-3-ols (catechins, proanthocyanidins). Those plants showed higher resistance against fire blight caused by the bacterium *Erwinia amylovora,* and against scab caused by the fungus *Venturia inaequalis*.

2.3 Metabolic engineering

Metabolic engineering in plants involves the modification of endogenous pathways to increase flux towards particularly desirable molecules. In some cases, the aim is to enhance the production of a natural product, whereas in others it is to synthesize a novel compound or macromolecule. There are three basic goals of metabolic engineering in plants: the production of more of a specific desired compound, the production of less of a specific unwanted compound, and the production of a novel compound (Verpoorte, 2000). Use of recombinant DNA distinguishes metabolic engineering from classical biochemical genetics

2.3.1 Metabolic engineering and flavonoids pathway

The metabolic engineering of flavonoid pathways began in 1987. In the first successful metabolic engineering experiments of the flavonoid pathway in plants, the maize *A1* gene encoding dihydroflavonol 4-reductase (DFR) was introduced in a chemogenetically characterized mutant line of *Petunia hybrida* accumulating kaempferol(Km) and dihydrokaempferol (DHK) in flowers, giving rise to a new orange *Petunia* phenotype not found in this species (Forkmann and Rahnau, 1987; Meyer *et al.*, 1987).

As the Flavonoid biosynthetic pathway was well known and the results could easily be observed by changes in flower colour (Dixon and Steele, 1999). Numerous experiments have been performed involving the overexpression of various pathway genes, aiming, for example, to produce new flower colours by introducing new compounds in the plant.

Because of their antioxidant activity, higher levels of anthocyanins and flavonoids in food are an interesting objective. Much work has focused on tomato. Chalcone isomerase (CHI), an

early enzyme of the flavonoid pathway, was found to be the key to increase flavonol production (Muir *et al.*, 2001). Overexpression of the *Petunia CHI* gene led to a 78-fold increase of flavonoid levels in the tomato peel. Upon processing such tomatoes, a 21-fold increase of flavonols in tomato paste was achieved, if compared with non-transgenic controls.

In legumes Isoflavones act as phytoalexins, that is, the biosynthesis of these antimicrobially active compounds is induced by microbial infection. These compounds could be produced in Arabidopsis, tobacco plants and maize, which normally lack the ability to synthesize these compounds, by overexpression of isoflavone synthase, a cytochrome P450 enzyme (Yu *et al.*, 2000; Jung *et al.*, 2000). The production of the isoflavones depends on the availability of precursors from the phenylpropanoid pathway.

Naturally, occurring flavonoid mutants and variants or genetically transformed plants have been important tools in several investigations clarifying the functions of the flavonoid pathway genes (Shirley *et al*.,1995; Tanaka *et al*., 1998). The expression of flavonoid pathway genes in fruit tissues has been studied on grape (*Vitis vinifera*) (Boss *et al*., 1996; Kobayashi *et al*., 2001), citrus (*Citrus unshiu* Marc.) (Moriguchi *et al*., 2001), and strawberry plants (*Fragaria* spp.) (Manning, 1998; Aharoni *et al*., 2001).

The scarcity of studies in this area may be due to a difficulty caused by the special features of the fruit tissues, e.g. the richness of different secondary metabolites and RNases, which may hinder the easy application of the molecular methods.

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, snapdragons, tobacco, Petunia and Arabidopsis (Holton and Cornisch, 1995).

2.4 Transcription factors

Transcription factors (TFs) are sequence-specific DNA binding proteins that are capable of activating and/or repressing transcription. In particular, they interact with the general transcription machinery, chromatin remodelling proteins and/or other transcription factors. They can act as activators or repressors of gene expression, mediating either an increase or a decrease in the accumulation of messenger RNA. Transcription factors have been isolated and characterized for two plant metabolic pathways, leading to biosynthesis of flavonoids and of terpenoid indole alkaloids (TIA), respectively.

Fig.4. Schematic diagram of the amino acid sequence (amino terminus to the left and carboxylic acid terminus to the right) of a prototypical transcription factor which contains (1) a DNA-binding domain (**DBD**), (2) signal sensing domain (**SSD**), and a transactivation domain (**TAD**). (http://en.wikipedia.org/wiki/Transcription factor)

2.4.1 Transcription factors involved in flavonoids pathway

Transcription factors (Regulatory proteins) of different families govern the synthesis of favonoids.

Early work on pathway transcription factors in plants began with the discovery of the maize flavonoid pathway regulators *Colorless1* (*C1*) gene belonging to Myb family and *Red(R)* gene belonging to bHLH family. Within a few years of their discovery, *C1* and *R* were shown to induce flavonoid gene expression and anthocyanin accumulation in transgenic plants (Goff *et al.*, 1990).

Muir *et al.,* (2001) overexpressed CHI, which encodes chalcone isomerase, an early flavonoid pathway enzyme that is expressed at low levels in tomato fruit. In a second experiment, (Bovy *et al.,* 2002) overexpressed the maize anthocyanin regulators Leaf colour (*Lc*) and *C1* in a fruit-specific manner. As anticipated, each of these approaches resulted in increased flux through the flavonoid pathway in the fruit, although rather unexpectedly, fruit flavonols rather than anthocyanins increased. In a striking contrast, overexpression of *CHI* enhanced flavonol production only in the peel, whereas *Lc* and *C1* caused an increase throughout the fruit.

Yu *et al.* (2003) exploited the effect of *R* and *C1* on early flavonoid pathway gene expression in soybean. They used *CRC*, a fusion of *C1* and *R*, rather than separate gene constructs to boost substrate availability for isoflavone synthase (IFS), which catalyses the first committed step into isoflavonoid biosynthesis.

2.4.1.1 The Myb domain factors

Myb factors represent a family of proteins that include the conserved Myb DNA-binding domain. The first *Myb* gene identified was the 'oncogene' *v-Myb* derived from the avian myeloblastosis virus (Klempnauer *et al.,* 1982). Evidence obtained from sequence comparisons indicates that *v-Myb* may have originated from a vertebrate gene, which mutated once, as it became part of the virus. Many vertebrates contain genes related to *v-Myb , c-Myb, a-Myb and b-Myb* (Westen, 1998) and other similar genes have been identified in insects,

plants, fungi and slime moulds (Lipsick, 1996).

Myb transcription factors have been isolated from various plant species. The best-characterized plant *Myb* genes involved in anthocyanin biosynthesis are *C1* and *PL* in maize. In maize, the accumulation of anthocyanin in competent tissues requires the presence of either *C1* in the seed or *PL* in the plant tissue (Cone *et al*., 1986). Moreover, *C1* and *PL* induce the expression of multiple anthocyanin biosynthetic genes such as *CHS*, *DFR* and *UFGT* (Cone *et al.*, 1986).

Myb transcription factors are potent candidates for regulatory factors in the upstream cascade because the expression of anthocyanin biosynthetic genes is thought to be under the control of Myb transcription factors (Stracke *et al*., 2001).

Accordingly, Myb-like proteins with one repeat have been designated as 'Myb1R', those with two repeats as 'R2R3Myb' and those with three repeats as 'Myb3R' (Stracke *et al*., 2001). Among these Myb transcription factors, R2R3Myb constitutes the largest Myb gene family in plants. In *Arabidopsis thaliana*, 125 distinct R2R3Myb genes have been detected within the complete genome sequence (http://www.ncbi.nlm.nih.gov/pubmed/11130711 Arabidopsis Genome Initiative 2000)

Up to now, no or only few functional data are available for the overwhelming majority of plant Myb genes. The functional data available indicate that Myb transcription factors are involved in a wide array of cellular processes. These include development (Oppenheimer *et al.*, 1991), signal transduction (Bendar and Fink, 1998), plant disease resistance (Daniel *et al.*, 1999), cell devision (Hirayama and Shinozaki, 1996) and secondary metabolism (Borevitz *et al.*, 2000).

2.4.1.2 The BHLH domain factors

Basic helix-loop-helix (bHLH) transcription factors represent a family of proteins that contain a bHLH domain, a motif involved in binding DNA. Regulation of the structural genes involved in anthocyanin biosynthesis is directly controlled by a combination of two distinct transcription factor families with homology to the protein encoded by the vertebrate proto-oncogene *c-Myb*, and the vertebrate basic-helix-loop-helix (bHLH) protein encoded by the proto-oncogene *c-Myc*, respectively (Mol *et al.*, 1998).

Stracke *et al.* (2001) mentioned that the common denominators in the regulation of anthocyanin biosynthetic genes are Myb transcription factors; basic helix–loop–helix (bHLH) called also (Myc) transcription factors and a WD40 protein. Yeast two-hybrid assays indicate that Myb, bHLH and WD40 can interact to form a protein with a complex three-dimensional

structure.

Three bHLH genes especially important for flavonoid synthesis have been identified: *EGL3, GL3* and *TT8*, and the triple mutant *egl3-1 gl3- 1 tt8-1* are essentially phenotypically indistinguishable from *ttg1* mutants (Zhang *et al.,* 2003)

However, Myb transcription factors such as *AtPAP1, AtPAP2, PhAN2, LeANT1* and *GMyb10* are sufficient for ectopic activation of anthocyanin biosynthetic genes in transgenic plants, possibly through the formation of complexes with endogenous bHLH and WD40 (Quattrocchio *et al*. 1999; Borevitz *et al.,* 2000; Elomaa *et al.*, 2003; Mathews *et al.*, 2003). Moreover, a retrotransposon insertion in *VvMybA1* is the molecular basis of the loss of pigmentation in a white grape cultivar of *Vitis vinifera* (Kobayashi *et al.,* 2004). These results indicated that modulation of Myb gene was enough to cause dramatic changes in anthocyanin accumulation; in other words, Myb transcription factors appear to be a dominant factor in anthocyanin accumulation.

The discovery of the proanthocyanidin (condensed tannin) pathway gene regulators transparent testa 2 /*TT2* which encodes an R2R3Myb domain protein and *TT8* which encodes a bHLH protein has opened the possibility of manipulating this branch of the flavonoid pathway (Nesi *et al*., 2000, 2001). Overexpression of *TT2* was shown to be sufficient to induce the accumulation of condensed tannin in tissues in which *TT8* is expressed (Nesi *et al.*, 2000). This result suggests that concurrent overexpression of *TT2* and *TT8* could be the key to controlling proanthocyanidin accumulation in other tissues.

Gonzalez *et al*. (2008) studied the regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings, they demonstrate that overexpression of *Myb113* or *Myb114* transscription factors results in substantial increases in pigment production, and pigment production in these overexpressors remains bHLH-dependent.

2.4.2 Transcription factor gene used in genetic transformation of apple *M. domestica* to regulate of flavonoids pathway

Takos *et al.* (2006b) isolated a gene encoding an R2R3Myb transcription factor from apple cultivar 'Cripps Pink' and designated it. They conclude that *MdMyb1* co-ordinately regulates genes in the anthocyanin pathway and the expression level of this regulator is the genetic basis for apple skin colour.

Ban *et al.* (2007) isolated and characterized a *Myb* transcription factor gene (*MdMybA*) from apple skin. Characterization of *MdMybA* demonstrated that *MdMybA* expression was specifically regulated depending on the tissue and cultivar/ species.

The *Lc* gene was transferred into the *M. domestica* cultivar Holsteiner Cox *via Agrobacterium tumefaciens*-mediated transformation, which resulted in enhanced anthocyanin accumulation in regenerated shoots. *Lc* overexpression in *M. domestica* resulted in enhanced biosynthesis of specific favonoid classes (Li *et al.*, 2007).

Lea *et al.* (2007) in their work show that three transcription factors, *PAP1, PAP2* and *GL3* known to be involved in the regulation of flavonoid synthesis respond to nitrogen deficiency by enhanced expression levels. In 4-day-old WT seedlings also a fourth transcript *Myb12* is significantly enhanced. *GL3* and *PAP2* transcripts showed the most profound responses to nitrogen depletion and their genes are therefore likely targets in the signal transduction chain responding to nitrogen deficiency and resulting in increased accumulation of flavonoids.

2.5 *MdMyb9* gene

The *MdMyb9* belongs to family of plant transcription factors that regulate the synthesis of anthocyanins or the anthocyanin biosynthetic pathway.

Chagné *et al.* (2007) chose it as one of four genes homologous to the production of anthocyanin pigments 1 (*PAP1*) gene (*MdMyb10*, transparent testa 2/*TT2* (*MdMyb9*), a colour inhibitor (*MdMyb17*) and a colour activator (*MdMyb12*).

2.6 *MdMyb10* gene

The *MdMyb10* a gene with sequence similarity to the Myb-class of transcription factors and known to regulate the biosynthetic steps in anthocyanin metabolism has been described and functionally characterized (Takos *et al.*, 2006b; Espley *et al.*, 2007).

Expression of several genes with sequence similarity to anthocyanin biosynthetic genes has been associated with red skin colour in apple *M. domestica* (Honda *et al.*, 2002; Takos *et al.*, 2006a). The *MdMyb10* gene showed high levels of expression in red-fleshed fruit and induced red colour when transiently infiltrated into tobacco leaves. Red foliage was also observed when a cDNA of the *MdMyb10* was over-expressed in transgenic 'Royal Gala' apple (Espley *et al.*, 2007). Recently, a gene with over 99% sequence identity to *MdMyb10* (*MdMyb1*) was shown to co-segregate with fruit skin colour in a population of 136 plants (Takos *et al.*, 2006b), demonstrating that allelic variation of *MdMyb10*-like genes are associated with alleles that influence anthocyanin distribution.

Chagné *et al.*(2007) have performed candidate gene mapping in a fruit tree crop and have provided genetic evidence that red colouration in the fruit core as well as red foliage are both controlled by a single locus named *Rni*. They have shown that the transcription factor *MdMyb10* may be the gene underlying *Rni* as there were no recombinants between the marker

for this gene and the red phenotype in a population of 516 individuals. Associating markers derived from candidate genes with a desirable phenotypic trait has demonstrated the application of genomic tools in a breeding programme of a horticultural crop species.

Lin-Wang *et al*. (2010) used gene specific primers to show that the three *Myb* activators of apple anthocyanin (*Myb10/Myb1/MybA*) are likely alleles of each other. *Myb* transcription factors, with high sequence identity to the apple gene were isolated from across the rosaceous family (e.g. apples, pears, plums, cherries, peaches, raspberries, rose, and strawberry). Key identifying amino acid residues were found in both the DNA-binding and C-terminal domains of these *Myb*s. The expression of these *Myb10* genes correlates with fruit and flower anthocyanin levels. Their function was tested in tobacco and strawberry. In tobacco, these *Myb*s were shown to induce the anthocyanin pathway when co-expressed with bHLHs, while over-expression of strawberry and apple genes in the crop of origin elevates anthocyanins.

Gao *et al.* (2011) introduced the *MdMyb10* transcription factor gene, into Arabidopsis and analyzed its function to osmotic stress in transgenic plants. Under high osmotic stress, the *MdMyb10* over-expressing plants exhibited growth better than wild-type plants. The elevated tolerance of the transgenic plants to osmotic stress was confirmed by the changes of flavonoids, chlorophyll, malondialdehyde and proline contents. These results preliminarily showed that the *MdMyb10* could possibly be used to enhance the high osmotic-tolerant ability of plants.

2.7 *MdMyb11* gene

MdMYB11 belongs to a similar family of plant transcription factors and has been found to upregulate several genes of the phenylpropanoid pathway. It has been shown that it is a transcriptional regulator of chalcone synthase and flavonols synthase in plants. *Myb11* belongs to the subgroup 7 of the R2R3Myb family, and other members of this subgroup are *Myb12* and *Myb111* (Stracke *et al*., 2001).

Stracke *et al*. (2007) determined the regulatory potential of these three transcription factors. They used a combination of genetic, functional genomics and metabolite analysis approaches for *Myb11*, *Myb12* and *Myb111*genes, show a high degree of functional similarity and display very similar target gene specificity for several genes of flavonoid biosynthesis, including chalcone synthase, chalcone isomerase, flavonone 3-hydroxylase and flavonol synthase 1. The analysis of the closely related *Myb* genes, *AtMyb11*, *AtMyb12*, and *AtMyb111* has been reported to show that in developing seedlings *AtMyb12* and *AtMyb111* are mainly responsible for the control of flavonol biosynthesis in roots and cotyledons, respectively, but no

significant differences in flavonol accumulation were detected in their *AtMyb11* mutant compared with wild type (Stracke *et al.*, 2010).

Petroni *et al*. (2008) reported that knock-out *AtMyb11-I* mutants and RNAi plants germinate faster, show a faster hypocotyl and primary root elongation, develop more lateral and adventitious roots, exhibit a faster development of the inflorescence and initiate more lateral inflorescences and fruits than wild-type plants. Plants overexpressing *AtMyb11* displayed the opposite phenotype. De novo formation of root meristemoids and, consequently, macroscopic roots from thin cell layers cultured *in vitro* was enhanced in explants from *AtMyb11-I* and reduced in those from lines overexpressing *AtMyb11*. These findings indicate that *AtMyb11* modulates overall growth in plants by reducing the proliferation activity of meristematic cells and delaying plant development.

The expression pattern of three genes was evaluated during all stages of fruit development by qRT-PCR and the results compared to those obtained from microarray analysis. The analysed *MdMyb11* show nearly the same expression pattern using the two approaches. *MdMyb11* is characterised by a progressive decrease of expression from May to September the biosynthetic pathways of two classes of flavonoids, flavonols and anthocyanins, are well known (Mehrtens *et al.* 2005; Newcomb *et al.* 2006).

Chalcone synthase I, chalcone-flavone isomerase and putative flavanone 3-beta-hydroxylase are three enzymes belonging to this pathway. The expression level of those enzymes decreases from May to September with the exception of the flavanone 3-beta-hydroxylase for which an increase in the transcript level is observed. cDNA microarray analysis showed a remarkable decrease of the *MdMyb11* expression during fruit ripening. Since transcription factors are key elements in controlling biosynthetic pathways (Soglio *et al*., 2009).

Feller *et al.* (2011) classified *MdMyb11* as one of several genes that play a role in anthocyanins biosynthetic pathwy.

2.8 Apple transformation

Genetic transformation of plants is the process where a defined fragment of DNA (a gene or a set of genes) is introduced and integrated into the genome of the plant, avoiding sexual reproduction. Genetic engineering enlarges the readiness of genes considerably, limited in conventional breeding programs, since genes isolated from other plants, animals or microorganisms can be transferred to plants (Brasileiro and Dusi, 1999). Apple was an early target for the emerging recombinant DNA technology. Transformation of *M.domestica* is

nowadays a common practice in several laboratories and the protocols have been constantly improved to enhance the transformation efficiency.

The most widely used method for introducing foreign genes into dicotyledoneous plants is the *Agrobacterium tumefaciens* mediated transformation. In this process, *Agrobacterium tumefaciens,* a disarmed Ti binary vector, and leaf fragments or callus cultures are the key component for an efficient transformation. Most studies started from wounded leaf sections, but apical intermodal explants from etiolated `Royal Gala′ apple shoots has produced a higher efficiency in producing transgenic shoots (Liu *et al.*, 1998).

James *et al*. (1989) described an agrobacterium-mediated method to produce transgenic apple (*Malus pumilla* Mill) plants with a normal phenotype except for a somewhat reduced capacity to root.

James *et al.* (1993) studied a number of factors affecting the transcription of the *vir* genes of *Agrobacterium tumefaciens* in the leaf disc transformation procedure and believed to limit the efficiency of regeneration of apples. The presence of acetosyringone and the osmoprotectant betaine phosphate in the virulence induction medium has been shown to increase the efficiency of transformation as monitored by fluorometric determinations of GUS activity in apple leaf discs infected with a disarmed strain of *Agrobacterium tumefaciens* harbouring the vector used.

De Bondt *et al*. (1994) studied the factors influencing transfer of an intron –containing ß-glucuronidase gene to apple leaf explants during early steps of an *Agrobacterium tumefaciens* mediated transformation procedure. They studied three different strains of *Agrobacterium tumefactions.*

Yao *et al.* (1999) developed a transformation system for the commercial apple (*M. domestica* Borkh.) cultivar 'Royal Gala'. Leaf pieces from *in vitro*-grown shoots were cocultivated for 2 days with *Agrobacterium tumefaciens* strain LBA4404 containing the binary vectors pKIWI105 or pKIWI110. Shoots were produced on a shooting medium containing kanamycin (50 $mg \cdot L^{-1}$). A 2-day incubation period on kanamycin-free medium prior to antibiotic selection enhanced the regeneration of kanamycin-resistant shoots.

De Bondt *et al.* (1996) worked on the optimization of postcultivation conditions for efficient and reproducible regeneration of transgenic shoots from the apple cultivar 'Jonagold '. Factors that were found to be essential for efficient shoot regeneration were the use of gelrite as a gelling agent and the use of the cytokinin-mimicking thidiazuron in the selective postcultivation medium. Improved transformation efficiencies were obtained by combining the hormones thidiazuron and zeatin and by using leaf explants from *in vitro* grown shoots not

older than 4 weeks after multiplication. Attempts to use phosphinotricin acetyl transferase as a selectable marker were not successful. Using selection on kanamycin under optimal postcultivation conditions, about 2% of file leaf explants developed transgenic shoots or shoot clusters.

Norelli *et al.* (1996) used leaves of *M. domestica* Borkh. cv. 'Royal Gala' were either crush-wounded with forceps, cut, or left whole, and then inoculated with *A. tumefaciens* strain EHA105 (p35SGUS-INT), with and without vacuum infiltration. Transformation was quantified 13 days after inoculation by determining the rate of β-glucuronidase (GUS) activity. Leaf wounding by crushing with nontraumatic forceps significantly increased transformation when compared to cutting the leaves. Vacuum infiltration of inoculum had no effect on transformation.

Puite and Schaart (1996) described an *Agrobacterium tumefaciens*-mediated transformation method for the commercial apple cultivars 'Gala', ' Golden Delicious' and ' Elstar'.

There are many of others researches and studies on apple genetic transformation (Hammerschlag *et al.,* 1997; Liu *et al.,* 1998; Sriskandarajah and Goodwin, 1998; Bolar *et al.,* 1999).

2.8.1 Genes used in genetic transformation of apple

Transformations are mostly based on traditional cultivars and they have been carried out using genes isolated from apple (Belfanti *et al.,* 2004b; Espley *et al.,* 2007; Malnoy *et al.,* 2007; 2008), or from other organisms (Wong *et al.,* 1999; Norelli *et al.,* 1994; 2000; Bolar *et al.,* 2000; 2001; Hanke *et al*., 2000; Liu *et al.,* 2001; Szankowski *et al.,* 2003; Markwick *et al.,* 2003; Faize *et al.,* 2004; Li *et al*., 2007). Genes affecting some physiological or morphological characters like growth (Holefors *et al.,* 2000), flowering (Yao *et al.,* 1999), early flowering (Flachowsky *et al*., 2007; 2010) and self-fertility (Van Nerum *et al.,* 2000) have also been incorporated into transgenic apple. Rootstock scions have also been used in transgenic assays to improve rooting rates and growth (Holefors *et al.,* 1998; Welander *et al.,* 1998; Sedira *et al.,* 2001; Pawlicki-Jullian *et al.,* 2002; Igarashi *et al.,* 2002). The function of some genes like sorbitol-6-phosphate (Kanamaru *et al.,* 2004; Cheng *et al.,* 2005), stilbene synthase (Rühmann *et al.,* 2006), polygalacturonase (Atkinson *et al.,* 2002) and from several promoters (Ko *et al.,* 2000; Gittins *et al.,* 2001; 2003; Szankowski *et al.,* 2009a) has also been studied using transgenic apple .

Table.3. some of recently genes used in genetic transformation of apple (polanco *et al.*, 2010)

		Gene	Donator	Results	Reference
Overexpression	Genes isolated from apple	*HcrVf2* (R-genes)	*Malus floribunda* 821	Scab-resistance	Belfanti *et al.*, 2004
		HcrVf1 (R-genes)	*M. floribunda* 821	Scab-resistance	Malnoy *et al.*, 2008
		MdPG1 (Polygalacturonase)	*Malus domestica* Borkh.	Changes in cell adhesion/ maduration	Atkinson *et al.*, 2002
		MpNPR1 (pathogenesis-related gene)	*M. domestica* Borkh.	Fungal disease resistance	Malnoy *et al.*, 2007a
		MdMYB10 (MYB transcription factor)	*M. domestica* Borkh.	Induce anthocyanin accumulation/red apple fruit color	Espley *et al.*, 2007
	Genes isolated from apple	*Attacin E*	*Hyalophora cecropia*	Fire blight resistance	Norelli *et al.*, 2000
		Cecropin MB39	*Hyalophora cecropia*	Fire blight resistance	Liu *et al.*, 2001
		ech42 (Endochitinase)	*Trichoderma harzianum*	Scab-resistance	Wong *et al.*, 1999
		ech42 (Endochitinase) *Nag70* (Exochitinase)	*Trichoderma harzianum*	Scab-resistance	Bolar *et al.*, 2001
		Vst1 (Stilbene synthase)	Grapevine (*Vitis vinifera* L.)	Antifungal activity	Szankowski *et al.*, 2003
		PGIP (Polygalacturonase inhibitor)	Kiwi (*Actinidia deliciosa*)	Antifungal activity	Szankowski *et al.*, 2003
		Avidin or Streptavidin	*Streptomyces avidinii*	Insect resistance (lightbrown apple moth)	Markwick *et al.*, 2003
		RolA (Hydrolyze phytohormone glucoside A)	*Agrobacterium rhizogenes*	Root induction	Holefors *et al.*, 2000
		RolB (Hydrolyze phytohormone glucoside B)	*A. rhizogenes*	Root induction	Welander *et al.*, 1998
		RolC (Hydrolyze phytohormone glucoside C)	*A. rhizogenes*	Root induction	Igarashi *et al.*, 2002
		PinB (puroindoline)	Wheat (*Triticum aestivum*)	Antifungal activity	Faize *et al.*, 2004
Silencing		*S-RNase* gene	*M. domestica* Borkh.	Self-fertility	Van Nerum *et al.*, 2000
		S6PDH (Sorbitol-6-phosphate dehydrogenase)	*M. domestica* Borkh.	Regulating partitioning between sorbitol and sucrose	Kanamaru *et al.*, 2004
Promotors		*ExtA* promotor	*Brassica napus* L.	Tissue-specific distribution	Gittins *et al.*, 2001
		RolC promotor	*A. rhizogenes*	Tissue-specific distribution	Gittins *et al.*, 2003
		HcrVf2 promotor	*M. floribunda* 821	Scab-resistance	Szankowski *et al.*, 2008

3 OBJECTIVES OF THIS STUDY

1- Establishment of an agrobacterium mediated transformation system for selected apple cultivars **(Gala, Holsateiner cox)** by using the vector pJAN harbouring *MdMyb9*, *MdMyb10*, *MdMyb11* transcription factor genes which are isolated from apple.

2- Improvement the selection system by cloning the MdMyb10, MdMyb11genes into the binary vector pGIIMH35S, and establishment of new transformation experiments with the new constructs.

3- Improve our knowledge of the role of flavonoid in plant resistance and may be helpful for use of their beneficial effects.

Hypothesis

Regulation of the flavonoid biosynthesis pathway through overexpresion of transcription factors in selected apple cultivars will increase resistance against disease.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

4.1.1.1 Growth media

Substance	Molecular weight	Company
MS basal salts mixture		Duchefa (Haarlem, Netherlands)
Plant agar		Duchefa (Haarlem, Netherlands)
D(+) Saccharose	342.3	Duchefa (Haarlem, Netherlands)
D-Sorbitol	182.18	Roth (Karlsruhe, Germany)
Gelrite		Duchefa (Haarlem, Netherlands)

4.1.1.2 Plant growth regulators and additives

Substance	Molecular weight	Company	Solvent
IBA	204.2	Duchefa (Haarlem, Netherlands)	KOH
NAA	186.2	Duchefa (Haarlem, Netherlands)	KOH
BAP	225.3	Duchefa (Haarlem, Netherlands)	KOH
TDZ	220.2	Duchefa (Haarlem, Netherlands)	KOH
GA3	346.4	Duchefa (Haarlem, Netherlands)	KOH
Glufosinate-ammonium (PPT)	238.8	Riedel de Häen (Seelze, Germany)	ddH_2O
BASTA® (200g/l)		Aventis GmbH (Frankfurt, Germany)	ddH_2O

4.1.1.3 Antibiotics

Substance	Molecular weight	Company	Solvent
Combactam		Pfitzer (Karlsruhe, Germany)	ddH_2O
Kanamycin	582.6	Duchefa (Haarlem, Netherlands)	ddH_2O
Ticarcillin	428.4	Duchefa (Haarlem, Netherlands)	ddH_2O
Ampicilin	371.39	Roth (Karlsruhe, Germany)	ddH_2O
Cefatoxime	477.4	Duchefa (Haarlem, Netherlands)	KOH
Rifampicin	823	Duchefa (Haarlem, Netherlands)	HCl

4.1.1.4 Restriction enzymes and buffers

Enzyme	10x Buffer	Company
*Eco*RI	10x EcoR1 buffer	MBI Fermentas (Leon-Rot, Germany)
*Bam*H1	10x BamH1buffer	MBI Fermentas (Leon-Rot, Germany)
*Bsu*15I (*Cla*I)	10X Buffer Tango™	MBI Fermentas (Leon-Rot, Germany)
*Xba*I	10X Buffer Tango™	MBI Fermentas (Leon-Rot, Germany)

*Nco*I	10X Buffer Tango™	MBI Fermentas (Leon-Rot, Germany)
*Nhe*I	10X Buffer Tango™	MBI Fermentas (Leon-Rot, Germany)
RNaseA		PEQLAB Biotechnology (Germany)
T4 ligase	10x ligation buffer	MBI Fermentas (Leon-Rot, Germany)
SAP(Shrimp Alkaline Phosphates	10X SAP buffer	MBI Fermentas (Leon-Rot, Germany)

4.1.1.5 Molecular biological kits

DNeasy® Plant Maxi Kit	Qiagen (Hilden, Germany)
GeneJET™ Gel Extraction Kit	MBI Fermentas (Leon-Rot, Germany)
GeneJET™ Plasmid Miniprep Kit	MBI Fermentas (Leon-Rot, Germany)
High Fidelity PCR Enzyme Mix	MBI Fermentas (Leon-Rot, Germany)
InviTrap® Spin Plant RNA Mini Kit	Invitek (Berlin, Germany)
PCR DIG Probe Synthesis Kit	Roche (Grenzach-Wyhlen, Germany)
Rapid DNA Ligation Kit	MBI Fermentas (Leon-Rot, Germany)
RevertAid™ H Minus First Strand cDNA Synthesis Kit	MBI Fermentas (Leon-Rot, Germany)
IQ™ SYBR® Green Super mix	Bio-Rad (California,USA)

4.1.1.6 DNA markers

DNA marker	**Concentration**	**Company**
Gene Ruler 100bp DNA ladder	0.5 mg/ml	MBI Fermentas (Leon-Rot, Germany)
Gene Ruler 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas (Leon-Rot, Germany)
DIG labelled DNA II Marker *	5 µg/µl	Roche (Grenzach-Wyhlen, Germany)

* Dig II marker: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 bp

4.1.1.7 Solvents and sterilizes and others

Substance	**Company**
Acetic acid	Roth (Karlsruhe, Germany)
Agarose for electrophoresis	Roth (Karlsruhe, Germany)
Ammonium nitrate	Duchefa (Haarlem, Netherlands)
CDP-Star chemiluminescence substrate	Roche (Grenzach-Wyhlen, Germany)
$CaCl_2$	Roth (Karlsruhe, Germany)
Cetyltrimethylammoniumbromid (CTAB)	Duchefa (Haarlem, Netherlands)
Chloroform	Roth (Karlsruhe, Germany)
D-Glucose	Duchefa (Haarlem, Netherlands)
Dimethyl sulfoxide (DMSO)	Riedel de Häen (Seelze, Germany)
2'-Desoxyribonucleosid-5'-triphosphate (dNTPs)	MBI-Fermentas (Leon- Rot, Germany)
EDTA	Roth (Karlsruhe, Germany)
Ethanol	Roth (Karlsruhe, Germany)
Ethidium bromide	Roth (Karlsruhe, Germany)
Glycerin	Roth (Karlsruhe, Germany)

HCl	Roth (Karlsruhe, Germany)
Isoamyl alcohol	Roth (Karlsruhe, Germany)
Isopropanol	Roth (Karlsruhe, Germany)
KOH	Roth (Karlsruhe, Germany)
Maleic acid	Roth (Karlsruhe, Germany)
$MgCl_2$	Roth (Karlsruhe, Germany)
$MgSO_4$ $X7H_2O$	Roth (Karlsruhe, Germany)
Myo-inositol	Duchefa (Haarlem, Netherlands)
2-Mercaptoethanol	Riedel-de Häen (Seelze, Germany)
NaOAc	Riedel de Häen (Seelze, Germany)
NaCl	Roth (Karlsruhe, Germany)
NaOH	Roth (Karlsruhe, Germany)
Peptone	Duchefa (Haarlem, Netherlands)
Primers	Roth (Karlsruhe, Germany)
Primers	Eurofins MWG Operon (Ebersberg, Germany)
PVP40	Roth (Karlsruhe, Germany)
1-Sodium dodecyl sulphate (SDS)	Roth (Karlsruhe, Germany)
Tircarcillin	Duchefa (Haarlem, Netherlands)
Tris-hydroxymethyl-amimomethane	Roth (Karlsruhe, Germany)
Tris-sodiumcitrate-dihydrat	Roth (Karlsruhe, Germany)
Tween	Roth (Karlsruhe, Germany)
Tryptone	Roth (Karlsruhe, Germany)
Yeast extract	Duchefa (Haarlem, Netherlands)

4.1.1.8 Primers

4.1.1.8.1 Primers used in gene cloning and vectors constructs

Primer	**Sequence 5'→3'**	**T_m (°C)**
Myb10-NcoI-for.	AA**TCCATG**GAGGGATATAACGAAAACCT	68
Myb10-Nhel-rev.	AA**TTGCTA**GCTTATTCTTCTTTTGAATGATTCC	
Myb11- NcoI-for.	ATT**TCCATG**GGAAGGAGTCCTTGTTGTTCAA	66
Myb11- XbaI-rev.	ATT**TCTAGA**TTAATTATCTACGAGCCAGCAGTCC	

4.1.1.8.2 Primers used in PCR and Reverse transcriptase PCR and Probes preparation

Primer	**Sequence 5'→3'**	**T_m (°C)**
167nptII-for.	CCACAGTCGATGAATCCAGA	64
367nptII-rev.	AGCACGTACTCGGATGGAAG	
MdMyb10-for.	ATGGAGGGATATAACGAAAAC	62
MdMyb10-rev.	ATGATTCCAAAGGTCCGTGCT	
35S promoter-for.	GTGGATTGATGTGATATCTCC	56 Or 58
Myb11-specific rev.	TTCCAGCTATCAAAGACCATCTG	
35S promoter-for.	GTGGATTGATGTGATATCTCC	58
Myb9-specific rev.	ATTAGTCCTCGGAAACTCTT	

bar sense	GCAGGAACCGCAGGAGTGGA	64
bar antisense	AGC CCG ATG ACA GCG ACCAC	
MdMyb10-for.	ATGGAGGGATATAACGAAAAC	62
PJan-polyA rev.	AGCTAATTACTCATGATCAGGTAC	
Myb11-specific for.	GTTGAACTACCTAAGACCTGAC	62
PJan-polyA rev.	AGCTAATTACTCATGATCAGGTAC	
Myb9-specific for.	CCTTCTTGGTAACAGATGG	62
PJan-polyA rev.	AGCTAATTACTCATGATCAGGTAC	
MdMyb10-for	ATGGAGGGATATAACGAAAAC	62
PGII Poly A rev.	AGA GAG ATA GAT TTG TAG AGA GA	
Myb11-specific for.	GTTGAACTACCTAAGACCTGAC	62
PGII Poly A rev.	AGA GAG ATA GAT TTG TAG AGA GA	
Myb10-Probe-for.	ATTAGACTTCACAGGCTTTTGGGA	60
Myb10-Probe-rev.	TTCTTCCTCTAACTCAATGCTGGG	
Myb9-probe-for.	CCTTCTTGGTAACAGATGG	58
Myb9-Probe-rev.	ATTAGTCCTCGGAAACTCTT	
Myb11-probe-for.	GTGGATTGATGTGATATCTCC	58
Myb11-Probe-rev.	TTCCAGCTATCAAAGACCATCTG	

4.1.1.8.3 Primers used in quantitative Real time PCR experiments

Primer	**Sequence 5'→3'**	**T_m (°C)**			**T_m (°C)**		
		PCR efficiency %			**Correlation Coefficient**		
Rubisco for.	GCTTGTCCAAGAGCAAGAGAAT	58°C	60°C	62°C	58°C	60°C	62°C
Rubisco rev.	CTCCCTCCCCTCAATTATAACC	80.0	95.3	100.3	0.999	0.999	0.999
Myb10-specific for.	GCGTTGAGATTCATGGAGAGG	62°C			62°C		
Myb10-specific rev.	CTAGCAATCAATGACCACCTGTT	94.7			0.999		
Myb11-specific for.	GTTGAACTACCTAAGACCTGAC	60°C			60°C		
Myb11-specific rev.	TTCCAGCTATCAAAGACCATCTG	97.7			0.997		
Myb9-specific for.	CCTTCTTGGTAACAGATGG	58°C			58°C		
Myb9-specific rev.	GTTGTATTCCAGTAATTCTTGATT	93.8			0.998		
RNAPOL II for.	ATATGCCACCCCGTTCTCTACT	58°C	60°C	62°C	58°C	60°C	62°C
RNAPOLII rev.	CACGTTCCATTTGTCCAAACTT	97.5	95.8	90.0	0.998	0.997	0.999

4.1.2 Equipment

Equipment	Manufacturer
Balances	Sartorius (Goettingen, Germany)
Biophotometer	Eppendorf (Hamburg, Germany)
Bio-Rad Gene Pulser Electroporation Porator System	Bio-Rad (California,USA)
CFX96™ Real-Time PCR Detection System	Bio-Rad (California,USA)
Centrifuge Z200 M/H	Hermle (Wehingen,Germany)
Centrifuge Z300	Hermle (Wehingen,Germany)
Centrifuge Z383K	Hermle (Wehingen,Germany)
Christ Alpha 1-4 LSC Freeze dryer	Martin Christ (Osterode, Germany)
Electrophoresis –system Elite 300 plus	Polymer (Germany)
Films	Kodak (Stuttgart, Germany)
Filter paper	Roth (Karlsruhe, Germany)
Gasprofis	WLD Tec (Germany)
Gene pulser Cuvette	Bio-Rad (California,USA)
Hybridization oven	Biometra (Göttingen, Germany)
Ice machine	ZIEGRA (Isernhagen,Germany)
IKA MAG® Surface heat plate	IKA labortechnik (Germany)
Incubator M500	Memmert (Germany)
Incubator III-178/13	Memmert (Germany)
Labcycler Thermoblock 96	Sensoquest (Göttingen, Germany)
Magnetic stirrer	Heidolph (Schwalbach, Germany)
Microwave	Panasonic (Germany)
Minispin centrifuge	Eppendorf (Hamburg, Germany)
NESCOFILM	Alfresa Pharma Coporation (Osaka, Japan
Nylon membrane positively charge	Roche (Grenzach-Wyhlen, Germany)
Refrigerator 4ºC	LIEBHERR (Germany)
PH meter	HANNA (Germany)
Pipette	Eppendorf (Hamburg, Germany)
Rinsed water station	MILLIPORE (Schwalbach, Germany)
RNA free tubes	Roth (Karlsruhe, Germany)
Rotary vane vacuum pump	VACUUBRAND GMBH + CO KG (Wertheim, Germany
Sterile bench Kojar®	Kojart (Finland)
Sterilization filter 0.22µM	Roth (Karlsruhe, Germany)
Surgical blades	Roth (Karlsruhe, Germany)
Thermo cycler PCR	Biometra (Göttingen, Germany)
Thermostat plus	Eppendorf (Hamburg, Germany)
UV-Transilluminator	Intas (Germany)
UV gel Image system	Intas (Germany)
Vacuum pump (~100 mbar)	ABM
Vacuum resistance container	Duran
Varioclave ® Steam sterilizer	H+P Labortechnik (Germany)
Variomag	H+P Labortechnik (Germany)
Vortex Reax Top	Heidolph (Schwalbach, Germany)
Water bath	GFL® (Germany)

Disposable plastic wares: - 2ml microtube-centrifuge - 1.5 ml and 2 ml Eppendorf-caps - Petri dishes - pipette tips	

4.1.3 Bacterial strains and genotype

***Agrobacterium tumefaciens* strain GV3101** (Holsters *et al*, 1980) harbouring the pMP90RK helper plasmid.

***Agrobacterium tumefaciens* strain EHA105** (Hood *et al*. 1993) was co-transformed with the pSoup helper plasmid according to the pGreenII system (pGreen website; Hellens *et al*., 2000).

Agrobacterium tumefaciens EHA105pSoup competent cells used for electroporation was provided by Dr.Fathi Hassan, Plant Biotechnology section, Leibniz Universität Hannover.

***E coli* strain TOP10** (Invitrogen)

Genotype: *F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*

4.1.4 Plasmids used in this study

Two binary vectors were used in this study:

- **PJan** harboring *MdMyb9* (GeneBank: DQ267900), *MdMyb10* (GeneBank: DQ267896) and *MdMyb11* (GeneBank: DQ074463) transcription factor genes, individually. (Fig.6). Those constructs ready to use for transformation experiments were provided by Julian Brüggemann, Ph.D., Chair of Genome Research, Faculty of Biology, Bielefeld University.
- **PGIIMHS35** harboring *MdMyb10, MdMyb11* transcription factor genes, individually. (Fig.5).

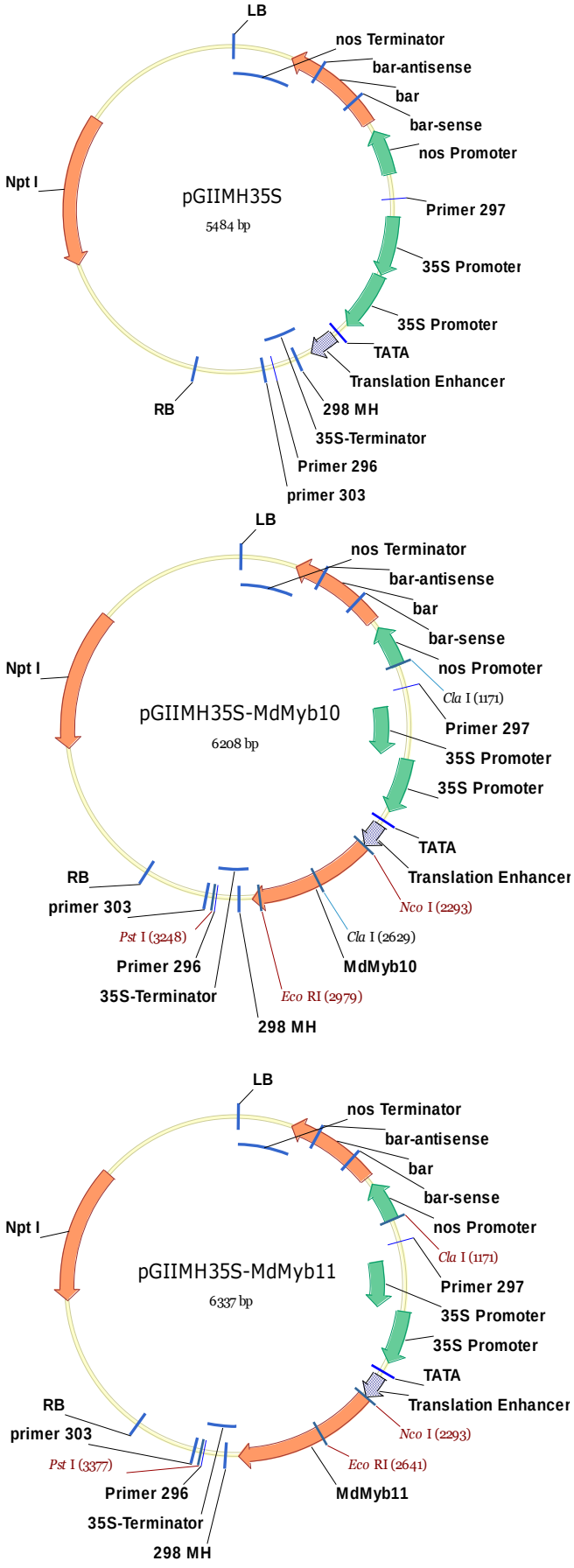
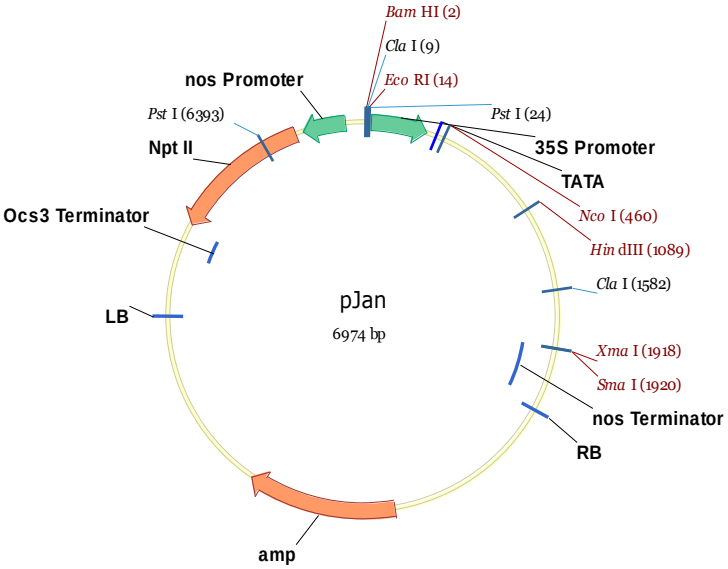
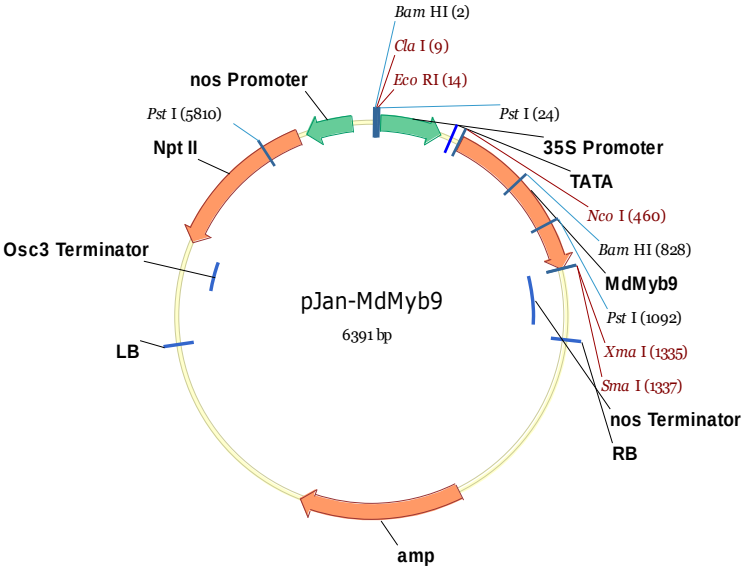
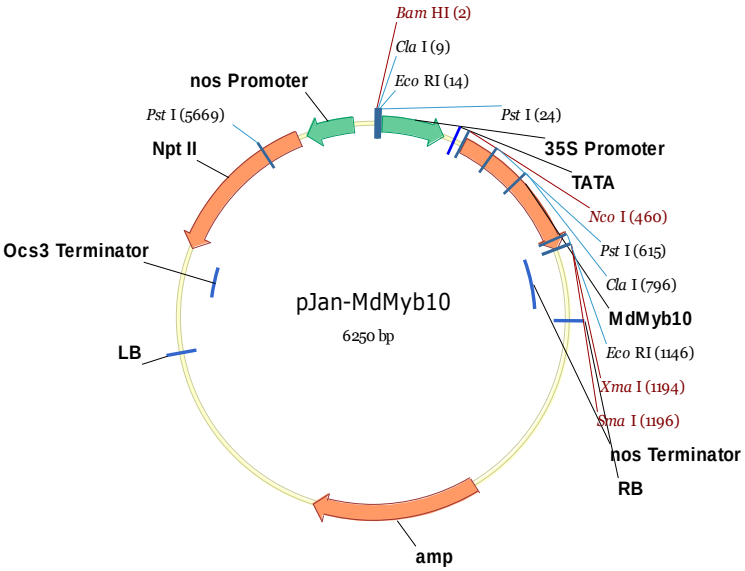


Fig.5. Functional maps of pGIIMH35S, pGIIMH35S-MdMyb10 and pGIIMH35S-MdMyb11 used in gene cloning, as well as in the transformation experiments


Bam HI (2)
Cla I (9)
Eco RI (14)
nos Promoter
Pst I (6393)
Pst I (24)
Npt II
35S Promoter
TATA
Ocs3 Terminator
Nco I (460)
Hin dIII (1089)
Cla I (1582)
pJan
6974 bp
LB
Xma I (1918)
Sma I (1920)
nos Terminator
RB
amp


Bam HI (2)
Cla I (9)
Eco RI (14)
nos Promoter
Pst I (5810)
Pst I (24)
Npt II
35S Promoter
TATA
Osc3 Terminator
Nco I (460)
Bam HI (828)
MdMyb9
pJan-MdMyb9
6391 bp
Pst I (1092)
LB
Xma I (1335)
Sma I (1337)
nos Terminator
RB
amp


Bam HI (2)
Cla I (9)
Eco RI (14)
nos Promoter
Pst I (5669)
Pst I (24)
Npt II
35S Promoter
TATA
Ocs3 Terminator
Nco I (460)
Pst I (615)
Cla I (796)
MdMyb10
pJan-MdMyb10
6250 bp
Eco RI (1146)
LB
Xma I (1194)
Sma I (1196)
nos Terminator
RB
amp

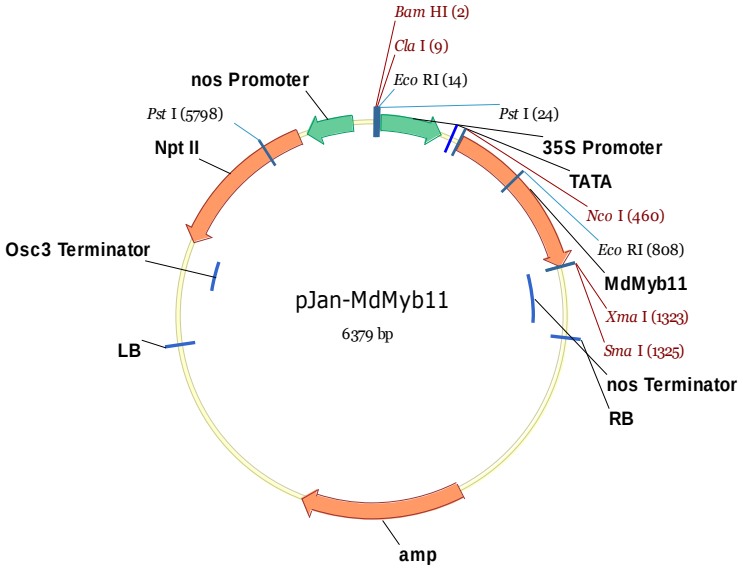


Fig.6. Functional maps of pJan, pJan-MdMyb9, pJan-MdMyb10 and pJan-MdMyb11 used in transformation experiments.

4.1.5 Plant material

As plant material, the following *in vitro*-cultivated shoots of the apple cultivars (*Malus domestica* Borkh.) 'Holsteiner Cox' or 'Gala' were used

4.1.6 Growth media

All media were sterilised by autoclaving at 121ºC, 20 min. If necessary, the media pH was adjusted as required using NaOH or HCl. Appropriate antibiotics were added as required. Media were stored at 4°C in darkness.

4.1.6.1 Bacteria media

Luria-Bertani (LB) broth: 1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. LB plates: solid LB broth medium, 1.5% (w/v) plant agar were added. pH was adjusted to 7.0

SOC medium: 2 % (w/v) tryptone, 0.5% (w/v) yeast extract , 0.5% (w/v) NaCl, 2.5mM KCl, 2.033g/l $MgCl_2$ x6H_2O, 10 mM $MgSO_4$ x7H_2O, 20 mM glucose (filter sterilized added before using). pH was adjusted to 7.0

YEP medium: 1% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl,
for solid medium 1.5% (w/v) agar were added. pH was adjusted to 7.0

4.1.6.2 Basic media for plant tissue culture

4.1.6.2.1 Basic tissue culture media for 'Holsteiner Cox'

Chemicals	Regeneration medium	Elongation medium	Propagation medium	Root induction medium
MS (Murashige and Skoog, 1962) salt mixture	5.3 g/l	5.3 g/l	5.3 g/l	5.3 g/l
Sucrose	-	3%	2%	2%
Sorbitol	3%	-	-	-
Myoinositol	0.1 g/l	-	0.1 g/l	0.1 g/l
IBA	1 µM		-	7 µM
BAP	-	5.3 µM	4.1 µM	
GA_3	-	0.28 µM	2.8 µM	
TDZ	3 µM	-	-	-
Plant Agar	-	0.8%	0.8%	0.7%
Gelrite	0.3 %	-	-	-
pH value	5.8	5.7-5.8	5.7- 5.8	5.7 -5.8
NAA	-		1 µM	-

4.1.6.2.2 Basic tissue culture media for 'Gala'

Chemicals	Regeneration medium	Elongation medium	Propagation medium	Root induction medium
MS (Murashige and Skoog, 1962) salt mixture	5.3 g/l	5.3 g/l	5.3 g/l	5.3 g/l
Sucrose	-	3%	2%	2%
Sorbitol	3%	-	-	-
Myoinositol	0.1 g/l	-	0.1 g/l	0.1 g/l
NAA	2.6µM		1 µM	7 µM
BAP	-	5.3 µM	4.1 µM	
GA_3	-	0.28 µM	2.8 µM	
TDZ	22.7µM	-	-	-
Plant Agar	-	0.8%	0.8%	0.7%
Gelrite	0.3 %	-	-	-
pH value	5.8	5.7-5.8	5.7-5.8	5.7-5.8
IBA	-		-	7 µM

4.2 Methods

4.2.1 Plant *in vitro* tissue culture

Shoot cultures were maintained on propagation medium (see 4.1.6.2.1) and incubated at 24°C under a 16/8-h (day/night) photoperiod. The plants were subcultured every 4-5 weeks.

For regeneration experiments, the four youngest unfolded leaves from 4-weeks-old micropropagated shoots were used as explants. Leaves were cut to strips, and only the middle part was used 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 2-4 weeks elongation, the shoots were transferred to propagation medium. 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 are given in %.
- The number of shoots per explant. The average number was identified and only explants with shoot regeneration were taken into consideration.

4.2.2 Root induction

Regenerated shoots (25-30 mm) were cultivated on rooting medium (see 4.1.6.2.1) to induce root formation and incubated at 25°C under a 16/8-h (day/night) photoperiod.

4.2.3 Transfer to soil (acclimatization)

The plantlets obtained *in vitro* were carefully washed under running water and set in pots filled with a mixture of peat moss and perlite (2:1v/v), covered with plastic bags and acclimatized gradually to greenhouse conditions throughout 4 weeks.

4.2.4 Plasmid construction and cloning

In order to improve the selection system, another marker gene (*bar*) replaced the marker gene *Npt* II, new constructs were developed as the following:

4.2.4.1 Preparation of *MdMyb10* and *MdMyb11* fragments (amplification)

In the Binary vectors PGIIMHS35, The T-DNA contains the *bar* gene fused between the nos promoter and terminator sequences of *Agrobacterium tumefaciens*. The *bar* gene encodes a phosphinothricin acetyltransferase (PAT) enzyme which confers resistance to bialaphos and

the related compounds phosphinothricin (PPT), the active ingredient of the herbicide BASTA® and glufosinate ammonium through acetylation.

The *MdMyb10* and MdMyb11 genes were cloned into the Ti-plasmid using PCR; the *MdMyb10* gene was amplified using two cloning primers Myb10-NcoI for., Myb10-Nhel-rev. flanking *Nco*I and *Nhe*I restriction sites, respectively (underlined, see 4.1.1.8.1) to the PCR products.

The *MdMyb11* gene was amplified using two cloning primers MYB11 –NcoI-for., MYB11–Xbal-rev. flanking *Nco*I and *Xba*I restriction sites, respectively (underlined, see 4.1.1.8.1) to the PCR products.

The PCR was performed using **High Fidelity PCR Enzyme Mix** (Fermentas, Germany). The template for the PCR were pJAN-MdMyb10, pJAN-MdMyb11.

The PCR mixture was prepared according to the manufacturer's protocol as follows:

4.2.4.1.1PCR Mixture

Reagent	**Quantity for 50µl**	**Final concentration**
Water, nuclease-free	variable	—
10 x High Fidelity PCR Buffer with $MgCl_2$	5 µl	1X
2 mM dNTP mix	5 µl	0.2 mM of each
Primer I	variable	0.2-1 µM
Primer II	variable	0.2-1 µM
Template DNA: Plasmids Genomic DNA	variable	 0.01-1 ng / 50 µl 0.1-1 µg / 50 µl
High Fidelity PCR Enzyme Mix	0.25-0.5 µl	1.25-2.5 u / 50 µl
Total volume	to 50 µl	

The PCR products were purified directly using **GeneJET™ Gel Extraction Kit** (Fermentas, Germany).

4.2.4.1.2 PCR Program

Segment	**Temperature**	**Duration**	**Number of cycles**
Initial denaturation	94°C	1-3 min	1
Denaturation	94-95°C	20- 60 s	25- 35
Annealing	Primer Ta	30- 60 s	
Elongation	72°C	1 min/kb PCR target	
Final elongation	72°C	10 min	1

4.2.4.2 Digestion of DNA by restriction endonucleases

DNA (plasmids, PCR products) was digested using different restriction endonucleases with

respective buffers as recommended by the supplier. When two enzymes had to be used for digest, the buffer was selected to be suitable for both enzymes; digestion was done at 37 °C for 2 h or overnight, and then enzymes were heat-inactivated for 15-20 min at 65 °C.

4.2.4.2.1 Compositions of DNA restriction digest reaction

Compound	**Amount per reaction**	**Compound**	**Amount per reaction**
Plasmid DNA (PGIIMHS35)	X µl (0.2-0.5µg)	PCR products *MdMyb10* or *MdMyb11* genes	32 µl
Enzyme1 (*Nco*I)	1 µl	Enzyme1(*Nco*I)	2 µl
Enzyme2 (*Xba*I)	1 µl	Enzyme2 (*Xba*I)	2 µl
H_2O	X µl	H_2O	---
10x buffer Tango	2.5 µl	10x buffer Tango	4 µl
Total volume	25 µl	Total volume	40 µl

4.2.4.3 Dephosphorylation of 5'-ends of digested vector DNA

Shrimp alkaline phosphate (SAP) was used for dephosphorylation of the 5'-ends of the digested vector to prevent re-ligation of the vector with the excised fragment as adapter. Dephosphorylation was done according to the manufacturer's protocol at 37°C for 1 h., and then the enzyme was heat-inactivated at 65°C for 15 min.

4.2.4.4 Ligation

DNA ligase catalyzes the phosphodiester binding between a free 5'-phosphate group and a free 3'-hydroxyl group of the same strands of a dsDNA. Intramolecular ligation results in a circularization of the DNA molecule. If an insertion is planned, self-circularization and oligomerization has to be prevented by dephosphorylation or eluting the fragment from the gel. Ligation of cohesive ends and the vector was done using **Rapid DNA Ligation Kit** (Fermentas, Germany), at a molar ratio of 3:1 in 5x ligation buffer, so 150 ng insert and 50 ng vector were mixed and 2U of T4 DNA ligase were added. The reaction was incubated at 22°C overnight, and then the ligase was heat-inactivated at 65°C for 15 min. Afterwards the ligation product was monitored by running on a gel to check the efficiency of ligation and then used for *E.coli* transformation.

4.2.4.4.1 Composition of ligation reaction

Compound	**Amount per reaction**
Restricted digested plasmid DNA (pGIIMHS35)	5 µl = 50ng
Restricted digested PCR products (*MdMyb10* or *MdMyb11* genes)	15 µl =150ng
Enzyme (T4 ligase)	2.5 µl

5 x T4 ligase buffer	2.5 µl
Total volume	25 µl

The plasmid integrity was checked by sequencing of *E.coli* derived plasmids after re-transformation.

4.2.4.5 Preparation of competent E. coli cells for heat shock transformation (Nakata et al., 1997 and Tang et al., 1994)

The required *E. coli* strains (Top10) were grown overnight in 1-5 ml of LB medium at 37 °C (without antibiotics) to stationary phase. The overnight culture was diluted in fresh LB 1:50 and grown at 37 °C until OD_{600} reached ~ 0.4. The cells were harvested by centrifugation at 4 °C, 4400 rpm, and re-suspended in 1/2 volume ice-cold 100 mM $CaCl_2$ and centrifuged again. The supernatant was discarded and the pellet was resuspended in 1/2 volume ice-cold 100 mM $CaCl_2$. Pelleted cells were re-suspended in 1/10 volume cold 100 mM $CaCl_2$ and incubated on ice for 1 hour and used immediately for heat shock transformation. Alternatively, 86 % sterile glycerol was added to a final concentration of 15 % and then aliquots of 100 µl in 1.5 ml tubes, which were put immediately in liquid nitrogen and stored at -80 °C for long-term storage.

4.2.4.6 Heat shock/calcium chloride method for *E. coli* transformation

Competent *E. coli* were transformed by the freeze-thaw method. A 10 µl aliquot of plasmid DNA or ligation reaction mixture was added to 50 µl thawed cells in a 1.5 ml tube and gently mixed. The cells were incubated on ice for 10 min before heat-shock at 42ºC for 45-60 sec in a water bath, followed by a further 3 min on ice. 500 µl of LB medium without antibiotics were added to develop antibiotic resistance and to reduce damage of *E.coli* cells. Finally, the tubes were incubated on a shaker at 240 rpm for 60 min at 37 °C. 200 µl of the resulting culture was spread on LB plates with the appropriate antibiotic added (kanamycin) and grown overnight at 37°C. The colonies were picked about 12-16 hours later.

4.2.4.7 Transformation of *Agrobacterium* through electroporation

Binary vectors were transferred into *Agrobacterium tumefaciens* (EHA105-pSoup) by electroporation. Competent cells (50 µl aliquots) were taken out from -80 °C freezer and were thawed on ice and plasmid DNA 50-200 ng in (1-5 µl)water was added, gently mixed and pipetted into a pre-cooled electroporation cuvette (0.2 cm gap, Bio-Rad, USA). The cuvette was placed in a GenePulser (Bio-Rad, USA) and electroporation was carried out at a voltage of 2.5 kV (capacitance 25 µFd, resistance: 200 Ohms) the field strength was between 6.25 – 12 kVwith a typical pulse time of 7-9 ms. The cells were recovered by addition of

500-1000 µl of pre-cooled SOC medium (without antibiotics), then transferred to 2 ml tubes and incubated at room temperature, with shaking (240rpm) at 28°C for 3 h. 200 µl of the transformed bacteria were spread onto separate LB plates containing the appropriate antibiotics. Plates were grown at 28°C; the colonies were picked about 2-3 days later.

4.2.4.8 Agrobacterium inoculation and harvesting

25 ml YEP medium in 100 ml Erlenmeyer flask including appropriate antibiotics for the respective plasmid (50 mg/l kanamycin for pGIIMH35S–MdMyb10 and pGIIMH35S – MdMyb11) were inoculated with 250 µl glycerol stock of *Agrobacterium tumefaciens*. The medium was inoculated and placed on a shaker at 240 rpm, at 28°C in the dark for 15 h. Bacteria were harvested by centrifugation at 4400 rpm, the supernatant was discarded and then the pellet was re-dissolved in liquid MS medium liquid, OD_{600} was measured using a photometer and adjusted to 1-1.3, and supplemented with the appropriate plant growth regulators, (depending on the used cultivar for transformation).

4.2.5 Transformation using the vector pJAN harboring *MdMyb9, MdMyb10, MdMyb11* transcription factor genes

The four youngest unfolded leaves from 4-weeks-old micropropagated shoots were used as explants. Leaves were cut to strips, and only the middle part was used for transformation.

For inoculation, 250 µl *Agrobacterium tumefaciens* from a glycerol stock were grown overnight (28ºC, 240 rpm) in 25 ml YEP medium (See 4.1.6.1) supplemented with 50 mg/l ampicillin and 50 mg/l rifampicin, pelleted (12 min, 4400 rpm) and resuspended in liquid MS medium to an OD_{600} of 0.8.

The explants were shaken gently in the bacterial solution for 15 min, blotted dry and cocultivated in the dark on shoot regeneration medium (See 4.1.6.2.1; 4.1.6.2.2).

After 3 days of coculture, the leaves were washed twice in water and once in liquid MS-medium supplemented with 150 mg/l ticarcillin and 50 mg/l kanamycin.

Explants were transferred with their adaxial side in contact with the shoot regeneration medium (same as for co-culture) supplemented with 150 mg/l ticarcillin for killing the agrobacteria, and 50 mg/l kanamycin. Subcultivation of the leaves was done in the dark for 2 weeks at 24ºC, then under a 16/8-h (day/night) photoperiod at the same temperature. The explants were transferred to fresh medium every 2 weeks. After 6 weeks on shoot-regeneration medium, regenerated shoots were transferred to elongation medium (see 4.1.6.2.2) supplemented with 150 mg/l ticarcillin and 50 mg/l kanamycin.

Putative transgenic shoots of ‘Gala’ and ‘Holsteiner Cox’ were micropropagated on

propagation medium (see 4.1.6.2.2) supplemented with 150 mg/l ticarcillin and 50 mg/l kanamycin as selection reagent for transgenic plants.

4.2.6 Transformation using the vector pGIIMH35S harboring *MdMyb10, MdMyb11* transcription factor genes

The explants were prepared as the same as used before.

For inoculation, 250 µl *Agrobacterium tumefaciens* from a glycerol stock were grown overnight (28ºC, 240 rpm) in 25 ml YEP medium supplemented with 50 mg/l kanamycin, pelleted (12 min, 4400 rpm) and resuspended in liquid MS medium to an OD_{600} of 0.8. The explants were shaken gently in the bacterial suspension for 15 min, blotted dry and cocultivated in the dark on shoot regeneration medium. The medium was the same as used before supplemented with 2.5 mg/l phosphinothricin (PPT).

After 3 days of coculture, the leaves were washed twice in water and once in liquid MS-medium supplemented with 150 mg/l ticarcillin and 2.5 mg/l phosphinothricin (PPT). Explants transferred with their adaxial side in contact with shoot regeneration medium (same as for co-culture) supplemented with 150 mg/l ticarcillin for killing the bacteria, 150 mg/l cefatoxime and 2.5 mg/l phosphinothricin (PPT). Subcultivation of the leaves was in the dark for 2 weeks at 24ºC, then under a 16/8-h (day/night) photoperiod at the same temperature.

The explants were transferred to fresh medium every 2 weeks; the concentration of phosphinothricin (PPT) was increased from 2.5 mg/l till 10mg/l during the work.

After 6 weeks on shoot regeneration medium, regenerated shoots were transferred to elongation medium (see 4.1.6.2.2), supplemented with 150 mg/l ticarcillin, 150 mg/l cefatoxime and 10 mg/l phosphinothricin (PPT). Shoots were transferred to the propagation medium (with 150 mg/l ticarcillin, 150 mg/l cefatoxime and 10 mg/l phosphinothricin (PPT) as selection reagent for transgenic plants).

4.2.7 Molecular biology methods

4.2.7.1 Agarose gel electrophoresis

Electrophoresis is used to separate molecules (DNA and RNA) based on their size. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field. In agarose gel electrophoresis, the DNA is forced to move through a sieve of molecular pores made by agarose. Large fragments of DNA move slower than small fragments of DNA. So the concentration of the gel depends on the fragment lengths to be separated. 0.8-1 % (w/v) agarose gel was prepared in 1x TAE buffer, where it melts in a microwave oven until the

agarose was totally dissolved. Then the agarose solution was cooled down until it reached 50°C and ethidium bromide (0.5 µg/ml) was added and the solution was casted into a gel mold to solidify. A suitable comb was used to make slots. The gel was transferred to the electrophoresis chamber containing running buffer (1x TAE buffer). Samples were mixed with 6x loading buffer and loaded together with molecular weight marker onto the wells for electrophoresis at a voltage of 60-100 V for 30-40 min after which the DNA fragments were observed and photographed under UV-light.

Buffers

6x loading buffer ready to use ((Fermentas, Germany)

TAE buffer (40 mM Tris-acetate, 20 mM glacial acetic acid, 1 mM EDTA), pH 7.5

4.2.7.2 Isolation of plasmid DNA from *E. coli* or *A. tumefaciens*

Plasmids of *E. coli* or *Agrobacterium tumefaciens* were isolated either with the **GENEJET™ Plasmid Miniprep Kit** (Fermentas, Germany) 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 for *E. coli* or 28°C for *Agrobacterium tumefaciens* in 25 mL LB medium supplemented with appropriate antibiotics. Two ml were centrifuged at 12000 rpm for 10 min. The obtained pellets were 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 into new tubes and 600 µl isopropanol were 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 50µL TE buffer or distilled water with 1 µl RNaseA (1 mg/ml). The isolated plasmid was stored at -20°C.

Plasmid isolation buffers:

Buffer A: 15 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose, 2 mg/ml fresh lysozyme.

Buffer B: 0.2 M NaOH, 1% SDS

Buffer C: 3M NaOAc PH 4.8

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

4.2.7.3 Isolation of total DNA from plants

Genomic DNA was isolated from apple using either the **DNeasy Plant Mini Kit** (Qiagen, Germany) according to the manufacturer's instructions, or the following method(Doyle and and Doyle, 1990)

1-1.5 g plant material was harvested and shock frozen in liquid nitrogen. The material was grinded as fine as possible in liquid nitrogen. 5 ml preheated CTAB solution and 2% mercaptoethanol were added in a 50 ml tube, which contained the grinded plant material. The tube was vortexed vigorously and incubated for 30 min in a preheated water bath at 65°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 4400 rpm for 10 min. The aqueous upper phase was carefully taken and pipetted to a new 50 ml tube. 2/3 volume of cold 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 for 20 min, and thereafter the tube was centrifuged at 4400 rpm for 5min, after 5-10 min drying, the pellet was dissolved in 3 ml TE buffer with 50 µg/ml RNAse. Incubation for 30 min at 37°C should remove remaining RNA. The precipitation was carried out by addition of 1/2 volume of 7.5 M-NH_4 acetate (1.5ml) and 2.5 volume absolute ethanol (7.5ml) and centrifuged at 4400 rpm for 10-20 min. The pellets were dried at 37°C for 1-1.5 hours and thereafter dissolved in 500 µl TE buffer or distillated water.

Buffers and solvents used for the DNA-Isolation

CTAB: 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

CI- mix: 24 ml chloroform, 1 ml iso-amylalcohol

DNA washing buffer: 76% (v/v) ethanol, 10 mM ammoniumacetate, dd 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

4.2.7.4 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 absorbance of the DNA preparations was determined at 260 nm and 280 nm where:

1O.D. at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA

1O.D. at 260 nm for single-stranded DNA = 20-33 ng/µl of ssDNA(cDNA)

1O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

The quotient OD_{260}/OD_{280} gives the level of DNA purity. If there is contaminating protein the OD ratio between 260 and 280 nm decreases. Pure DNA has an OD_{260}/OD_{280} between 1.8 and 2.0. If this quotient is below 1.8, it indicates a protein contamination. The respective RNA-concentration was calculated as follows:

RNA concentration µg/ml = (OD_{260} x dilution factor x 40), the purity of the RNA was determined using the ratio of OD_{260}/OD_{280}, which should be between 1.9 and 2.0 for pure RNA. All measurements were accomplished in UVette® (Eppendorf), which is light transparent in the range of 220 nm to 1600 nm.

4.2.7.5 Polymerase chain reactions (PCR)

All regenerated plants were analysed by PCR. The PCR reaction contains 1U (0.2 µl of *Taq* polymerase (Promega, Germany), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.4 µM of each primer in a total volume of 25 µl.

4.2.7.5.1 PCR Mixture

Compound and concentration	**Amount per reaction**
H_2O	13.3 µl
5*buffer (GoTaq-Promega)	5 µl
dNTP (50 mM)	1 µl
Primer For (10 pMole)	1 µl
Primer Reverse (10 pMole)	1 µl
Taq polymerase (1 µ/ml)	0.2 µl
Template (50-100 ng)	1 µl
25mM $MgCL_2$(1-4mM)	2.5
Total volume	25 µl

4.2.7.5.2 PCR program

PCR step	**Temperature (°C)**	**Time (s)**	**No. of cycles**
Initial denaturation	94	240	1
Denaturation	94	60	30-35
Annealing	Specific for primer	Specific for primer	30-35
Extension	72	30	30-35
Final extension	72	600	1
Cooling down	10 or 4	-	1

4.2.7.6 Southern blot using non-radioactive probe

4.2.7.6.1 Buffers and solutions

Pre-hybridization solution: Dig Easy Hyb. (Roche Diagnostics, Germany)

Hybridization solution: 45 µl probe + 33 ml Dig Easy Hyb.

Blocking Solution: 1 % blocking solution (Roche) in maleic acid buffer.

Antibody solution: (Anti-Digoxigenin-alkaline phosphatase conjugate Fab Fragments) (Roche Diagnostics) 1:20000 in 1% blocking solution.

Depurinizing solution: 0.25 M HCl

Denaturation Solution: 0.5 M NaOH, 1.5 M NaCl

Neutralization Solution pH 7.5: 0.5 M Tris-HCl, 3 M NaCl

10 % SDS (Filter sterilized) (Sodium dodecyl sulfate (or sulphate) (SDS or NaDS) ($C_{12}H_{25}NaO_4S$)

Maleic acid buffer pH 7.5: 0.1 M maleic acid, 0.15 M NaCl

Detection buffer pH 9.5: 100 mM Tris-HCl, 100 mM NaCl

20x SSC buffer pH 7: 3 M NaCl, 0.3 M Sodium citrate

Washing buffer: Maleic acid buffer, 0.3 % Tween 20 (do not autoclave)

Stripping buffer: 0.2 M NaOH, 0.1 % SDS

Developing solution: 1: 3.5 dilution of Tetenal Roentogen developer

Fixation solution: 1:4 dilution of Tetenal Roentogen Superfix

The blot was performed according to Southern (1975) to confirm integration patterns of T-DNA and to determine the copy number of the integrated transgenes using the genomic DNA of transformed plants. Genomic DNA was isolated from transgenic and non-transgenic plants by large-scale DNA preparation. Non radioactive detection methods were used and DIG labelled PCR products for the different genes were prepared as probe (as described below).

4.2.7.6.2 Production of DIG labelled probes

DIG labelled probes were produced by PCR with the substances listed in (see 4.2.7.6.2.1), 5µl PCR reaction mix was tested by gel electrophoresis to confirm the successful labelling. Compared to the control, the probe was larger and moved slower in the gel because of the DIG labelling. For labeling PCR products, **DIG Probe Synthesis Kit** (Roche, Germany) was used; the PCR mixtures for probe synthesis and the control were prepared according to the manufacturer's protocol as follows:

4.2.7.6.2.1 PCR Mixture for probe preparation

Reagent	Volume required for		Final concentration
	control	probe	
Water, dd H_2O	add up to 50 µl	add up to50 µl	
PCR buffer with $MgCl_2$ 10× conc.	5µl	5µl	
dNTP stock solution	5µl	---	200 µM each dNTP
PCR DIG Probe Synthesis Mix	---	5µl	200 µM dATP, dCTP, dGTP, 130 µM dTTP, 70 µM DIG-dUTP
Forward PCR primer, 10 µM	5µl	5µl	0.1 - 1 µM
Reverse PCR primer, 10 µM	5µl	5µl	0.1 - 1 µM
Enzyme mix	0.75µl	0.75µl	
Template DNA	variable	variable	10 - 100 pg plasmid DNA
Total volume	50 µl	50 µl	

4.2.7.6.3 Restriction digests of genomic DNA for Southern blot

20-30 µg of gDNA were digested by *Eco*RI or *Bam*H1 in the respective buffer at 37°C overnight. A second amount of the enzyme was added, tubes were incubated for further 3-4 h to ensure complete digest, followed by heat inactivation for 10-15 min at 65°C.

4.2.7.6.4 Precipitation of the digest

In order to precipitate the digest, 1 volume of 7.5 M NH_4-acetate (100 µl) and 2.5 volume of EtOH abs. were added and gently mixed, followed by centrifugation at full-speed (14400 rpm) in a lab centrifuge for 10 min at room-temperature. Supernatants were discarded completely and the pellet was dried for 1 h at 37°C and finally re-dissolved in 40µl TE buffer at 4°C overnight.

4.2.7.6.5 Electrophoresis

8µl of 6x loading buffer were added to the restriction digest (40 µl), mixed and briefly centrifuged. Then samples, DIG-labelled-DNA Molecular Weight Marker II (Roche) and 10 µl positive control (plasmid DNA) were loaded on a 1% agarose gel containing 0.5 µg/ml EtBr in 1x TAE buffer. The gel was run overnight at 0.6 V/cm (20-30 V). The next day, the gel was monitored under a UV-transilluminator, and then rinsed in ddH_2O, followed by submerging in 250 ml of depurinizing sol. for 7 min to nick the DNA and thereby facilitating the transfer of large fragments. After that, the gel was rinsed in ddH_2O twice to remove the acid followed by submerging in denaturation sol. for 2x15 min at RT on a shaker. The gel was rinsed in ddH_2O and then neutralized in neutralization sol. for 2x15 min at RT, followed by submerging in 250ml of 20xSSC for 10min.

4.2.7.6.6 Capillary Southern-transfer (overnight)

20x SSC solutions were placed in a tray where filter paper bridges were built on a glass plate, and 3 filter papers were soaked in 20 x SSC solutions and then placed on the top of the bridge (avoiding any air bubbles under the paper). A plastic wrap was placed in between to prevent by-pass between filter papers that were placed on top of the gel and the filter papers under the gel. A piece of positively charged nylon membrane (Roche) was first wetted in ddH_2O, then in 20xSSC and placed on top of the gel. Another 3 filter papers were soaked in 20 x SSC solutions, and then placed on the membrane to avoid air bubbles.

Tissue papers stacks were loaded onto the filter papers and a glass plate centered on top of the paper towels. 500 ml bottle full of water was placed in the center of the glass plate to distribute the weight evenly across the gel, papers and membrane. Transfer by capillary form would take place over night. When the transfer was completed, the membrane was rinsed 3 times in 2 x SSC and then air dried. The membrane was placed between two filter papers for 30 min at 120°C in the oven, then covered with foil and stored at RT.

4.2.7.6.7 Pre-hybridization and hybridization

The dry blot was placed in an autoclaved hybridization tube and 30 ml of pre-hybridization solution was added and incubated for 3 h at 42°C, and then the pre- denaturized probe (95°C for 5 min and cooled at 4°C) was added (for the first use), or a preheated (68°C) probe was added and incubated overnight at 42°C.

The membrane was washed as follow: 2 x 10 min in 2 x SSC + 0.1 % SDS at 42°C, then 1 x 15 min in preheated (65°C) 0.5 x SSC + 0.1 % SDS at 65°C, followed by 1 x 15 min in 0.1 x SSC + 0.1 % SDS at 65°C, and then 1 min in maleic acid buffer at RT, then the membrane incubated in blocking solution for 30 min followed by incubation with antibody solution for 30 min. Afterwards the blot was rinsed in washing buffer for 2 x 15 min at RT, and equilibrated for 3-5 min in detection buffer.

4.2.7.6.8 Non-radioactive detection

500 µl CDP star ready to use (Roche, Germany) were dropped by pipetting on a wrap foil. The membrane was removed from the detection buffer, transferred immediately onto the substrate, and incubated for 5 min at RT. The excess substrate was removed and Biomax-Luminescence-film (Kodak) was laid on the membrane and incubated for 30-120 min and then developed.

4.2.7.6.9 Stripping of the membrane

After usage, the membrane can be stored in 2x SSC buffer for a second hybridization. The membrane was rinsed in sterile H_2O and incubated twice for 15 min in stripping buffer at

65°C in the hybridization tube followed by rinsing in ddH_2O. The membrane could be stored in 2x SSC buffer at 4°C.

4.2.7.7 Reverse transcriptase – PCR:

4.2.7.7.1 Isolation of RNA:

Total RNA was isolated using **Pure Link™ Plant RNA Reagent** (Invitrogen, Paisley, Scotland) from 100 mg of young leaves harvested from transgenic lines and control 'Holsteiner Cox' and 'Gala' plants, as the following:

0.5 ml of cold (+4°C) Plant RNA Reagent were added to 100 mg of frozen, ground tissue. Mix by brief vortex or flicking the bottom of the tube until the sample is thoroughly re-suspended. The tubes were incubated for 5 minutes at room temperature. **Note**: Lay the tube down horizontally to maximize surface area during RNA extraction. The solution was clarified by centrifuging for 2 minutes at 13000 rpm in a microcentrifuge at room temperature. The supernatant was transfered to an RNase-free tube (Roth, Germany). 0.1 ml of 5 M NaCl was added to the clarified extract and tap tube to mix, and then 0.3 ml of chloroform were added, tubes were mixed thoroughly by inversion. The samples were centrifuged at +4°C for 10 minutes at 13000 rpm to separate the phases; the aqueous phase was transferd to an RNase-free tube. An equal volume of isopropyl alcohol was added to the aqueous phase, and then tubes were mixed and were left at room temperature for 10 minutes. The samples were centrifuged at +4°C for 10 minutes at 13000 rpm, the supernatants were decanted, and taking care not to lose the pellet and 1 ml of 75% ethanol were added to the pellet. **Note**: pellet may be difficult to see. The samples were centrifuged at room temperature for 1 minute at 13000 rpm. The liquid was decanted carefully again, taking care not to lose the pellet. Briefly centrifuge to collect the residual liquid and remove it with a pipette. To dissolve the RNA 10-30 µl RNase-free water were added, Pipetted up and down over the pellet to dissolve the RNA. RNA was stored at -70°C.

4.2.7.7.2 cDNA synthesis:

2 µg of total isolated RNA was treated with *DNaseI* (Fermentas, Germany) to eliminate genomic DNA contaminations, as the following:

2 µg RNA
2 µl DNAse buffer
2 µl DNAse (2U)
0.5 µl RNAse inhibitor

Incubate for 30 min at 37°C then add 2 µl EDTA to prevent RNA degradation.

Incubate for 10 min at 65°C to stop the reaction.

The treated RNA was reversed transcribed using of **RevertAid™ H Minus First Strand cDNA Synthesis Kit** (Fermentas, Germany), as the following:

Total treated RNA

1 µl oligo$(dT)_{18}$ primers

Complete the volume up to12 µl by using DEPC water.

Incubated for 10 min at 70°C, followed by a reverse transcriptase reaction prepared on ice as follows:

4 µl 5 x reaction buffer

1 µl RNAse inhibitor (40 U/µl)

2 µl dNTPs (5mM)

The mixture was incubated for 5 min at 37°C in a thermo block, and then 1 µl reverse transcriptase polymerase (200 U/µl) was added and incubated for 1 hour at 42°C followed by 10 min inactivation at 70°C, cDNA was used directly or stored at -20°C while RNA was stored at -80°C.

To screen for DNA contaminations, specific primers for the apple gene *M.domestica* ribulose-1, 5-bisphosphate carboxylase/oxygenase activase mRNA**, (**GenBank: Z21794) http://www.ncbi.nlm.nih.gov/nuccore/415851 were used.

The success of reverse transcription was tested by PCR with 1 µl of cDNA and the primers Rubisco forw and Rubisco rev as described.

4.2.7.7.3 Reverse transcriptase – PCR conditions:

Total RNA was isolated from non-transgenic'Holsteiner' Cox and 'Gala' plants, as well as from all transgenic lines. The cDNA was synthesized, and tested by using the primers Rubisco forw. and Rubisco rev. as described.

The PCR reaction contains 1U (0.2 µl) of *Taq* polymerase (Promega, Germany), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 µM of each primer in a total volume of 25 µl. Two pairs of primers were used, one to test the success of reverse transcription (Rubisco for. and Rubisco rev.), the second to confirm transcription of the transgenes (depending on the used gene in the transformation).

4.2.7.7.3.1 PCR Mixture for RT-PCR

Compound and concentration	Amount per reaction
H_2O	13.3 µl
5X buffer (GoTaq-Promega)	5 µl
dNTP (50 mM)	1 µl
Primer For (10 pMole)	0.5 µl
Primer Reverse (10 pMole)	0.5 µl
Primer For 2 (10 pMole)	0.5 µl
Primer Reverse2 (10 pMole)	0.5 µl
Taq polymerase (1µ/ml)	0.2 µl
Template cDNA (50-100 ng)	1 µl
25mM $MgCL_2$(1-4mM)	2.5 µl
Total volume	25 µl

4.2.7.8 Real time (qPCR) expression analysis

Real time or quantitative PCR (qPCR) was performed on cDNA isolated from apple to detect relative transcript expression levels of both native genes and transgenes. The primer sets used for each gene are listed in (see 4.1.1.8.3). Gene specific primers, corresponding to these genes were designed using Vector NTI version11 (www.invitrogen.com) to a stringent set of criteria, including a T_m of between 58ºC and 62ºC, an amplicon length between 100 and 200 bp, avoidance of GC clamp at 3′ end and limited predicted secondary structure or dimer formation. Adherence to these criteria enabled the application of universal reaction conditions. Real-time amplifications were run in triplicate on 96-well reaction plates "low Profile" (kisker, Germany), reactions were prepared in a total volume of 15 µl containing: 1 µl cDNA, 0.3 µl of each 10 pMole primer (200 mM each), 7.5µl of IQ™ SYBR® Green Supermix (Bio-Rad, USA), 5.9µl RNase/DNase-free sterile water (Qiagen, Germany). All the samples and blank controls were run in triplicate for each master mix.

Amplification and analysis by qPCR were carried out using CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). All reactions were performed with a thermal profile as follows: initial template denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 sec, and combined primer annealing/elongation at three difference temperature(58,60, 62°C) depending on the used primers for 20 sec, extension at 72°C for 30 sec, then one cycle at 95°C for 10 sec. This cycle was followed by a melting curve analysis, ranging from 65°C to 95°C, with temperature increasing by steps of 0.5°C every 10 sec. A negative control with no cDNA template (NTC) was included in each run. Fluorescence was measured at the end of each annealing step.

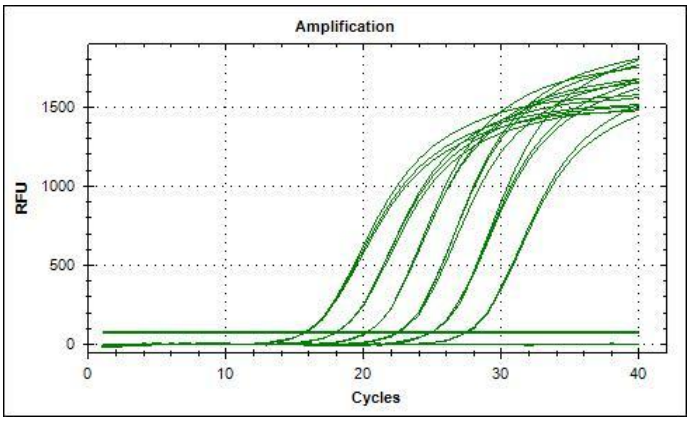
The raw data were analysed with the CFX software version 1.5 and expression was

normalized to two reference genes, the apple gene *M.domestica* ribulose-1, 5-bisphosphate carboxylase/oxygenase activase mRNA, **(**GenBank: Z21794) http://www.ncbi.nlm.nih.gov/nuccore/415851 and mRNA of the *M. domestica* cDNA clone Mdfw2033f21.y1 (similar to the RNA polymerase subunit II), (GeneBank : CN579456) http://www.ncbi.nlm.nih.gov/nucest/46991006.

a

	1	2	3	4	5	6	7	8
A				Std-1 1,00E+06	Std-1 1,00E+06	Std-1 1,00E+06		
B				Std-2 2,00E+05	Std-2 2,00E+05	Std-2 2,00E+05		
C				Std-3 4,00E+04	Std-3 4,00E+04	Std-3 4,00E+04		
D				Std-4 8,00E+03	Std-4 8,00E+03	Std-4 8,00E+03		
E				Std-5 1,60E+03	Std-5 1,60E+03	Std-5 1,60E+03		
F				Std-6 3,20E+02	Std-6 3,20E+02	Std-6 3,20E+02		
G								
H				NTC-1 SYBR	NTC-1 SYBR	NTC-1 SYBR		

b



c

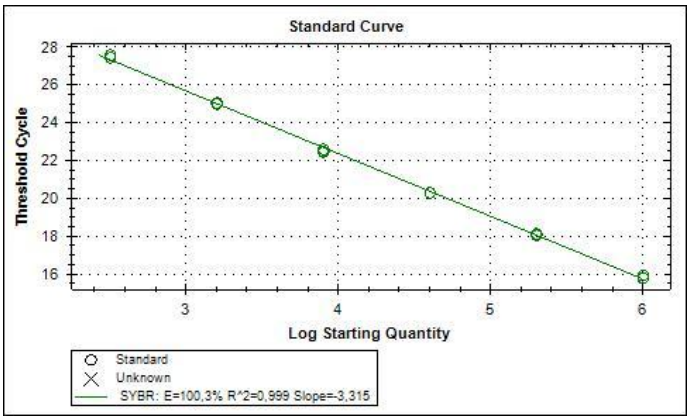


Fig.7. Serial dilutions of cDNA template used to generate a standard curve representing primer efficiency in CFX software version 1.5. (a) Serial of used dilutions (b) Representative example for MdMyb10 shows a 5-fold dilution series over 6 orders of magnitude. Efficient amplification of the template is seen at all six dilutions over the 40 cycle PCR. (c) Raw qPCR data was used to construct a curve representing the efficiency of the primer pair to amplify the target DNA.

For each gene, a standard curve was generated with a cDNA serial dilution (1:5) (Fig.7), and

the resultant PCR efficiency calculations (80% - 100%) were imported into relative expression data analysis, the relative expression ratio of a target gene is calculated on the reaction efficiency (E) and the crossing point deviation of the target gene and reference gene, normalised to the calibrator sample.

PCR products were sequenced to confirm authenticity.

4.2.7.9 HPLC analysis

A Bruker Daltonics esquire 3000plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and equipped with a quaternary pump and a variable wavelength detector was utilized for all experiments. Components were separated with a Phenomenex (Aschaffenburg, Germany) Luna C-18 column (150 mm long x 2.0 mm inner diameter, particle size 5 µm) that was held at 25°C. The electrospray ionization voltage of the capillary was set to -4000 V and the end plate to -500 V. Nitrogen was used as dry gas at a temperature of 300°C and a flow rate of 10 l/ min. The full scan mass spectra were measured in a scan range from 50 to 800 m/z with a scan resolution of 13000 m/z/s until the ICC target reached 20000 or 200 ms, whichever was achieved first. Tandem mass spectrometry was carried out using helium as the collision gas (3.56 x 10-6 mbar) with the collision voltage set at 1 V. Spectra were acquired in the positive and negative ionization mode. The LC parameters went from 100% A (0.1% formic acid in water) to 50 % B (0.1% formic acid in methanol) in 30 min, then in 5 minutes to 100% B, held for 15 min at these conditions, then returned to 100% A in 5 min at a flow rate of 0.2 ml min-1. The detection wavelength was 280 nm.

4.2.7.9.1 Metabolite analysis

About 100 mg of apple leaves were exactly weighed and extracted with 200 µl methanol containing 0.2 mg/ml Biochanin A as an internal standard. Methanol was removed in a rotary vacuum concentrator (Christ RVC 2-18, Osterode, Germany) and the extract was re-dissolved in 45 µl water for analysis by LC-ESI-MSn. Metabolites were tentatively identified by their retention times, mass spectra and product ion spectra in comparison with the data determined for authentic reference material or published data. Relative metabolite quantification was performed using the Data Analysis 4.1 and Quant Analysis 1.5 software (Bruker Daltonics, Bremen, Germany) normalizing all results to the internal standard.

Several different polyphenolic secondary metabolites were analysed; including many of sub-groups belonging to the family of flavonoids (monomeric and polymeric flavon 3-ols, flavonols, dihydrochalcone, anthocyanidin, hydroxycinnamic acid).

Prof. Dr. Thomas Hofmann, Technical University Munich**,** kindly carried out the HPLC analysis.

4.2.7.10 Leaf painting assay for detection of *bar* gene-based herbicide resistance

In the constructs used for transformation, (Fig.5) the *bar* gene was used as selectable marker gene. The *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase (PAT) enzyme, isolated from *Streptomyces hygroscopicus*. It is analogous to *pat* gene isolated from *S. viridochromogenes* (Murakami *et al*., 1986; Thompson *et al*., 1987; Strauch *et al*., 1988), which confers resistance to bialaphos and the related compounds phosphinothricin (PPT), the active ingredient of herbicide BASTA®, Liberty® and glufosinate ammonium.

Phosphinotricin inhibits Glutamine Synthetase (GS), the enzyme that incorporates NH_3 into amino acids. When glutamine synthetase is blocked, the plants runs out of amino acids and pH of the cell rises causing the plant/tissue death due to accumulation of NH_3.

Transgenic plants expressing the *bar* gene are resistance to BASTA® through PAT enzyme by covalently linking an acetyl group to PPT to inactivate and detoxify compound to acetyl-PPT (De Block *et al*., 1987; Murakami *et al*., 1986) (Fig.8).

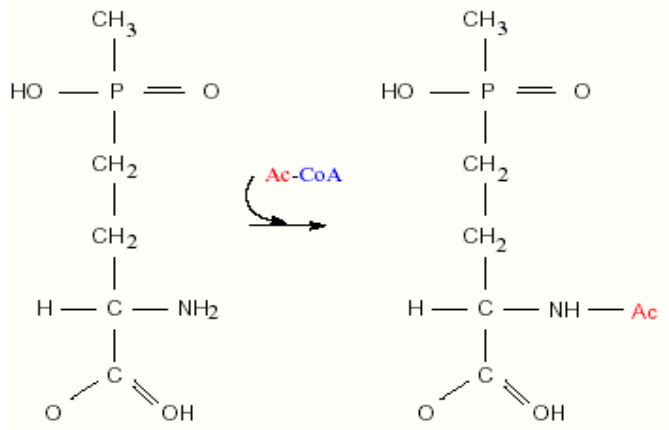


Fig.8. Detoxification and inactivation of PPT by acetylation. (Droege *et al.*, 1992).

BASTA® is a non-selective herbicide with no residual activity and has been regarded as environmentally safe (Nap and Metz, 1996). The *bar* gene offers an efficient and cheap selection system since all plants not containing or expressing *bar* will die.

BASTA® (Aventis GmbH, Germany) at a dilution of 600 mg/l (stock 200 g/l) was painted on 3 months old plants (plants have 9-11 leaves) to identify transformants. It was also applied to the upper surface of one marked apple leaf. Transgenic plants and control plants were treated in the same way, and BASTA® effect was controlled after one week.

5 Results

5.1 Phenotype

The results of transformation experiments showed changes in the phenotype in the case of 'HC' transgenic plants transformed with the binary vectors pJan-MdMyb10 and pGIIMH35S-MdMyb10. Starting from the red calli formed, red colour was observed on the veins of leaves of regenerated shoots, the *in vitro* plants, and on the plants grown in the green house (Table.4 , Fig.9; 10). With a notice that the red colour appeared on the leaves of HC' transgenic plants transformed with the binary vector pGIIMH35S-MdMyb10 appeared to be stronger. Roots of transgenic plants did not show any changes in the phenotype in comparison to roots of non-transgenic plants.

Calli and regenerated shoots formed from 'Gala' transformation by using the *MdMyb10* gene showed high red colour, while there was any difference between the *in vitro* plants and the grown plants in the green house in comparison to non- transgenic 'Gala' plants (Table.4).

There was no changes in the phenotype when the constructs contain *MdMyb9*, *MdMyb11* genes were used (in the case of two cultivars used) (Table.4).

Table.4. Effect of genetic transformation of apple *M.domestica* using MdMyb9, MdMyb10 MdMyb11 transcription factor genes on the phenotype (No: No effects, +: red colour)

	Holsteiner Cox				Gala			
	Calli and explant	Regenerated shoots and *in vitro* plants	Roots of *in vitro* plants	Acclimatized plants	Calli and explant	Regenerated shoots and *in vitro* plants	Roots of *in vitro* plants	Acclimatized plants
pJan-MdMyb9	No	No	No	No	No	No	No	No
pJan-MdMyb10	+	+	No	+	+	No	No	No
pJan-MdMyb11	No	No	No	No	No	No	No	No
pGIIMH35S-MdMyb10	+	++	No	++				
pGIIMH35S-MdMyb11	No	No	No	No				

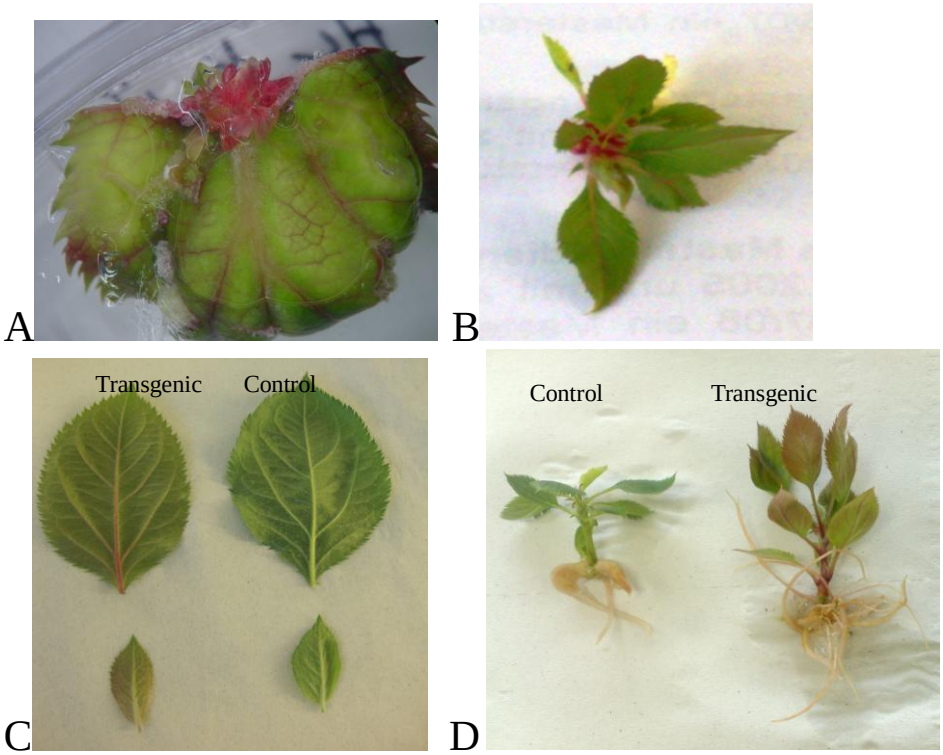


Fig.9. Change of phenotype in different stages of transformation of HC using pJan-MdMyb10 binary vector (A: explant on regeneration medium; B: *in vitro* regenerated shoots; C: leaves of acclimatized plants; D: rooted plants)

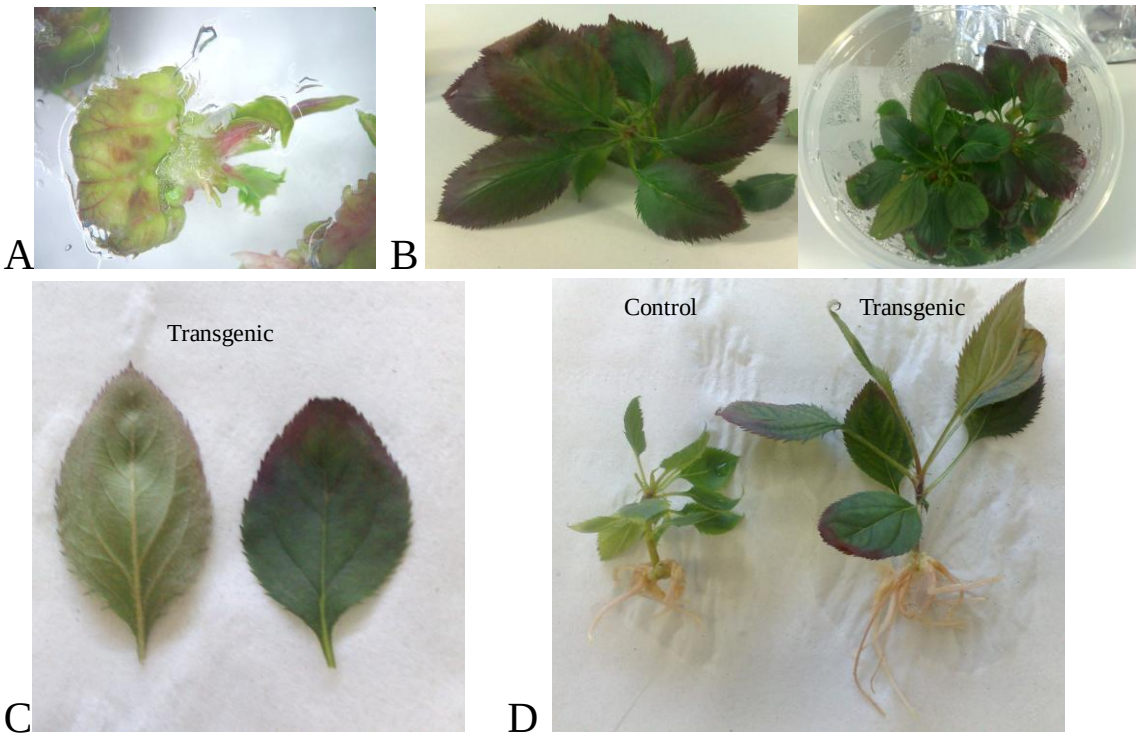


Fig.10. Change of phenotype in different stages of transformation of HC using pGIIMH35S-MdMyb10 binary vector (A: explant on regeneration medium; B: *in vitro* regenerated shoots; C: leaves of acclimatized plants; D: rooted plants).

To confirm the transgenic nature, the transgenic plants using the vector pGIIMH35S harboring *MdMyb10*, *MdMyb11* transcription factor genes**,** were tested with leaf paint assay for their tolerance to commercially available form of PPT, 600mg/l BASTA® was applied using a siring to the leaves of transgenic and control plants. The results (Fig.11) clearly indicated that the transgenic plants could tolerate the applied herbicide treatment without any significant observable lesion contrary to the lesions and necrosis-taking place at the leaves of control plants.

Fig.11. Leaf paint assay on apple plants cv. HC after one week treatment using 600 mg/l BASTA® (A: nom-transgenic plants (-); B: transgenic plant using pGIIMH35S-MdMyb10 binary vector (+); C: transgenic plant using pGIIMH35S-MdMyb11 binary vector (+))

5.2 Transformation of *M. domestica* CVs 'HC' and 'Gala' using the binary vector pJAN harboring *MdMyb9*, *MdMyb10*, *MdMyb11* Transcription factor genes

5.2.1 Molecular analysis

5.2.1.1 PCR and southern blot analysis

5.2.1.1.1 Transformed plants using the vector pJAN harboring *MdMyb9* transcription factor gene

One transgenic line from 'HC' and three transgenic lines from 'Gala' were obtained. The integration of the *MdMyb9* transcription factor gene into genomic DNA of transgenic plants was confirmed by using the primers 35S prom-for., and Myb9-specific rev. (see 4.1.1.8.2), which amplify a 726-bp specific fragment. The results clearly indicate and confirm the successful integration of T–DNA into genomic DNA of transgenic apple plants (Fig.12).

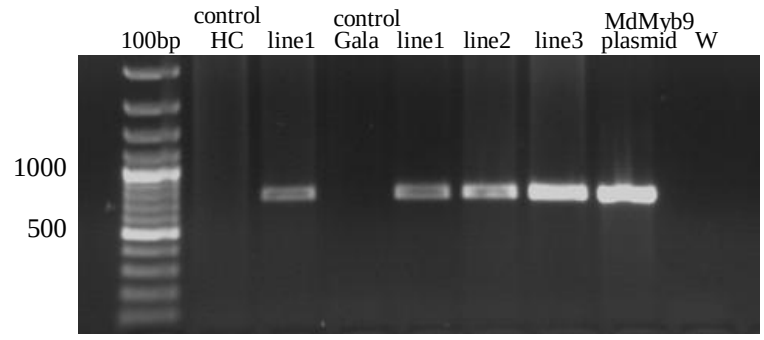


Fig.12. PCR of different transgenic lines of HC and Gala (transformed with PJan-MdMyb9) using 35S prom-for. and Myb9-specific rev. primers (spanning a 726bp DNA fragment).

In order to confirm the integration and to know the copy numbers of *MdMyb9* in the transgenic lines, the isolated genomic DNA from both non-transgenic and transgenic plants were digested with *Eco*RI restriction enzyme. Southern blot hybridization was performed using a DIG labeled PCR amplified probe. The result showed that one copy of a *MdMyb9* analog was found in all plants analysed (non-transgenic and transgenic plants). The *MdMyb9* gene was successfully integrated into the genome of the 'HC' transgenic plants as one copy of *MdMyb9* gene was detected in the 'HC' transgenic plants (line1), in addition to that found in the wild type. The *MdMyb9* gene was successfully integrated in the genome of the 'Gala' transgenic plants; one additional copy of *MdMyb9* gene was detected in the 'Gala' transgenic line1, while two gene copies with different places were detected in line2 and line 3, in addition to that found in the wild type (Fig.13).

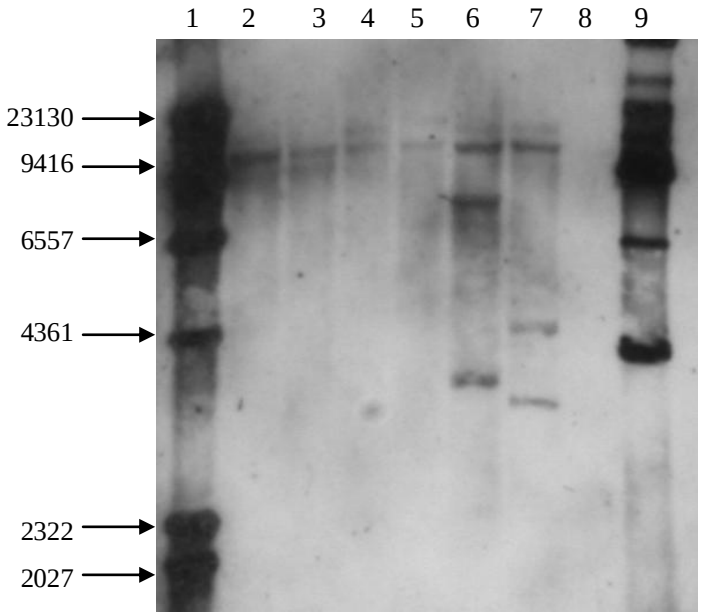


Fig.13. Southern blot analysis of g DNA for HC and Gala transgenic plants transformed with pJan-MdMyb9 digested with *Eco* RI, using MdMyb9 probe (1: Marker; 2: HC non-transgenic;3:HC transgenic line1; 4:Gala non transgenic;5,6,7: Gala transgenic lines1, 2, 3; 9: positive control(plasmid))

5.2.1.1.2 Transformed plants using the vector pJAN harboring *MdMyb10* transcription factor gene

One transgenic line from 'HC' and four transgenic lines from 'Gala' were obtained. The stable integration of the *MdMyb10* transcription factor gene into genomic DNA of transgenic plants was confirmed by using the primers Myb10 for., and Myb10 reav. (see 4.1.1.8.2), which amplify a 717-bp *Myb10* specific fragment (Fig.14).

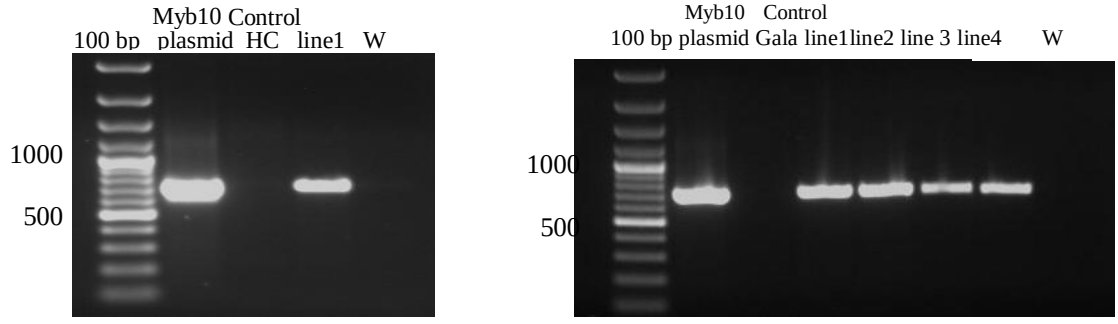


Fig.14. PCR of different transgenic lines of HC and Gala (transformed with PJan-MdMyb10) using MYB10 primers (spanning a 717 bp DNA fragment)

The genomic DNA from both non-transgenic and transgenic Plants was digested with *Bam*H1. Southern blot hybridization was performed using a DIG labeled PCR amplified probe. The result showed that one copy of a *MdMyb10* analog was found in all analysed plants (non-transgenic and transgenic plants). The *MdMyb10* gene was successfully integrated into the genome of the 'HC' transgenic plants; one copy was detected in line1, in addition to that

found in the wild type. The *MdMyb10* gene was successfully integrated in the genome of the 'Gala' transgenic plants; one additional copy was detected in the 'Gala' transgenic plants (line1, line 4), while two gene copies were detected in line2, line 3, in addition to that found in the wild type (Fig.15).

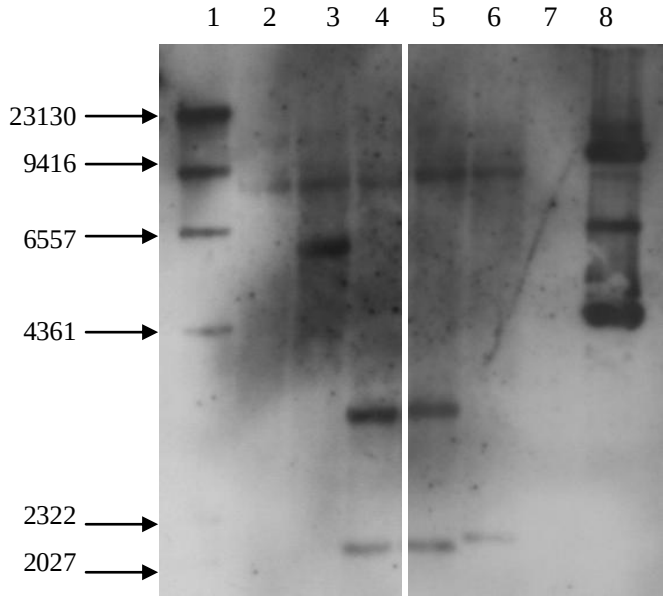


Fig.15. Southern blot analysis of g DNA for Gala transgenic plants transformed with pJan-MdMyb10 digested with *Bam* H1, using MdMyb10 probe (1: Marker; 2: Gala non-transgenic; 3, 4, 5, 6: Gala transgenic lines 1-4; 8: positive control (plasmid))

5.2.1.1.3 Transformed plants using the vector pJAN harboring *MdMyb11* transcription factor gene

Two transgenic lines from 'HC' and one transgenic line from 'Gala' were obtained. The stable integration of the *MdMyb11* transcription factor gene into genomic DNA of transgenic plants was confirmed by using the primers 35S prom-for., and Myb11-specific rev. (see 4.1.1.8.2), which amplify a 474-bp specific fragment (Fig.16).

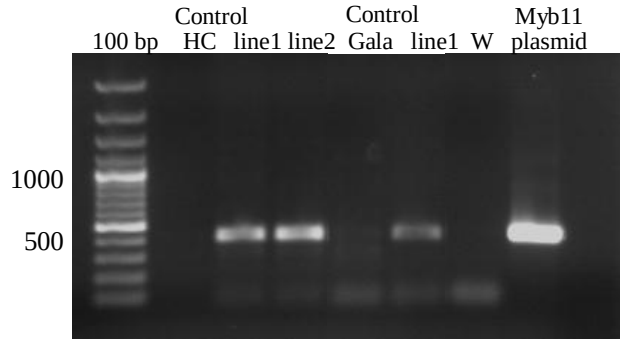


Fig.16. PCR of different transgenic lines of HC and Gala (transformed with PJan-MdMyb11) using 35S promoter-for and MYB11-specific rev primers (spanning a 474 bp DNA fragment).

The genomic DNA from both non-transgenic and transgenic Plants was digested with *Eco*RI. Southern blot hybridization was performed using a DIG labeled PCR amplified probe. The

result showed that one copy of an *MdMyb11* analog was found in all analysed plants (non - transgenic and transgenic plants from both cultivars used).

The *MdMyb11* gene was successfully integrated in the genome of the 'HC' transgenic plants; one additional copy was detected in line1, while four copies were found in the case of line2, in addition to that found in the wild type.

The *MdMyb11* gene was successfully integrated in the genome of the 'Gala' transgenic plants; one additional copy of *MdMyb11* gene was detected in the 'Gala' transgenic plants (line1), in addition to that found in the wild type (Fig.17).

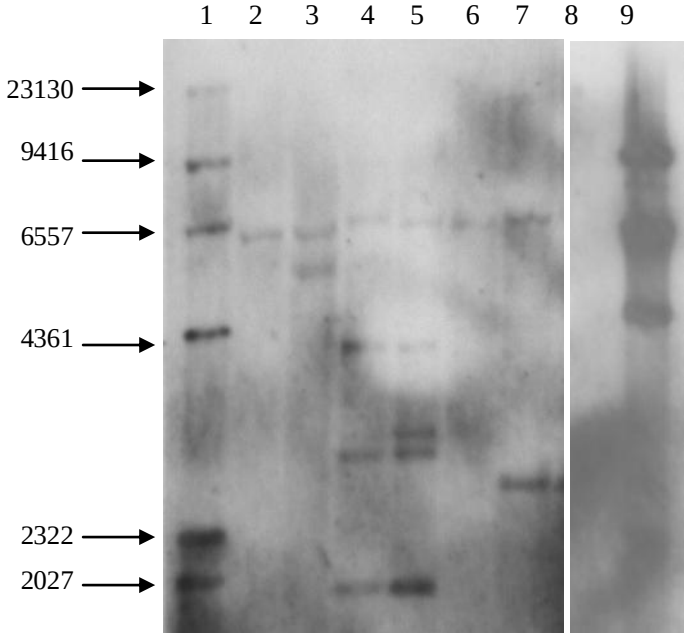


Fig.17. Southern blot analysis of g DNA for HC and Gala transgenic plants transformed with pJan-MdMyb11 digested with *Eco*RI, using MdMyb11 probe (1: Marker; 2: HC non-transgenic; 3, 4, 5: HC transgenic lines 1-2; 6: Gala non transgenic; 7: Gala transgenic line1; 9: positive control (plasmid))

The presence of the *npt* II gene in all transgenic plants using the vector pJAN harboring *MdMyb9*, *MdMyb10*, *MdMyb11* transcription factor genes was confirmed by using the primers 167 nptII-for and 367nptII-rev (see 4.1.1.8.2), (Result not shown).

5.2.1.2 Reverse transcriptase PCR

5.2.1.2.1.Transformed plants using the vector pJAN harboring *MdMyb9* transcription factor gene

RT-PCR was performed to confirm transcription of the transgene using myb9 specific primer for and Pjan-poly-A rev. primer (the reverse primer from the vector after the stop codon and before the polyadenylation region) (see 4.1.1.8.2); the expected fragment with the size of 651bp was amplified from all regenerated transgenic lines(Fig.18).

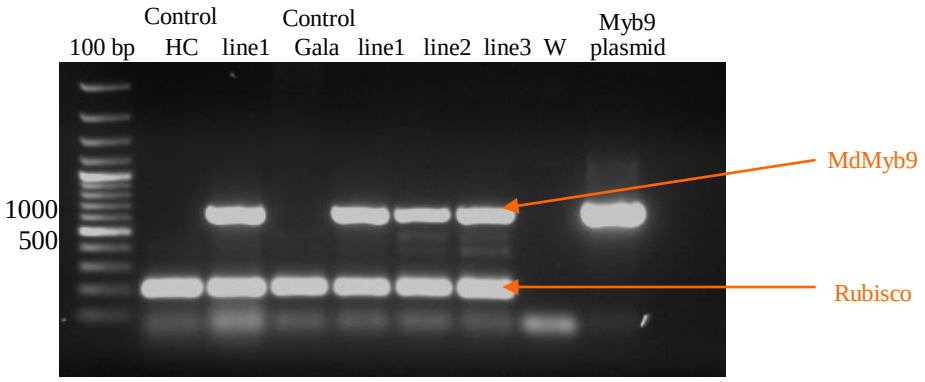


Fig.18. RT-PCR of different transgenic lines of HC and Gala (transformed with PJan- MdMyb9) using Myb9 specific-for., pJan-polyA-rev. primers (spanning a 651bp cDNA fragment of the *MdMyb9* gene). Rubisco-for. and Rubisco rev. primers (spanning a 200bp cDNA fragment of the *Rubisco* gene) were used as a control.

5.2.1.2.2 Transformed plants using the vector pJAN harboring *MdMyb10* transcription factor gene

RT-PCR was performed to confirm transcription of the transgene using myb10 specific primer for and pJan-poly-A rev. primer (the reverse primer from the vector after the stop codon and before the polyadenylation region) (see 4.1.1.8.2), the expected fragment with the size of 729 bp was amplified from all the regenerated transgenic lines (Fig.19).

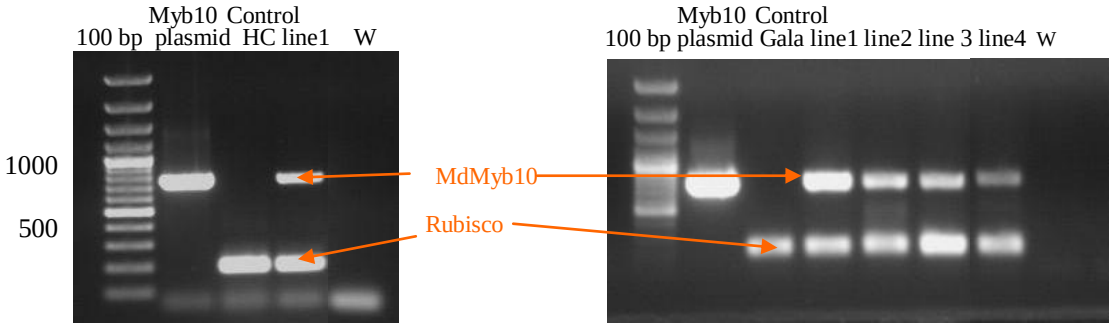


Fig.19. RT-PCR of different transgenic lines of HC and Gala (transformed with PJan- MdMyb10) using Myb10 specific-for., pJan-polyA-rev. primers (spanning a 729bp cDNA fragment of the *MdMyb10* gene). Rubisco-for. and Rubisco rev. primers (spanning a 200bp cDNA fragment of the *Rubisco* gene) were used as a control.

5.2.1.2.3 Transformed plants using the vector pJAN harboring *MdMyb11* transcription factor gene

RT-PCR was performed to confirm transcription of the transgene using myb11 specific primer for and pJan-poly-A rev. primer (the reverse primer from the vector after the stop codon and before Polyadenylation region) (see 4.1.1.8.2), the expected fragment with the size of 714bp was amplified from all the regenerated transgenic lines (Fig.20).

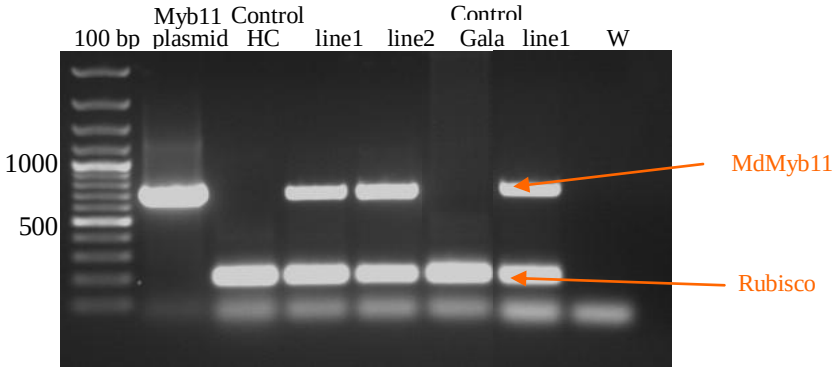


Fig.20. RT-PCR of different transgenic lines of HC and Gala (transformed with PJan- MdMyb11) using Myb11specific-for., pJan-polyA-rev. primers (spanning a 714bp cDNA fragment of the *MdMyb11* gene). Rubisco-for. and Rubisco rev. primers (spanning a 200bp cDNA fragment of the *Rubisco* gene) were used as a control.

5.2.2 Quantitative-RT-PCR analysis

The Real Time PCR results of samples taken from transgenic 'HC' and 'Gala' lines showed that the transcript levels of the *MdMyb9* ,*MdMyb10*, *MdMyb11* transcription factor genes were increased in all transgenic plants in comparision to the non-transgenic plants.

5.2.2.1 Transformed plants using the vector pJAN harboring *MdMyb9*

Real Time PCR was performed using *MdMyb9* gene specific primers (see 4.1.1.8.3) to obtain transcription profiles for the transgene *MdMyb9*. Expression levels of *MdMyb9* gene was determined and expressed relative to the *Rubisco* and the *RNA polymerase* genes. These two genes were chosen as reference genes because SYBR-Green RT-PCR analysis showed that they have a high and stable mRNA expression level in apple leaves (Flachowsky, unpublished). The transcripts of the transgene *MdMyb9* were detected in both transgenic and non-transgenic control plants.

The *MdMyb9* gene transcription level of the transgenic line was 47- fold in relation to non-transgenic 'HC' plants (Fig.21).

The *MdMyb9* gene transcription levels of the transgenic lines ranged between 3.7- fold (line1) and 1451-fold (line2) in relation to non- transgenic 'Gala' plants (Fig.22).

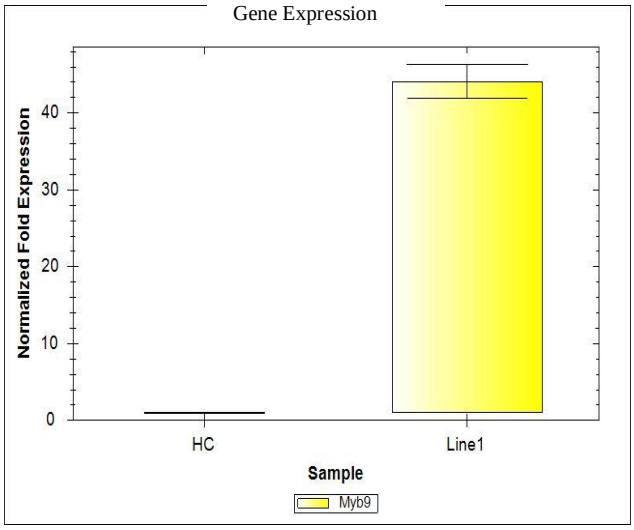


Fig.21. Expression of *MdMyb9* gene in HC transgenic line (transformed using pJan–MdMyb9 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates. (HC: non transgenic plant, Line1: transgenic plant)

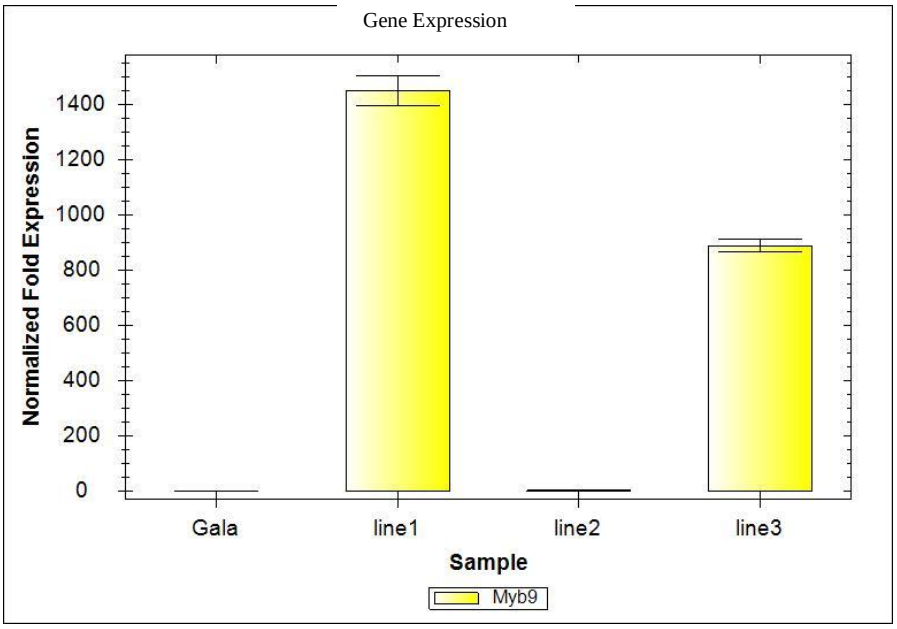


Fig.22. Expression of *MdMyb9* gene in Gala transgenic lines (transformed using pJan–MdMyb9 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates (Gala: non-transgenic plant, Line1, Line2, Line3: transgenic plants).

5.2.2.2 Transformed plants using the vector pJAN harboring *MdMyb10*

Real-time PCR was performed using *MdMyb10* gene specific primers (see 4.1.1.8.3) to obtain transcription profiles for the transgenes *MdMyb10*. Expression levels of *MdMyb10* gene were determined and expressed relative to the *Rubisco* and the *RNA polymerase* genes. The transcripts of the transgenes *MdMyb10* was detected in both transgenic and non-transgenic

control plants. The *MdMyb10* gene transcription level of the transgenic line was 1261-fold,in relation to non-transgenic 'HC' plants (Fig.23).

The *MdMyb10* gene transcription level of the Gala transgenic lines ranged between 86- fold (line2) and 847-fold (line1), in relation to non- transgenic 'Gala' plants (Fig.24).

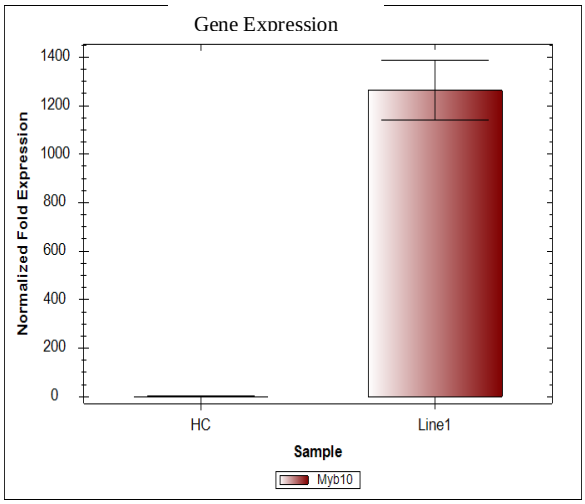


Fig.23. Expression of *MdMyb10* gene in HC transgenic line (transformed using pJan–MdMyb10 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates. (HC: non transgenic plant, Line1: transgenic plant)

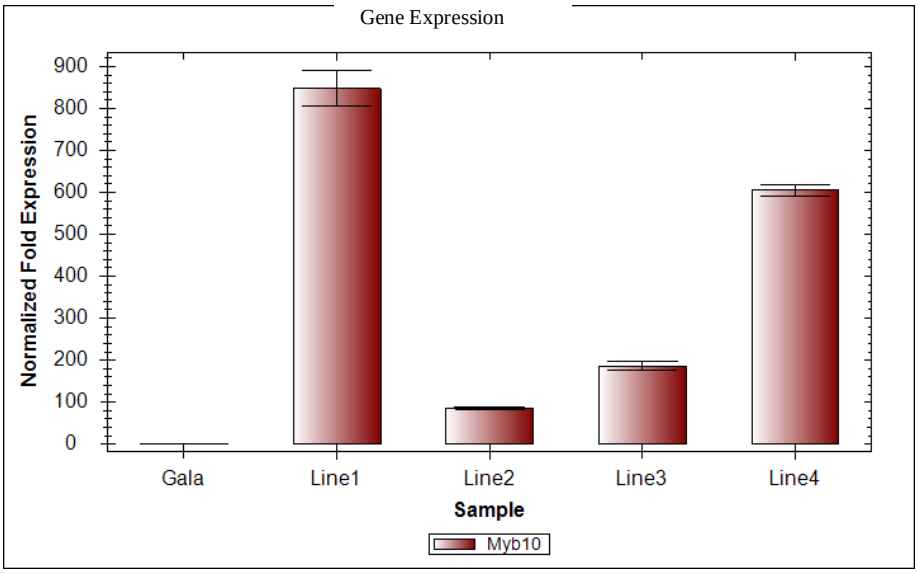


Fig.24 . Expression of *MdMyb10* gene in Gala transgenic lines (transformed using pJan–MdMyb10 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates (Gala: non-transgenic plant, Line1, Line2, Line3, Line4: transgenic plants).

5.2.2.3 Transformed plants using the vector pJAN harboring *MdMyb11*

Real-time PCR was performed using *MdMyb11* gene specific primers (see 4.1.1.8.3) to obtain transcription profiles for the transgenes *MdMyb11*. Expression levels of *MdMyb11* gene were

determined and expressed relative to the *Rubisco* and the *RNA polymerase* genes. The transcripts of the transgenes *MdMyb11* was detected in both transgenic and non-transgenic control plants. The *MdMyb11* gene transcription level of the 'HC' transgenic lines ranged between 1.15- fold (line1) and 5.99-fold (line2), in relation to non- transgenic 'HC' plants (Fig.25).

The *MdMyb11*gene transcription level of the transgenic line was 9.6-fold, in relation to non-transgenic 'Gala' plants (Fig.26).

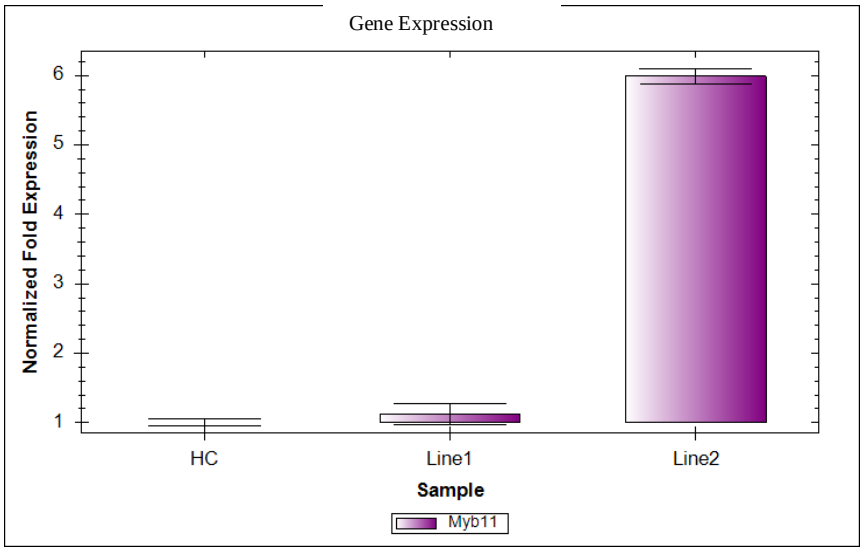


Fig. 25. Expression of *MdMyb11*gene in HC transgenic lines (transformed using pJan–MdMyb11 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates (HC: non-transgenic plant, Line1, Line2: transgenic plants).

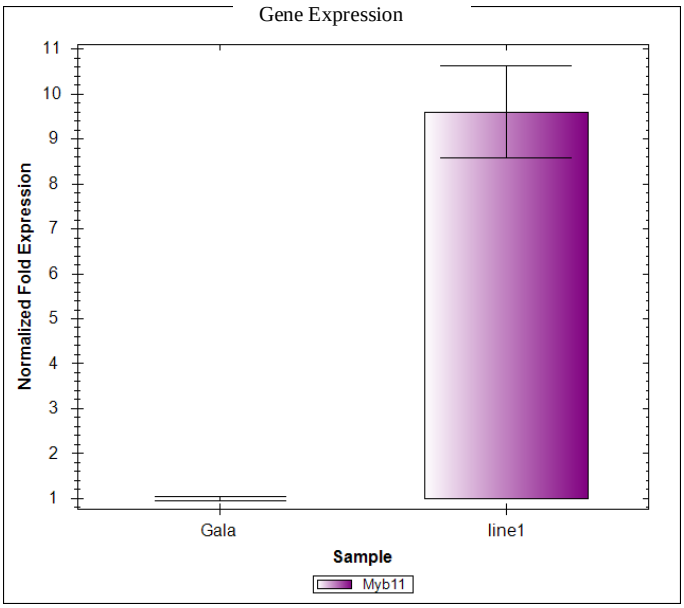


Fig.26. Expression of *MdMyb11* gene in Gala transgenic line (transformed using pJan–MdMyb11 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates (Gala: non-transgenic plant, Line1: transgenic plant).

5.2.3 Metabolic profiling

5.2.3.1 Quantitative analysis of flavonoid levels in *MdMyb9* transgenic plants

In *MdMyb9* 'HC' transgenic plants, some of the polyphenol classes analysed were enhanced and others were reduced (Fig.27, 30). The total content levels of flavan-3-ols were 1.21 times in comparison to non-transgenic plants, strongest rises were observed for catechin 3 times, epicatechin 2.34 times. The level of Cyanidin 3-O-galactoside in *MdMyb9* 'HC' transgenic plants was reduced to 0.42 in comparison to non-transgenic plants. The total content levels of hydroxycinnamic acids were increased 2 times in comparison to their levels in the non-transgenic plants, caffeic acid-glucose- ester showed the highest raises up to 4 times. The total content levels of dihydrochalcones (phloretin, phloridzin and phloretin 2'-xyloglucoside) -as the dominant *M. domestica* polyphenol class- were decreased to 0.71 in *MdMyb9* 'HC' transgenic plants in comparison to non-transgenic plants. The total content levels of flavonols were reduced to 0.89 in comparison to their levels in non-transgenic plants.

In *MdMyb9* 'Gala' transgenic plants, all the polyphenol classes analysed were reduced in the case of line 2. With a decrease to 0.49 for the total content levels of flavon 3-ols, 0.26 for the total content levels of hydroxycinnamic acids, 0.72 for the total content levels of dihydrochalcones, and 0.57 for the total content levels of flavonols.

In *MdMyb9* 'Gala' transgenic plants (line1, line3), some of the polyphenol classes analysed were induced and others were reduced (Fig.27, 31). The total content levels of flavon 3-ols were 1.02, 1.41 times for line1 and 3, respectively, in comparison to non-transgenic plants, with an increase 3.6, 7 times for epicatechin, 4.1, 2.4 times for catechin for line1 and 3, respectively. The level of Cyanidin 3-O-galactoside in *MdMyb9* 'Gala' transgenic plants was nearly the same in the case of line1, and increased up to 2.2 times in comparison to non-transgenic plants in the case of line3. For the hydroxycinnamic acids, the total content of their levels in *MdMyb9* 'Gala' transgenic plants were 1.56 and 1.92 times increases in comparison to non-transgenic plants for line1 and line3, respectively, strongest rises were observed in the case of chlorogenic acid (3.4, 4.36) times, and caffeic acid-glucose-ester 2.896, 2.856 times.

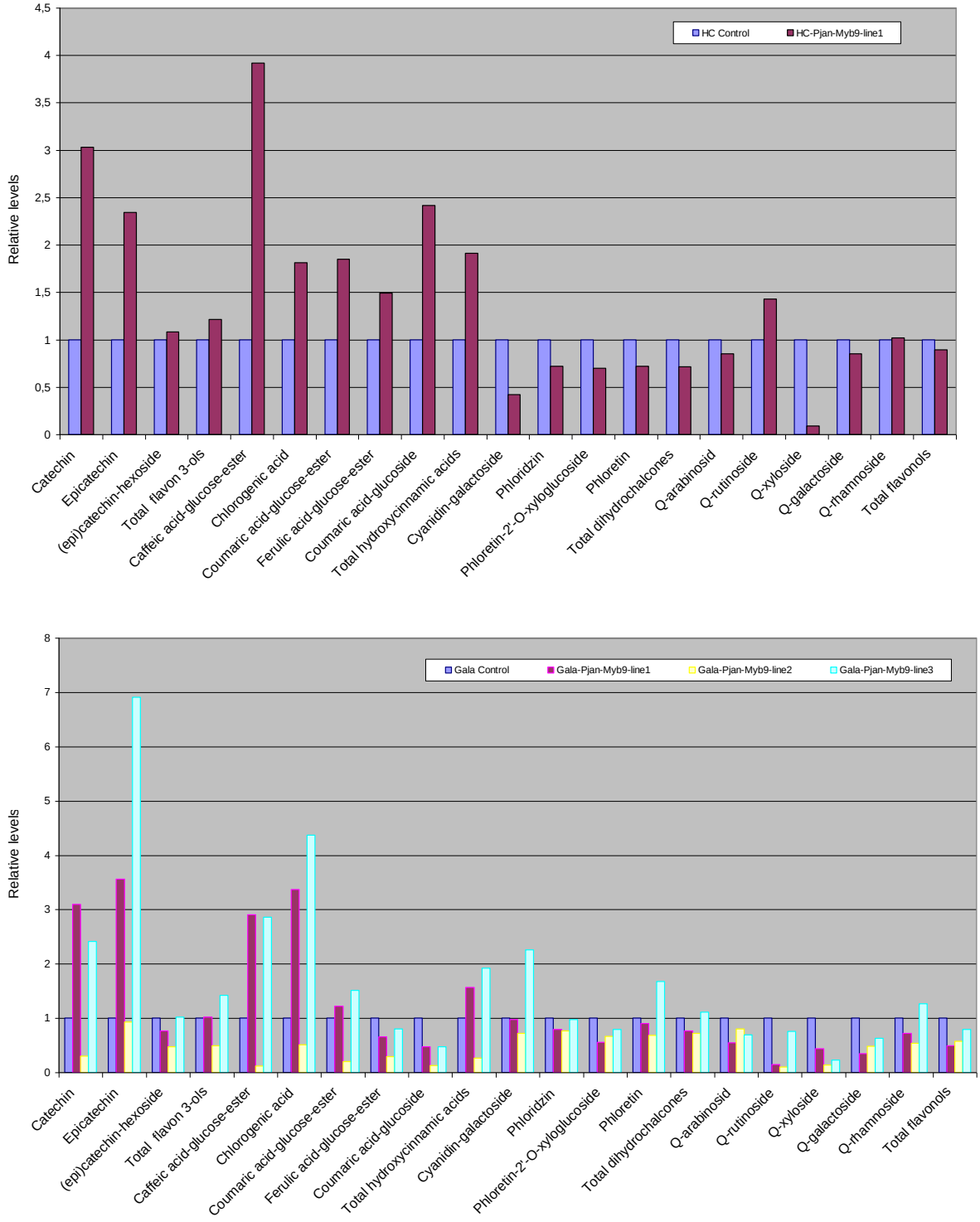


Fig.27. Relative levels of selected phenolic compounds (values are metabolite levels of each compound in the transgenic plants transformed with the binary vector pJan-MdMyb9 comparing to the levels in non-transgenic plants), (a: HC transgenic plants, b: Gala transgenic plants)

The total content levels of dihydrochalcones were 1.1 times more in the case of line3 in comparison to non-transgenic plants, with an increase of 1.67 times for phloretin. In the case of line1, the total content levels of dihydrochalcones were reduced to 0.76 times in

comparison to non-transgenic plants. The total content levels of flavonols in *MdMyb9* 'Gala' transgenic plants were reduced to 0.49 and 0.77 times for line1 and line3, respectively, in comparison to their levels in the non-transgenic plants.

5.2.3.2 Quantitative analysis of flavonoid levels in *MdMyb*10 transgenic plants

In *MdMyb10* 'HC' transgenic plants, some of the polyphenol classes analysed were enhanced and others were reduced (Fig.28, 32). The total content levels of flavan-3-ols were increased to 1.4 times, strongest rises were observed for (epi) catechin-hexoside 1.44 times in comparison to their levels in non-transgenic plants. The level of cyanidin–galactoside, was nearly the same in *MdMyb10* 'HC' transgenic plants as well as in non-transgenic plants. For the hydroxycinnamic acids, the total content of their levels were reduced to 0.76 times in comparison to non-transgenic plants. For the dihydrochalcones, the total content of their levels were nearly the same in *MdMyb10* 'HC' transgenic plants and 'HC' non-transgenic plants, only the level of phloretin was 1.32 times higher in *MdMyb10* 'HC' transgenic plants. The total content levels of flavonols were higher 1.68 times in comparison to their levels in the non-transgenic plants, Q-xyloside showed rises 2.7 times in comparison to non-transgenic plants.

In *MdMyb10* 'Gala' transgenic plants, most of the analysed polyphenol classes were enhanced (Fig.28, 33). The total content levels of flavon 3-ols were increased 2.3, 2.5 and 1.85 times for the lines 1,2 and 3, respectively, the levels were nearly the same for the line 4 in *MdMyb10* 'Gala' transgenic as well as in non-transgenic plants. Strongest rises were observed for (epi) catechin-hexoside 2.45, 2.74 and 1.83 times for the lines 1, 2 and 3, respectively. The total content levels of hydroxycinnamic acids in *MdMyb10* 'Gala' transgenic plants were increased 1.2 and 1.24 times for the lines1 and 3, respectively, the levels were decreased 0.81 and 0.69 times for the lines 2 and 4, respectively, in comparison to non-transgenic plants . The level of Cyanidin 3-O-galactoside in *MdMyb10* 'Gala' transgenic plants was increased 5.3, 1.7 and 1.95 times for the lines 1, 2 and 3, respectively, in comparison to non-transgenic plants. For the line 4 the level of Cyanidin 3-O-galactoside was nearly the same in *MdMyb10* 'Gala' transgenic plants as well as in non-transgenic plants.

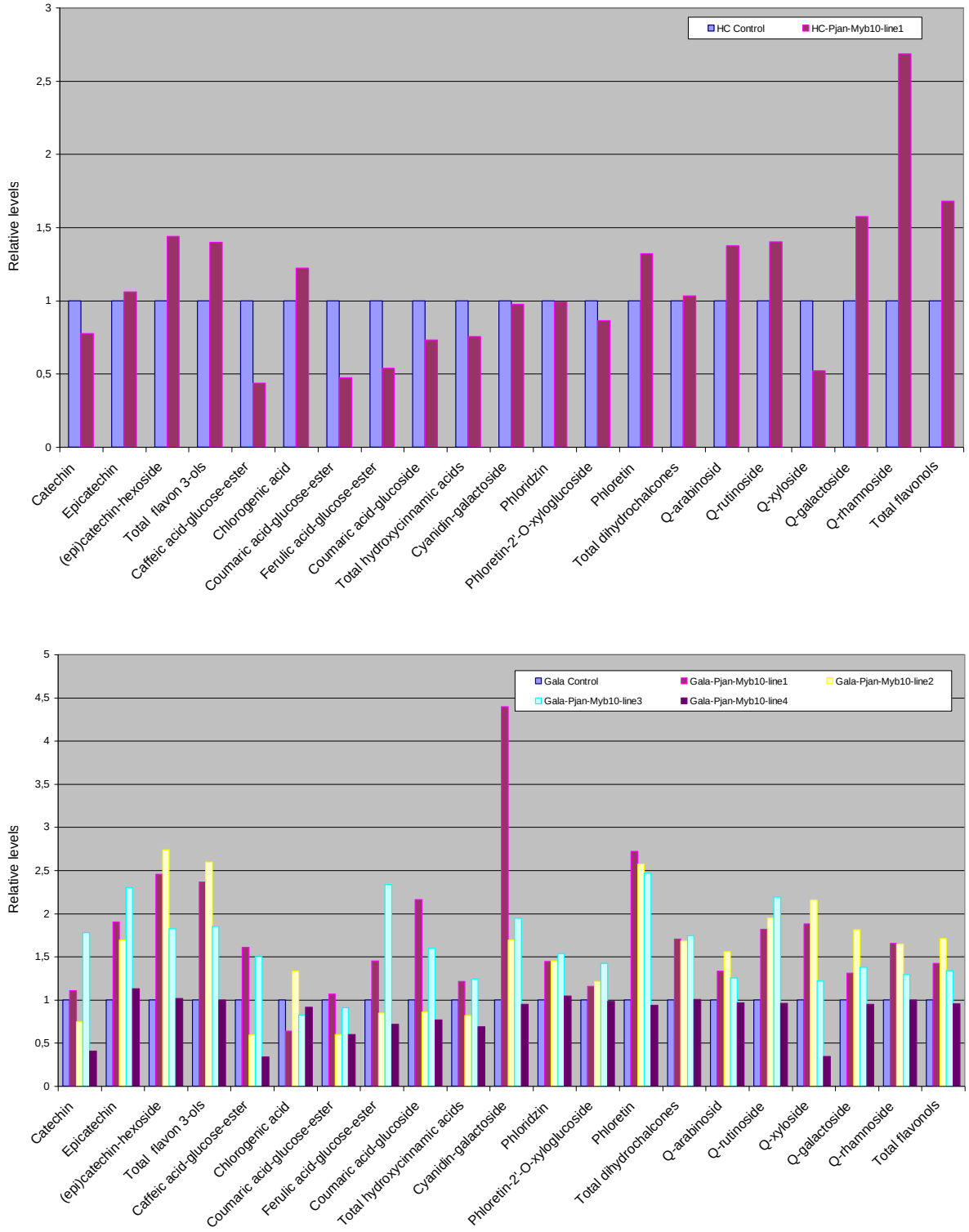


Fig.28. Relative levels of selected phenolic compounds (values are metabolite level of each compound in the transgenic plants transformed with the binary vector pJan-MdMyb10 comparing to the levels in non-transgenic plants), (a: HC transgenic plants, b: Gala transgenic plants)

For the dihydrochalcones (phloretin, phloridzin and phloretin 2'-xyloglucoside), as the dominant *M. domestica* polyphenol class, the total content levels in *MdMyb10* 'Gala' transgenic plants increased up to 1.7 times for the lines 1, 2 and 3 in comparison to non-

transgenic plants. Strongest rises were observed for phloretin 2.73, 2.57 and 2.47 times for the lines 1, 2 and 3, respectively, for the line 4 the total content levels of dihydrochalcones was nearly the same in *MdMyb10* 'Gala' transgenic plants as well as in non-transgenic plants.

The total content levels of flavonols were increased 1.42, 1.7 and 1.39 times for the lines 1, 2 and 3, respectively, in comparison to their levels in non-transgenic plants, for the line 4 the total content levels of flavonols was nearly the same in *MdMyb10* 'Gala' transgenic plants and non-transgenic plants.

5.2.3.3 Quantitative analysis of flavonoid levels in *MdMyb11* transgenic plants

In *MdMyb11* 'HC' transgenic plants, (Fig.29, 34)., the total content levels of flavan-3-olswere increased 1.53 and 1.3 times for the lines 1 and 2, respectively, in comparison to non-transgenic plants, strongest rises were observed for catechin 4.87 times and (epi)catechin-hexoside 2.89 times in the case of line 2. The level of Cyanidin 3-O-galactoside in *MdMyb11* 'HC' transgenic plants were reduced to 0.83 and 0.56 times for line1 and line 2, respectively, in comparison to non-transgenic plants. The total content levels of hydroxycinnamic acids in *MdMyb11* 'HC' transgenic plants were increased up to 2 times for the line 2 , the levels were nearly the same for line1 in comparison to the non-transgenic plants . Strongest rises were observed for Caffeic acid-glucose-ester up to 4 times for line 1 and 2, chlorogenic acid showed rises 1.43 and 2.6 times for the line 1 and 2, respectively. For the dihydrochalcones, the total content levels in *MdMyb11* 'HC' transgenic plants reduced to 0.39 and 0.81 times for the lines 1 and 2, respectively, in comparison to non-transgenic plants. Also all the flavonols analysed were reduced, only observed increase was in Q-xyloside level up to 3 times in the case of line 1, Q-rhamnoside showed rises 1.7 and 2.1 times for line 1 and 2, respectively, the total content levels of flavonols in *MdMyb11* 'HC' transgenic plants were nearly the same as it is in non-transgenic plants.

In *MdMyb11* 'Gala' transgenic plants, most of the polyphenol classes analysed were reduced (Fig.29, 35). All the analysed flavon 3-ols were reduced, only epicatechein showed rises up to 1.2 times, the total content levels of the flavon 3-ols were 0.60 times in comparison to non-transgenic plants. The level of Cyanidin 3-O-galactoside in *MdMyb11* 'Gala' transgenic plants reduced to 0.58 times in comparison to non-transgenic plants. For the hydroxycinnamic acids, all of the analysed compounds were reduced, only chlorogenic acid level showed rises 1.5 times, the total content levels of the hydroxycinnamic acids reduced to 0.6 times in comparison to non-transgenic plants. For the dihydrochalcones, the total content levels in

MdMyb11 'Gala' transgenic plants reduced to 0.51 times in comparison to non-transgenic plants. Also all the flavonols analysed were reduced. The total content levels of flavonols in *MdMyb11* 'Gala' transgenic plants reduced to 0.53 times in comparison to non-transgenic plants.

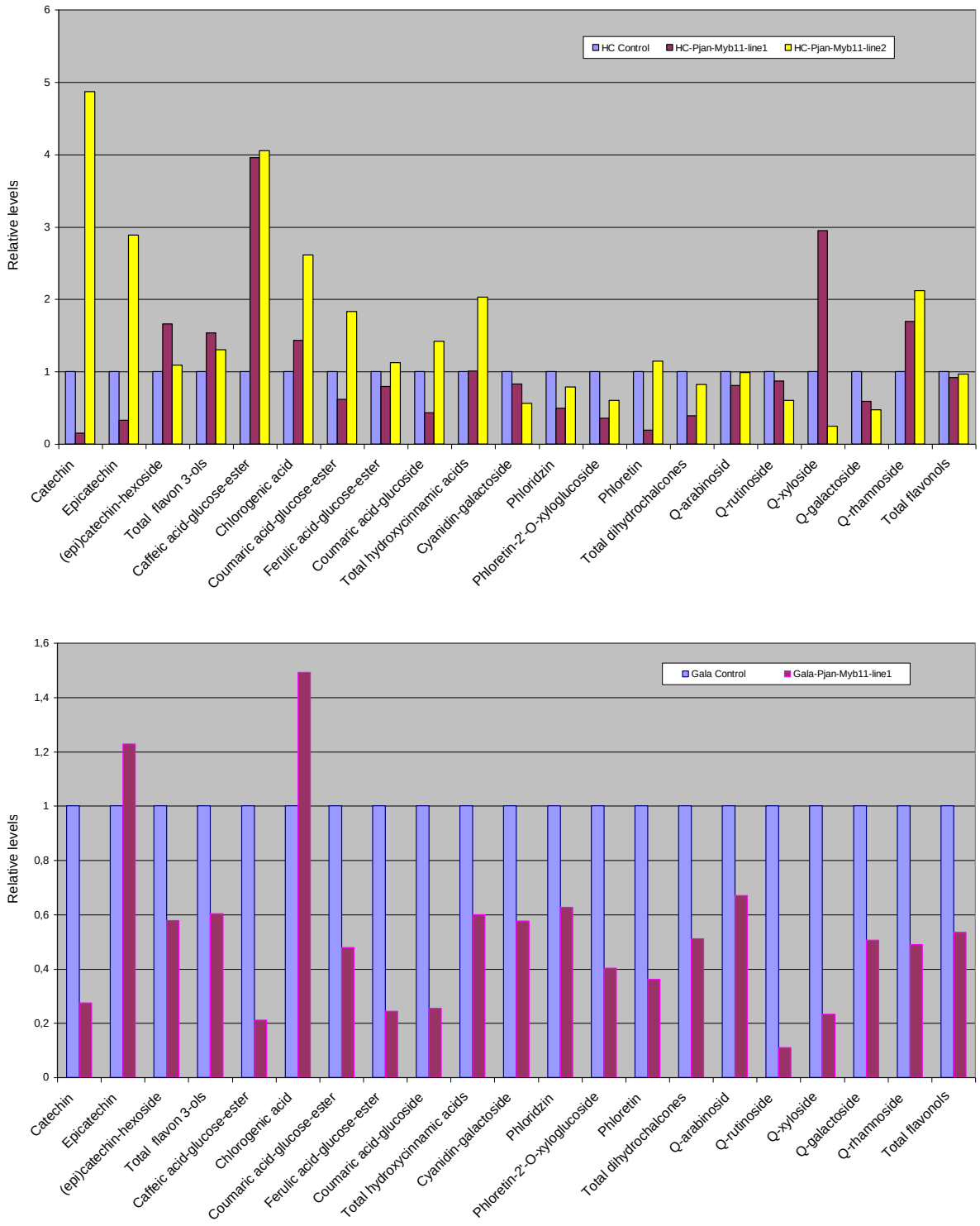


Fig.29. Relative levels of selected phenolic compounds (values are metabolite levels of each compound in the transgenic plants transformed with the binary vector pJan-MdMyb11comparing to the levels in non-transgenic plants), (a: HC transgenic plants, b: Gala transgenic plants)

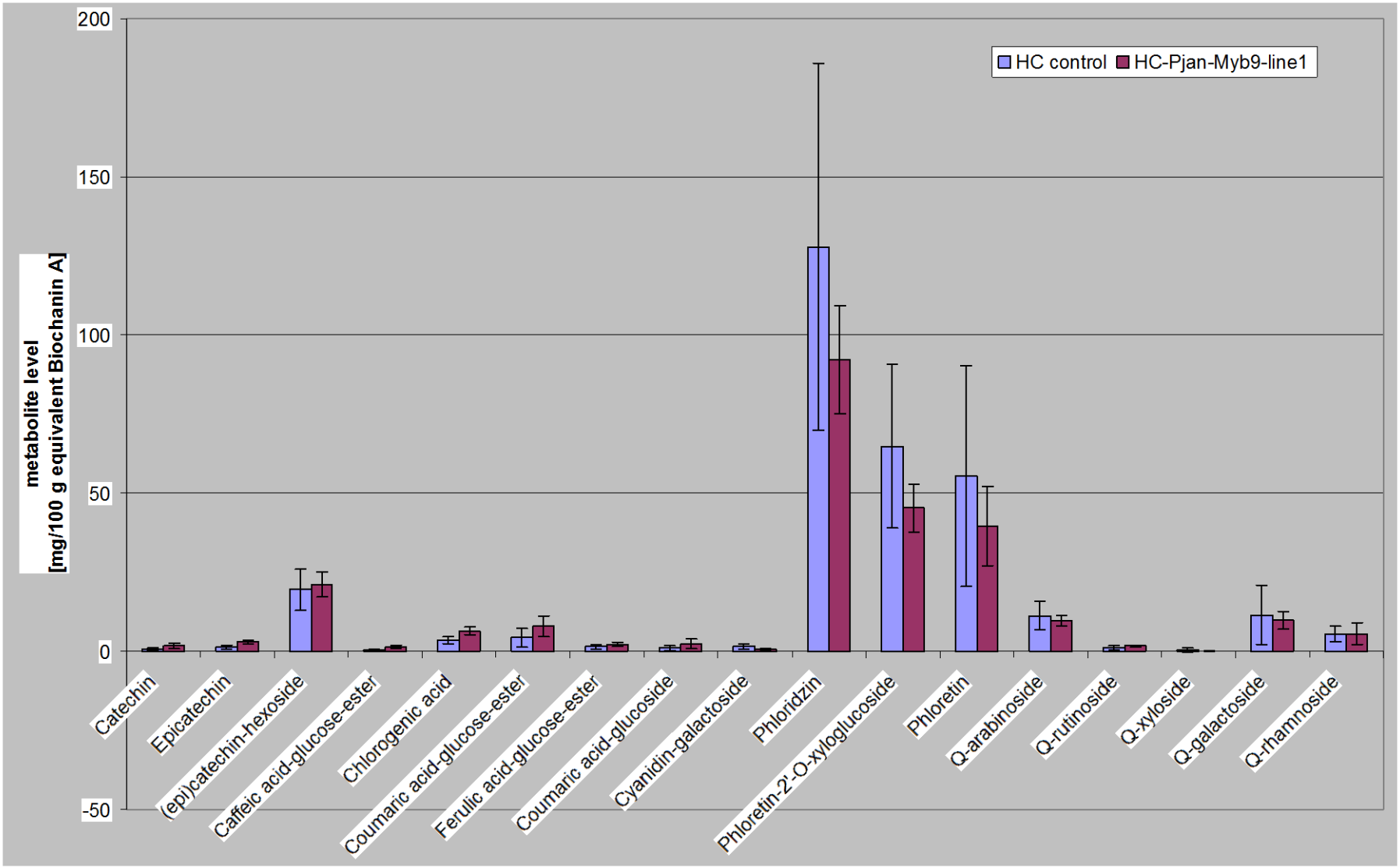


Fig.30. Metabolite levels of selected phenolic compounds in leaves taken from‘HC’ plants transformed with the binary vector pJan- MdMyb9, relative to the levels in leaves from‘HC’ non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

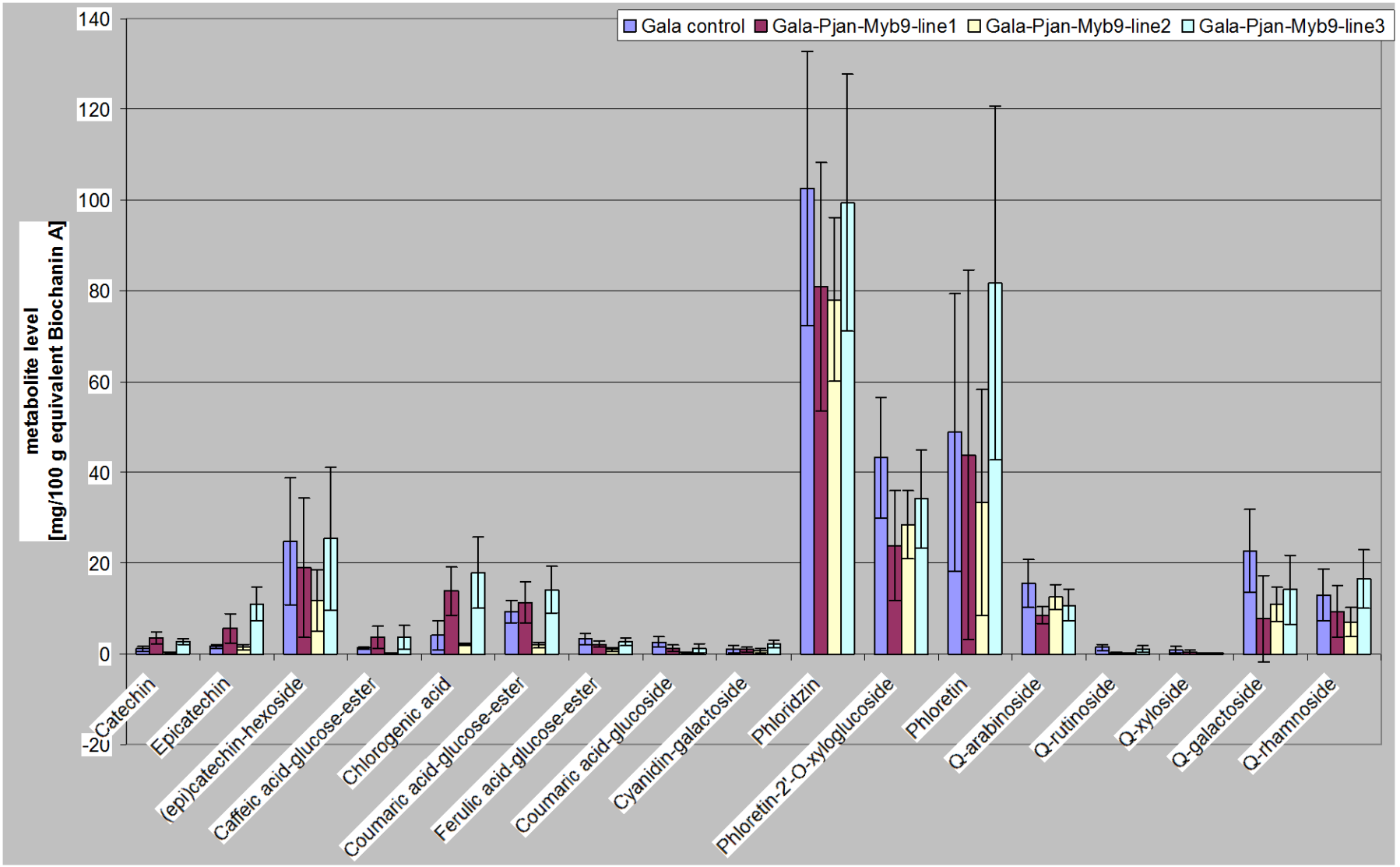


Fig.31. Metabolite levels of selected phenolic compounds in leaves taken from‘Gala’ plants transformed with the binary vector pJan- MdMyb9, relative to the levels in leaves from‘Gala’ non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

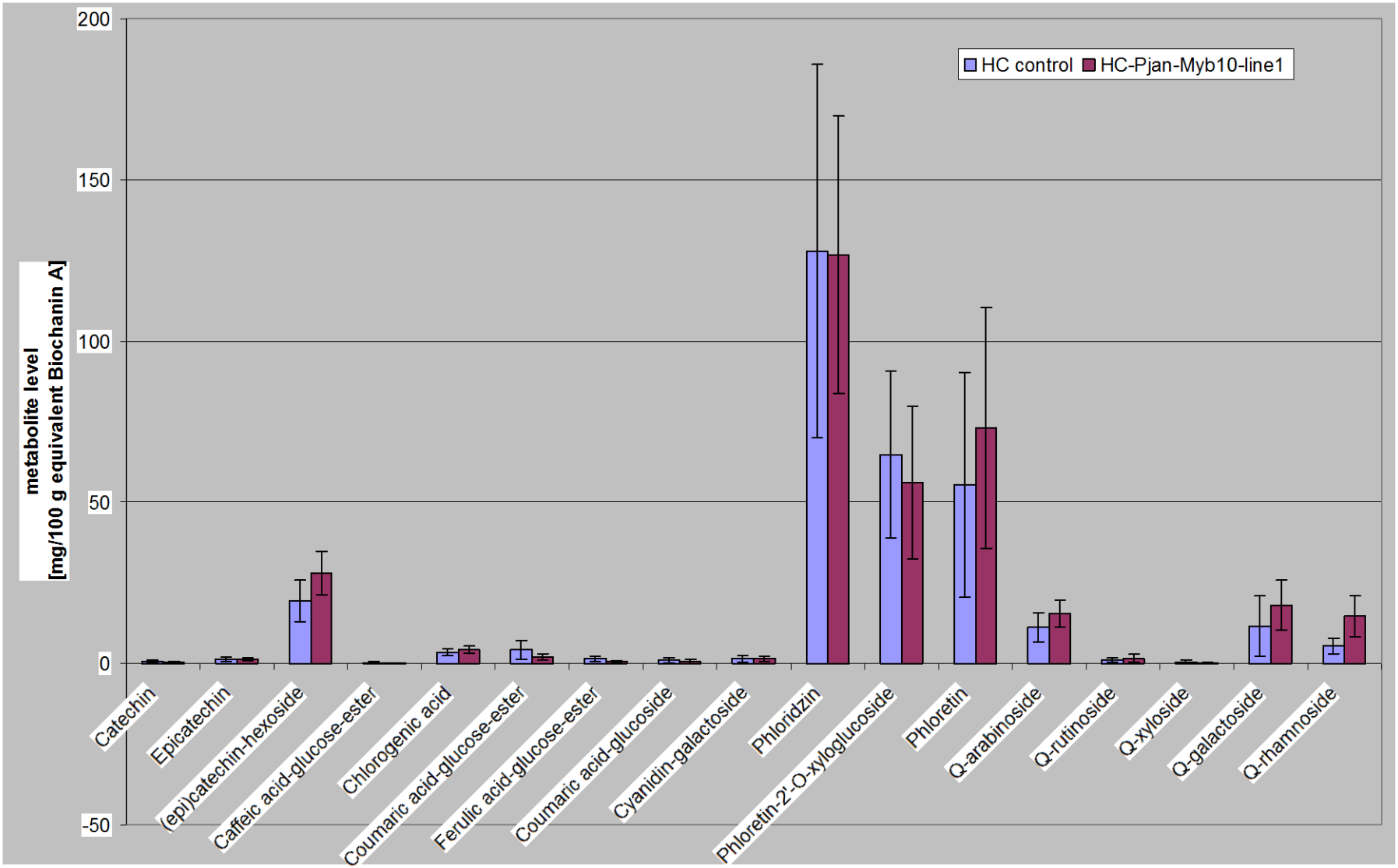


Fig.32. Metabolite levels of selected phenolic compounds in leaves taken from'HC' plants transformed with the binary vector pJan- MdMyb10, relative to the levels in leaves from'HC' non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

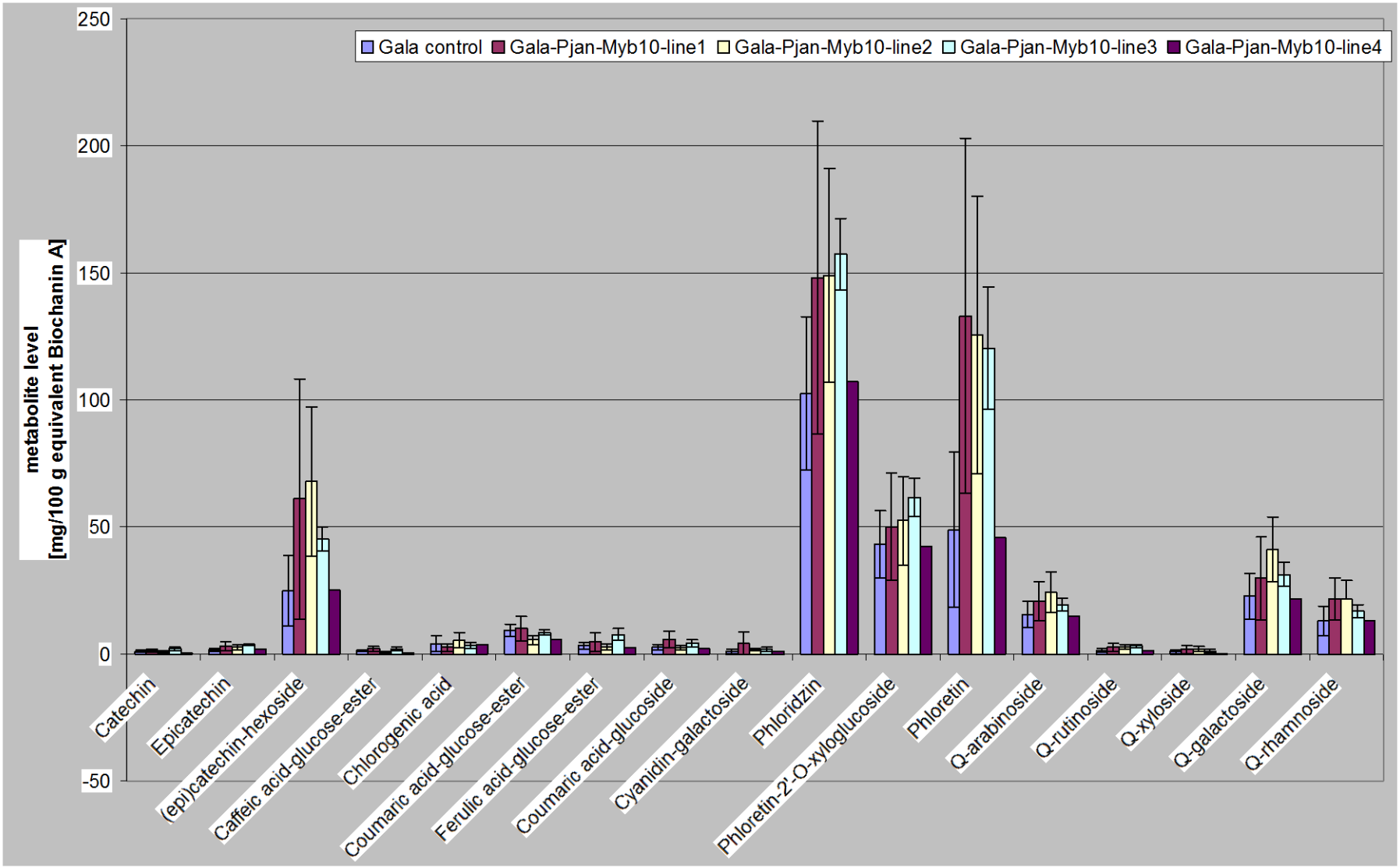


Fig.33. Metabolite levels of selected phenolic compounds in leaves taken from‘Gala’ plants transformed with the binary vector pJan- MdMyb10, relative to the levels in leaves from‘Gala’ non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

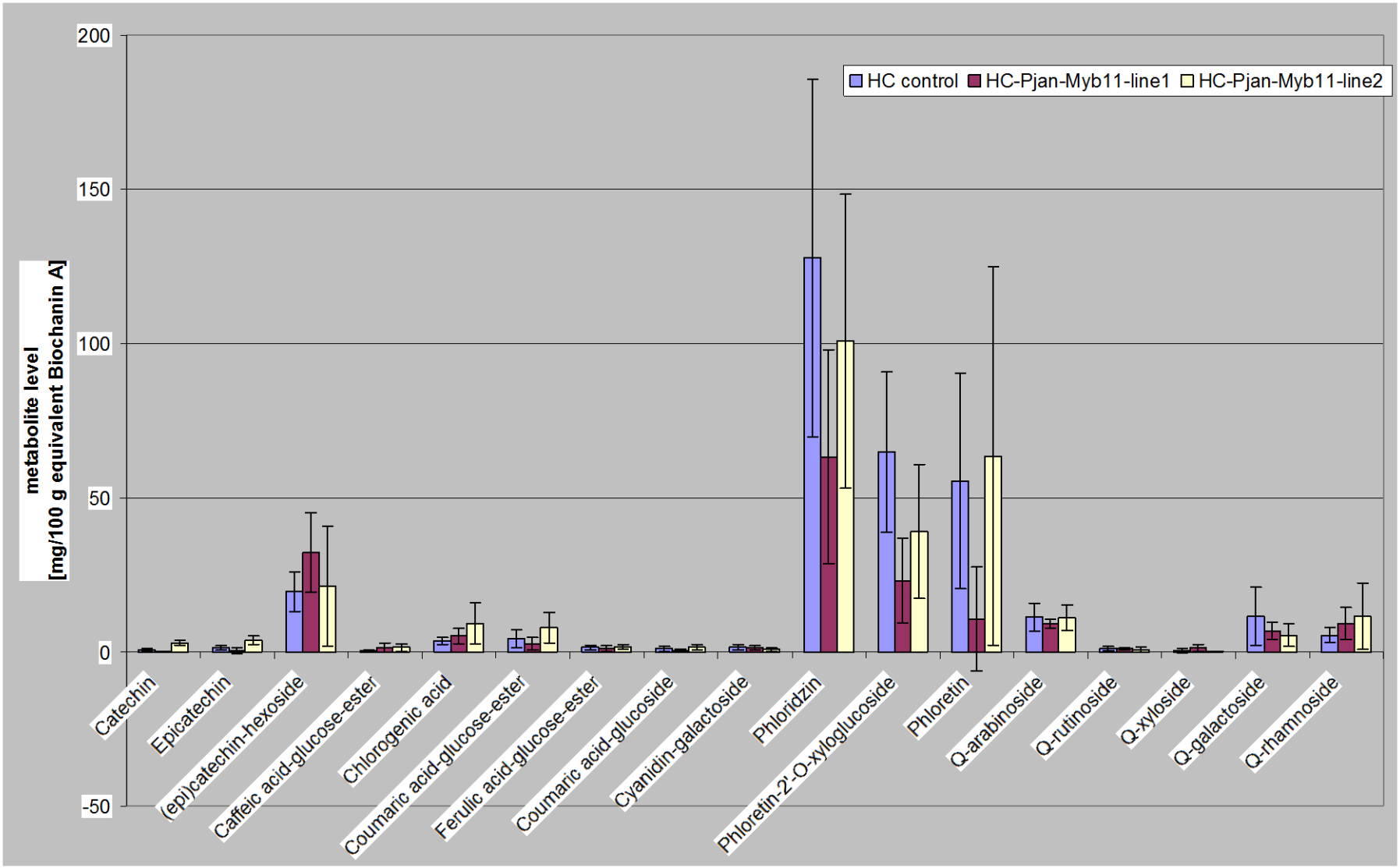


Fig.34. Metabolite levels of selected phenolic compounds in leaves taken from‘HC’ plants transformed with the binary vector pJan- MdMyb11, relative to the levels in leaves from‘HC’ non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

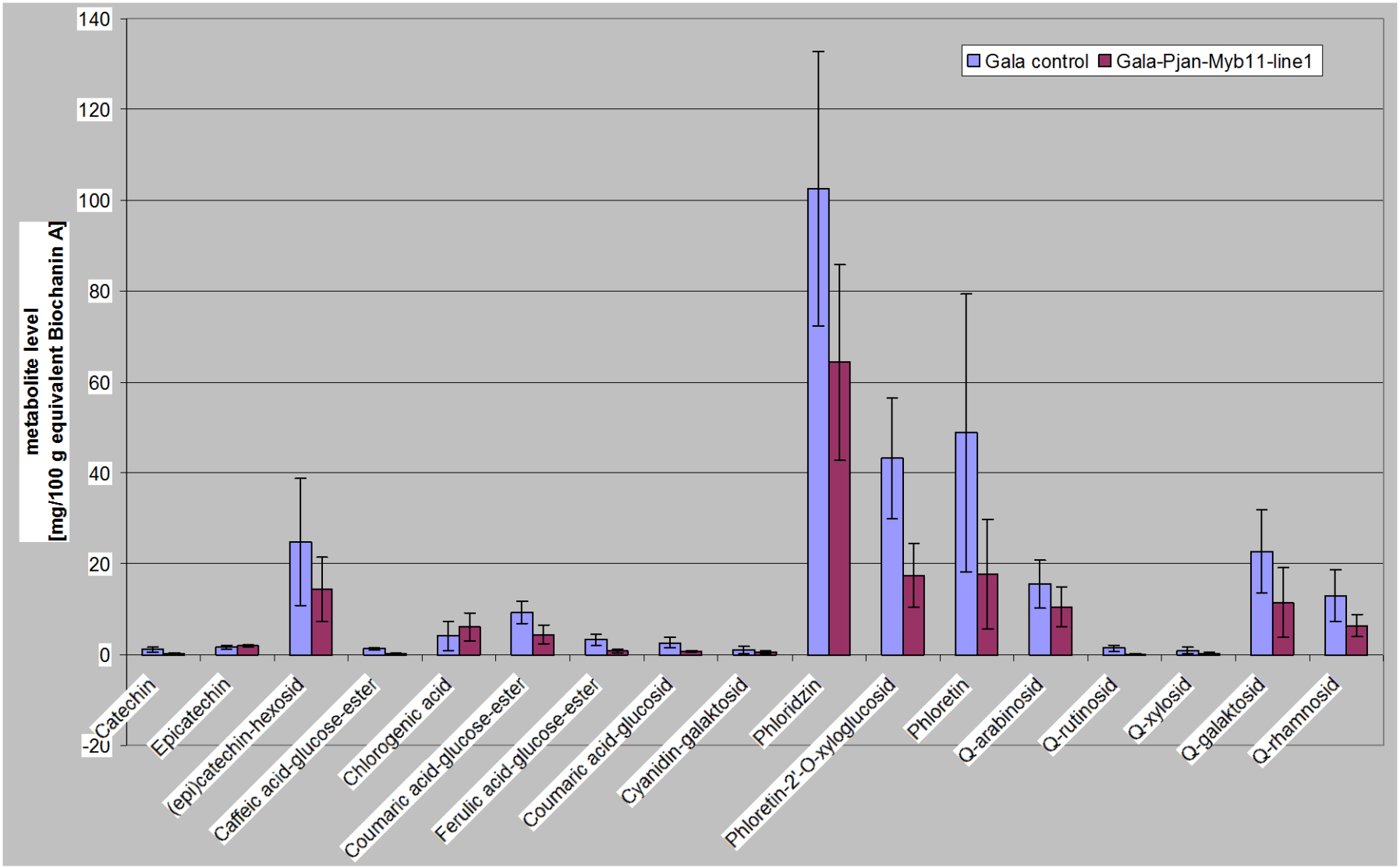


Fig.35. Metabolite levels of selected phenolic compounds in leaves taken from‘Gala’ plants transformed with the binary vector pJan- MdMyb11, relative to the levels in leaves from‘Gala’ non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

5.3 Transformation of *M.domestica* CV. 'HC' using the vector pGIIMH35S harboring *MdMyb10, MdMyb11* transcription factor genes

5.3.1 Cloning the *MdMyb10, MdMyb11* genes into the vector pGIIMH35S

The *MdMyb10, MdMyb11* genes were amplified from the vectors PJan-MdMyb10, PJan-MdMyb11, respectively, the amplified fragments were introduced into the new vector PGIIMH35S successfully, the integration of new fragments was confirmed by a restriction digest (Fig.36, 37,38) .

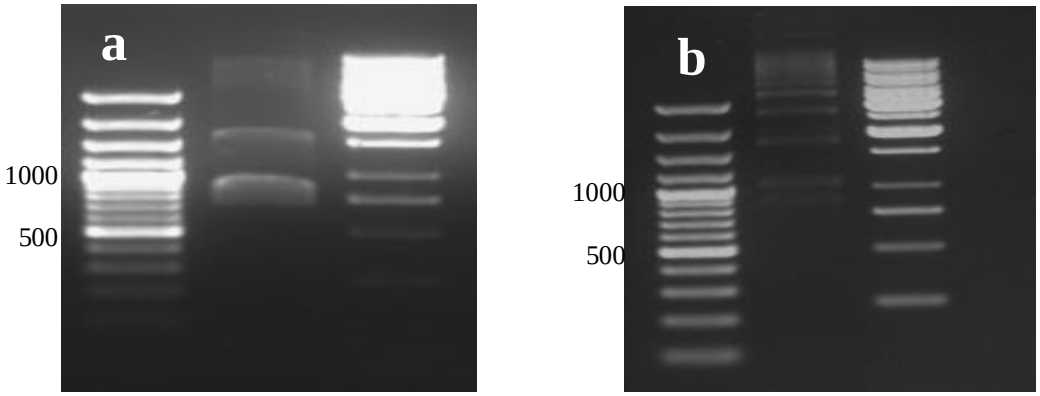


Fig.36. Ligation results for the new fragments with the new vector PGIIMH35S (a: MdMyb10, b: MdMyb11)

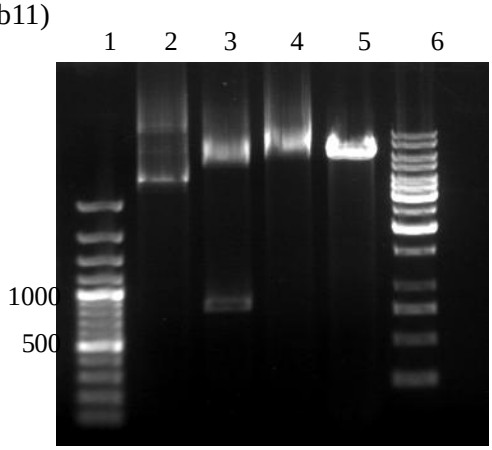


Fig.37. Restriction digest of the PGIIMH35S-MdMyb11 vector by using different restriction enzymes (lane1: 100bp marker, lane2: undigested plasmid, lane3: digested plasmid with *Xba*I and *Nco*I, lane4: digested plasmid with *Nco*I, lane5: digested plasmid with *Eco*RI, lane 6: 1Kb marker)

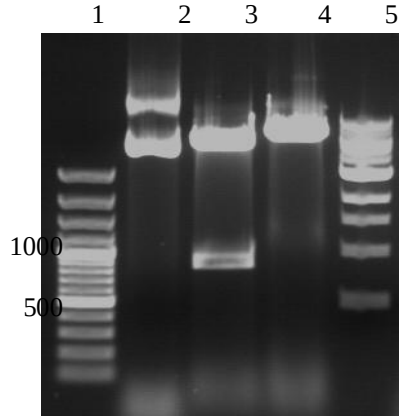


Fig.38. Restriction digest of the PGIIMH35S-MdMyb10 vector by using different restriction enzymes (lane1: 100bp marker, lane2: undigested plasmid, lane3: digested plasmid with *Xba*I and *Nco*I, lane4: digested plasmid with *Nco*I, lane 5: 1Kb marker)

5.3.2 Molecular analysis

5.3.2.1 PCR and southern blot analysis

5.3.2.1.1 Transformed plants using the vector pGIIMH35S harboring *MdMyb10*

The stable integration of the *MdMyb10* transcription factor gene into genomic DNA of transgenic plants was confirmed by using the primers Myb10 for., poly A-PGII rev. (see 4.1.1.8.2), which amplifies a 782-bp *MdMyb10* specific fragment(Fig.39).

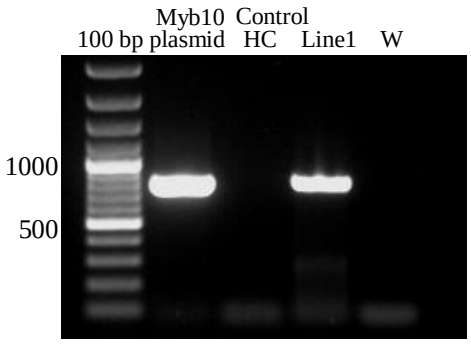


Fig.39. PCR of transgenic line of HC (transformed with PGIIMH35S-MdMyb10) using MYB10 for., and poly A -PGII rev. primers (spanning a 782 bp DNA fragment).

The genomic DNA from both non-transgenic and transgenic Plants was digested with *Eco*RI. Southern blot hybridization was performed using a DIG labeled PCR amplified probe. The result showed that one copy of a *MdMyb10* analog was found in all plants analysed (non – transgenic and transgenic plants). The *MdMyb10* gene was successfully integrated into the genome of the 'HC' transgenic plants; one copy was detected in line1, in addition to that found in the wild type (Fig.40).

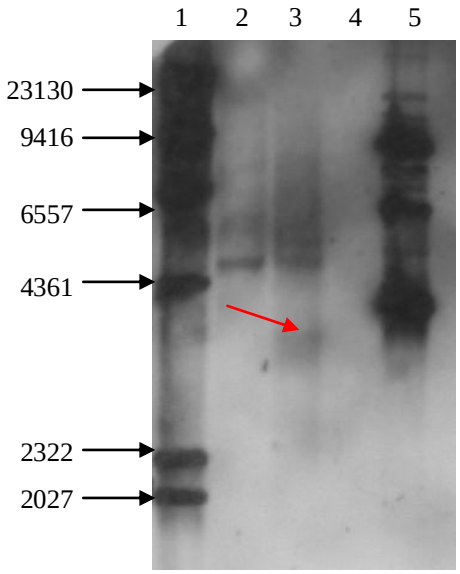


Fig.40. Southern blot analysis of g DNA for Hc transgenic plants transformed with pGIIMH35S-MdMyb10 digested with *Eco* RI, using MdMyb10 probe (1: Marker; 2: HC non-transgenic; 3: HC transgenic line1; 5: positive control (plasmid))

5.3.2.1.2 Transformed plants using the vector pGIIMH35S harboring *MdMyb11*

The stable integration of the *MdMyb11* transcription factor gene was confirmed by using the primers Myb11 for., poly A-PGII rev. (see 4.1.1.8.2), which amplify a 904-bp *MdMyb11* specific fragment (Fig.41).

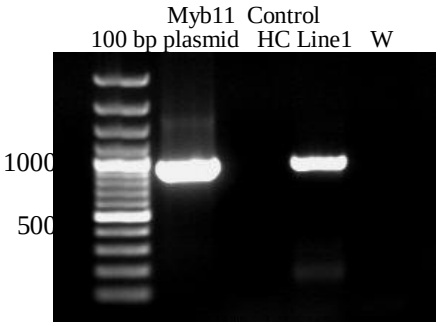


Fig.41.PCR of transgenic line of HC (transformed with PGIIMH35S-MdMyb11) using MYB11 primer forward and poly A- PGII reverse (spanning a 904 bp DNA fragment).

The genomic DNA from both non-transgenic and transgenic Plants was isolated and digested with *Eco*RI. Southern blot hybridization was performed using a DIG labeled PCR amplified probe. The result showed that one copy of *MdMyb11* analog was found in all analysed plants (non –transgenic and transgenic plants).

The *MdMyb11* gene was successfully integrated into the genome of the 'HC' transgenic plants; one gene copy was detected in line1, in addition to that found in the wild type (Fig.42).

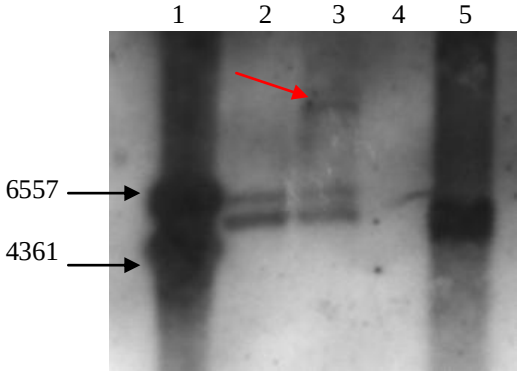


Fig.42. Southern blot analysis of g DNA for Hc transgenic plants transformed with pGIIMH35S-MdMyb11 digested with *Eco* RI, using MdMyb11 probe (1: Marker; 2: HC non-transgenic; 3: HC transgenic line1; 5: positive control (plasmid))

The presence of the *bar* gene was confirmed by using the primers bar-sense and bar-antisense (see 4.1.1.8.2), which amplify a 250-bp *bar*-specific fragment (Fig.43).

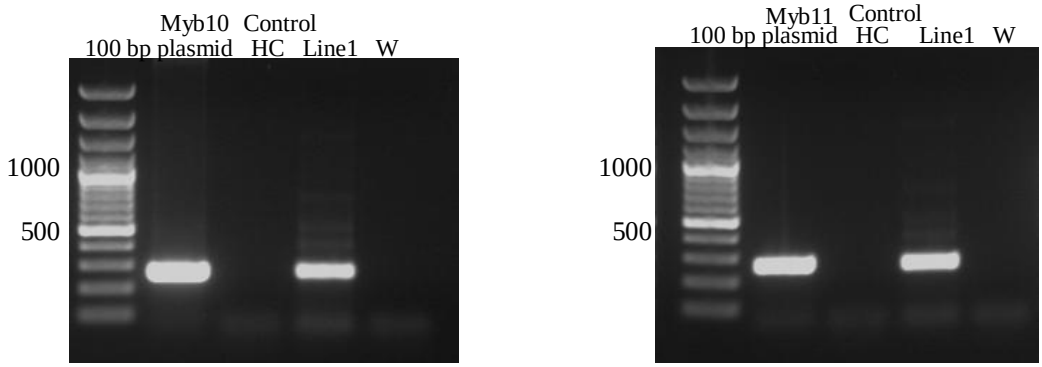


Fig.43. PCR of transgenic lines of HC (transformed with PGIIMH35S-MdMyb11, PGIIMH35S-MdMyb10) using bar sense and bar antisense primers (spanning a 250 bp DNA fragment of the *bar* gene).

5.3.2.2 Reverse transcriptase PCR analysis

5.3.2.2.1 Transformed plants using the vector pGIIMH35S harboring *MdMyb10*

RT-PCR was performed to confirm transcription of the transgenes using Myb10 specific for. and poly A-PGII rev. primers (the reverse primer from the vector after the stop codon and before Polyadenylation region) (see 4.1.1.8.2), the expected fragment with the size of 782 bp was amplified (Fig.44).

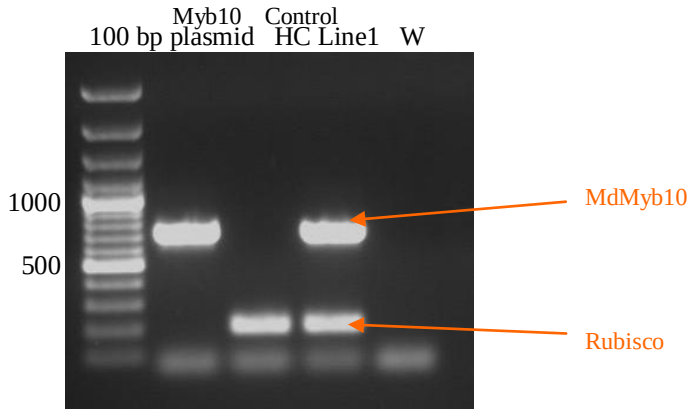


Fig.44. RT-PCR of transgenic lineof HC (transformed with pGIIMH35S - MdMyb10) using Myb10 for. and Poly-A-PGII rev. primers (spanning a 782bp cDNA fragment). Rubisco-for. and Rubisco rev. primers (spanning a 200bp cDNA fragment of the *Rubisco* gene) were used as a control.

5.3.2.2.2 Transformed plants using the vector pGIIMH35S harboring *MdMyb11*

RT-PCR was performed to confirm transcription of the transgenes using myb11 specific for. and poly A-PGII rev. primers (the reverse primer from the vector after the stop codon and before Polyadenylation region) (see 4.1.1.8.2), the expected fragment with the size of 904 bp was amplified (Fig.45).

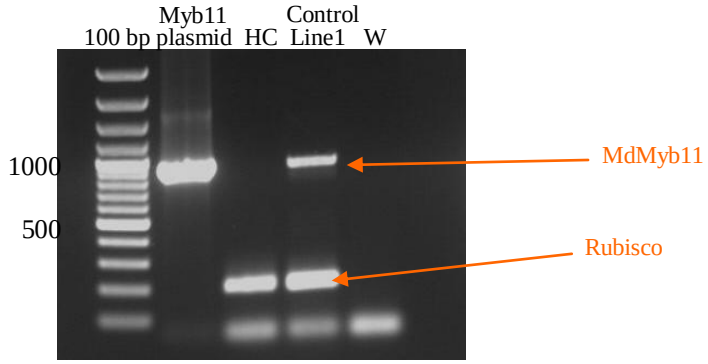


Fig.45. RT-PCR of transgenic line of HC (transformed with pGIIMH35S-MdMyb11) using Myb11 specific for. and poly-A-PGII rev. primers (spanning a 904bp cDNA fragment). Rubisco-for. and Rubisco rev. primers (spanning a 200bp cDNA fragment of the *Rubisco* gene) were used as a control.

5.3.3 Real Time -RT-PCR analysis

The Real time PCR results of samples taken from transgenic 'Holsteiner Cox' lines showed that the transcript levels of the *MdMyb10, MdMyb11* genes were increased in comparison to non-transgenic plants

5.3.3.1 Transformed plants using the vector pGIIMH35S harboring *MdMyb10*

Real-time PCR was performed using *MdMyb10* gene specific primers (see 4.1.1.8.3) to obtain transcription profiles for the transgene *MdMyb10*. Expression levels of *MdMyb10* gene were

determined and expressed relative to the *Rubisco* and the RNA *polymerase* genes. The transcripts of the transgene *MdMyb10* were detected in both transgenic and non-transgenic control plants.

The *MdMyb10* gene transcription level of the 'HC' transgenic line was 881.8-fold, in relation to non-transgenic 'HC' plants (Fig.46).

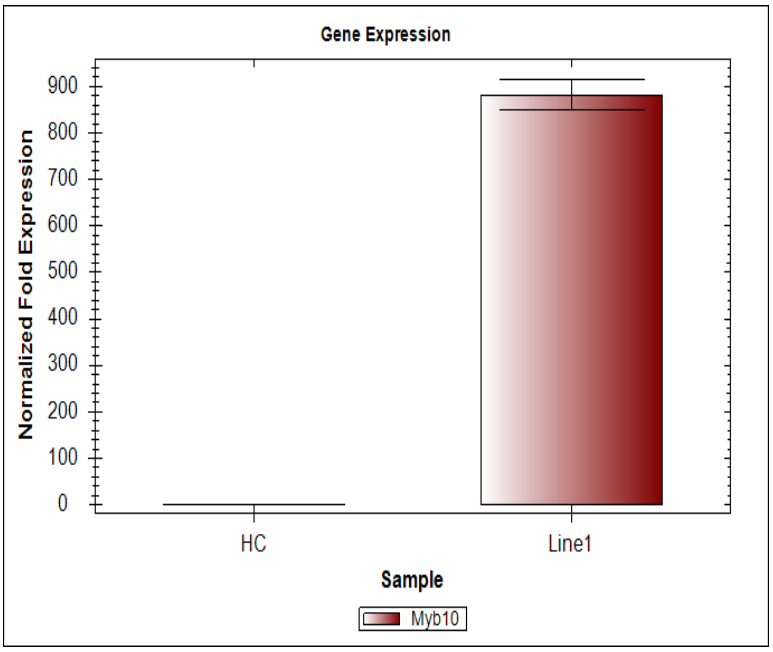


Fig.46. Expression of *MdMyb10* gene in HC transgenic line (transformed using pGTTMH35S – MdMyb10 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates.

5.3.3.2 Transformed plants using the vector pGIIMH35S harboring *MdMyb11*

Real Time PCR was performed using *MdMyb11* gene specific primers (see 4.1.1.8.3) to obtain transcription profiles for the transgene *MdMyb11*. Expression levels of *MdMyb11* gene were determined and expressed relative to the *Rubisco* and the *RNA polymerase* genes. The transcripts of the transgene *MdMyb11* were detected in both transgenic and non-transgenic control plants.

The *MdMyb11* gene transcription level of the 'HC' transgenic line was 5.3-fold, in relation to non-transgenic 'HC' plants (Fig.46).

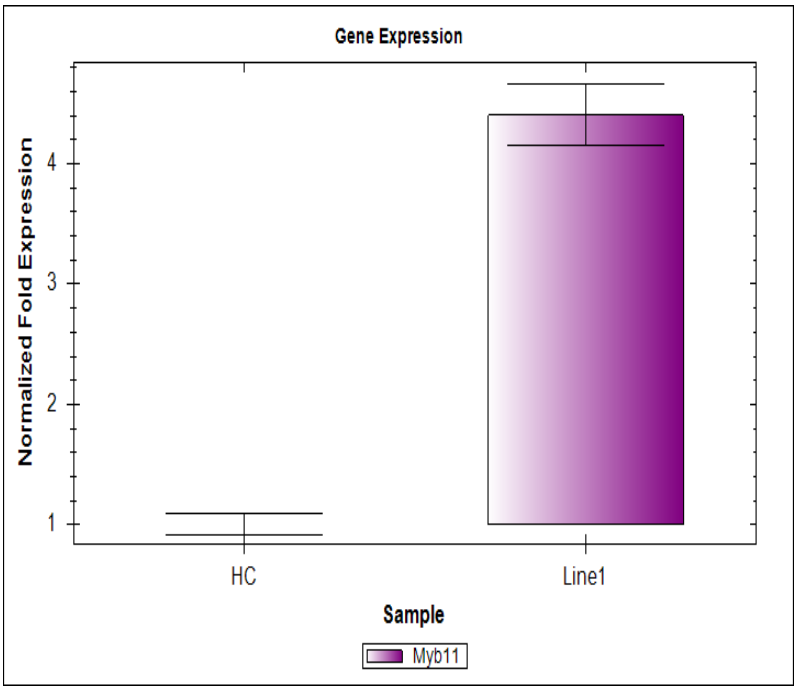


Fig.47. Expression of *MdMyb11* gene in HC transgenic line (transformed using pGTTMH35S – MdMyb11 vector) and respective control. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are mean and standard error (error bars ±1 SE) of three replicates.

5.3.4 Metabolic profiling

5.3.4.1 Quantitative analysis of flavonoid levels in *MdMyb10* transgenic plants

In *MdMyb10* 'HC' transgenic plants, some of the polyphenol classes analysed were induced and others were reduced (Fig.48, 50). The total content levels of flavan-3-ols were increased to 1.26 times in comparison to non-transgenic plants. Strongest rises were observed for Cyanidin 3-O-galactoside 11.56 times in *MdMyb10* 'HC' transgenic plants in comparison to non-transgenic plants. For the hydroxycinnamic acids, the levels in *MdMyb10* 'HC' transgenic plants were slightly increased, caffeic acid-glucose-ester showed rises 2.32 times, chlorogenic acid increased 2.47 times, the total content levels of the hydroxycinnamic acids were 1.6 times in comparison to non-transgenic plants. All of dihydrochalcones analysed were reduced, the total content levels in *MdMyb10* 'HC' transgenic plants were 0.57 times in comparison to non-transgenic plants. For the flavonols, all the flavonols analysed were reduced, the total content levels of the flavonols in *MdMyb10* 'HC' transgenic plants were 0.76 times in comparison to non-transgenic plants.

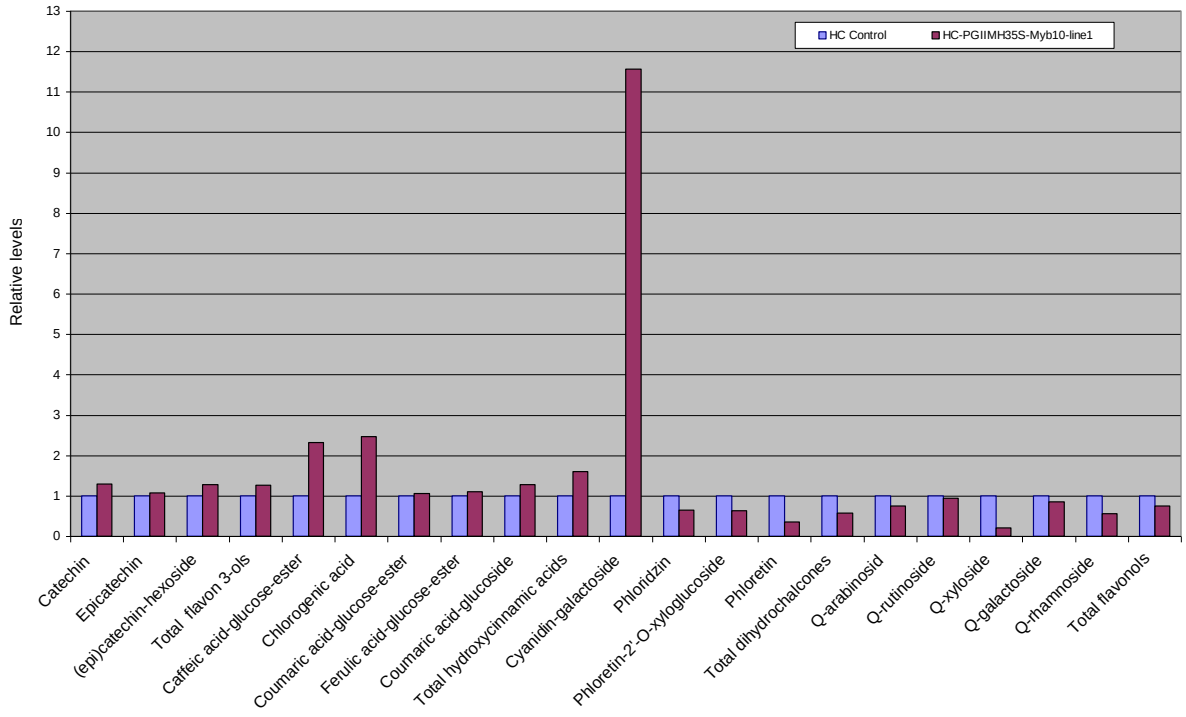


Fig.48. Relative levels of seleted phenolic compounds (values are metabolite levels of each compound in HC transgenic plants transformed with the binary vector pGIIMH35S-MdMyb10 comparing to the levels in non-transgenic plants).

5.3.4.2 Quantitative analysis of flavonoid levels in *MdMyb11* transgenic plants

In *MdMyb11* 'HC' transgenic plants, most of the polyphenol classes analysed were induced (Fig.49, 51). The total content levels of flavan-3-ols were increased 2.82 times, (epi)-catechin–hexoside and catechin showed raises 3 and 1.2 times, respectively, in comparison to non-transgenic plants. The level of Cyanidin 3-O-galactoside in *MdMyb11* 'Gala' transgenic plants were increased 2.6 times in comparison to non-transgenic plants. For the hydroxycinnamic acids the levels in *MdMyb11* 'HC' transgenic plants were increased in the case of chlorogenic acid 7 times , caffeic acid-glucose-ester showed rises 9.9 times , coumaric acid increased 1.53 times. The total content levels of the hydroxycinnamic acids were 3.46 times in comparison to non-transgenic plants. For the dihydrochalcones, the level of phloridzin was nearly the same as it is in non-transgenic plants, the total content levels of the dihydrochalcones were 0.76 times in comparison to non-transgenic plants. All of the flavonols analysed were reduced, the total content levels of the flavonols in *MdMyb11* 'HC' transgenic plants were 0.46 times in comparison to non-transgenic plants.

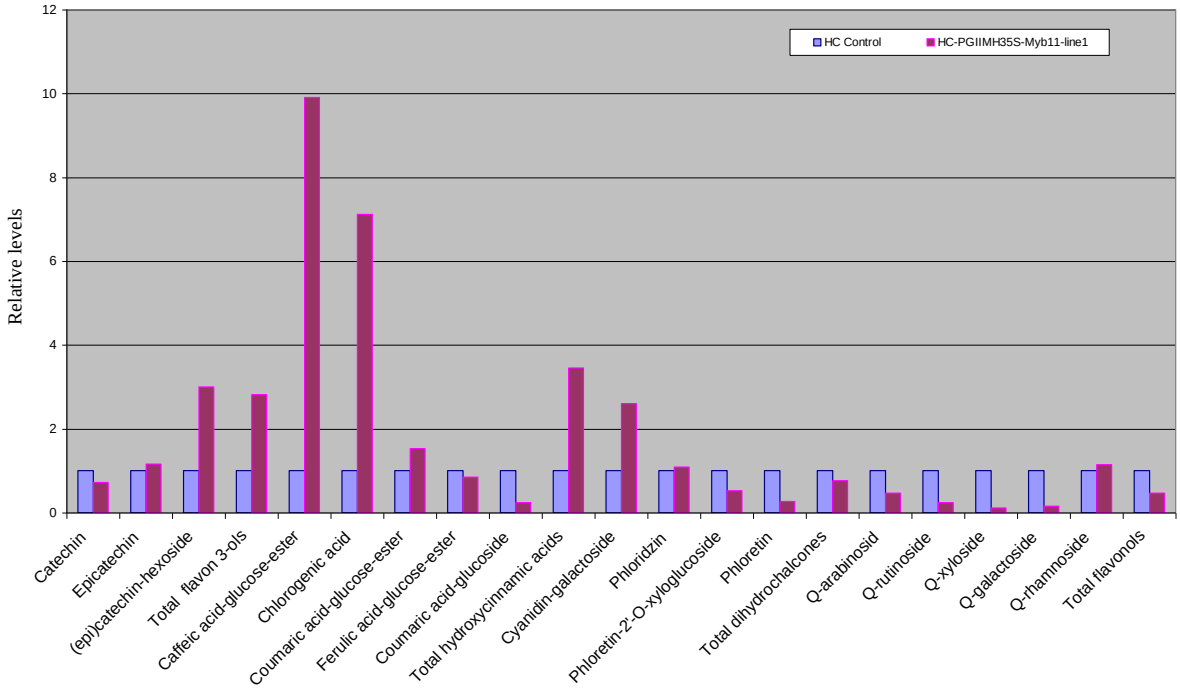


Fig.49. Relative level of selected phenolic compounds (values are metabolite levels of each compound in HC transgenic plants transformed with the binary vector pGIIMH35S-MdMyb11 comparing to the levels in non-transgenic plants).

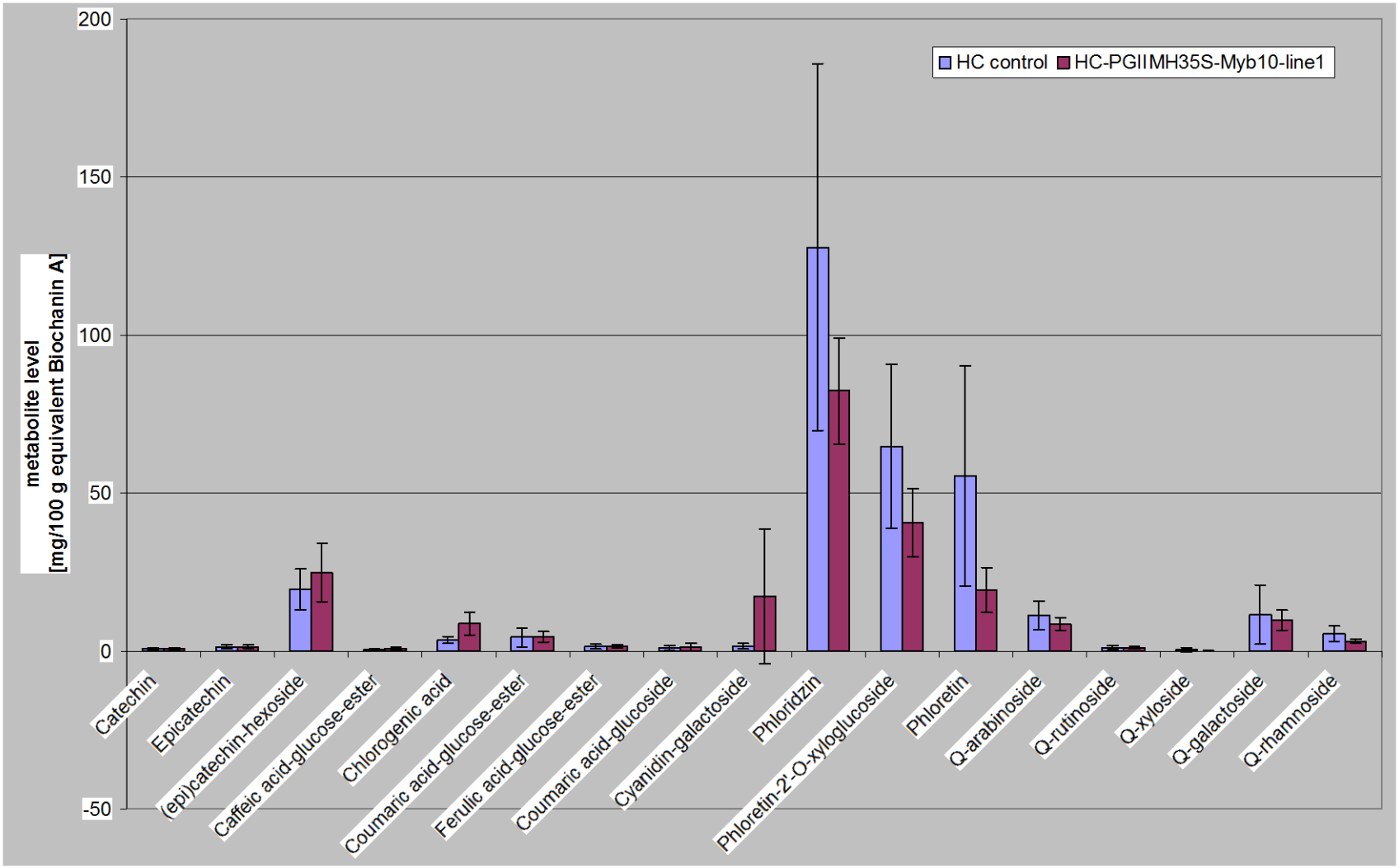


Fig.50. Metabolite levels of selected phenolic compounds in leaves taken from'HC' plants transformed with the binary vector pGIIMH35S- MdMyb10, relative to the levels in leaves from'HC' non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

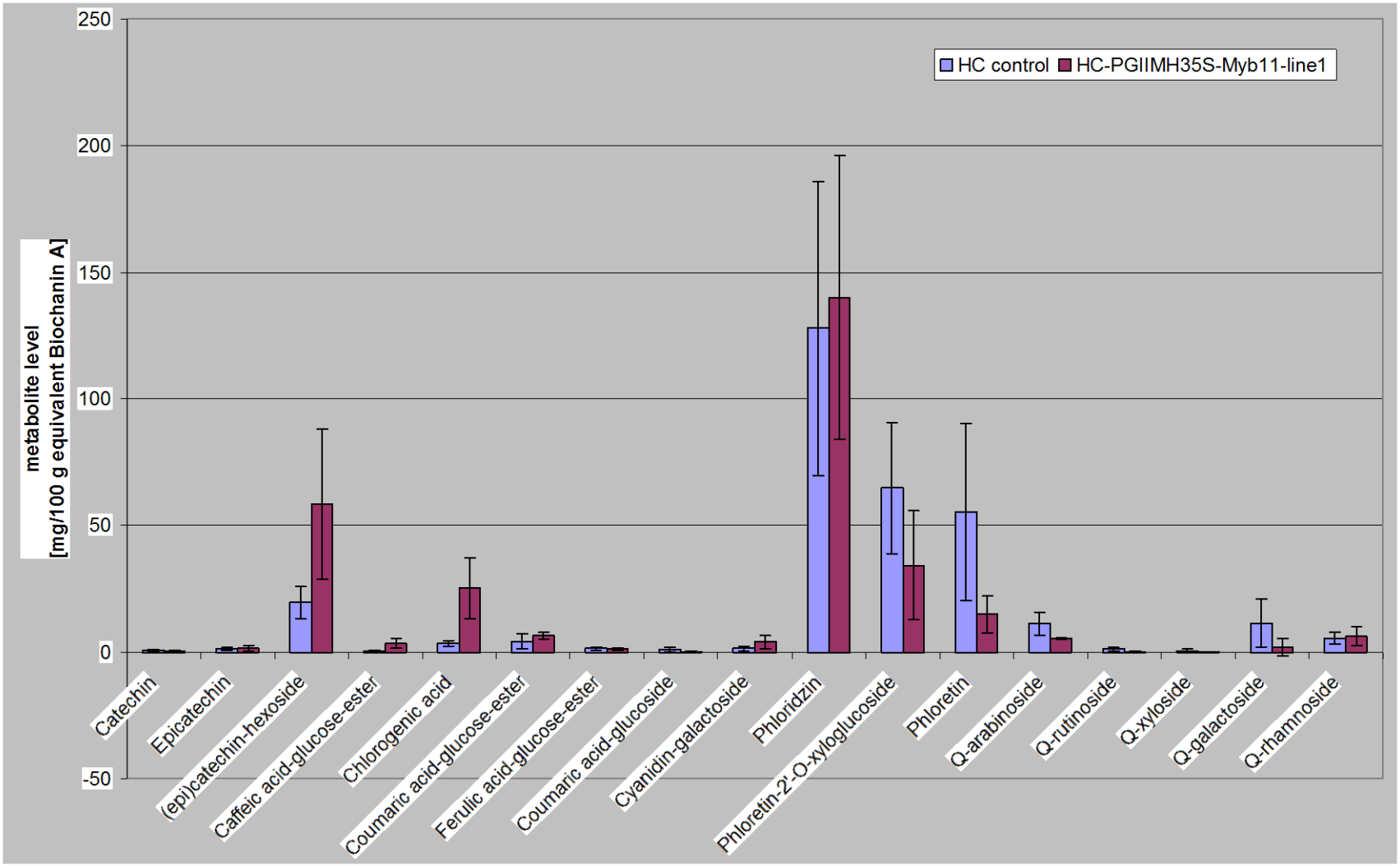


Fig.51. Metabolite levels of selected phenolic compounds in leaves taken from'HC' plants transformed with the binary vector pGIIMH35S- MdMyb11, relative to the levels in leaves from'HC' non- transgenic plants (values are expressed as mg equivalent Biochanin A per 100g of dry wt., values are mean of 5 replicates ±SD)

6 Discussion

6.1 Apple transformation and regeneration

In this study, direct shoot organogenesis was used from strips of the youngest leaves, after inoculation with an *Agrobacterium tumefaciens* harboring the binary vectors pJan-MdMyb9, pJan-MdMyb10, pJan-MdMyb11, PGIIMH35S-MdMyb10 and PGIIMH35S-MdMyb11.

6.1.1 Transformation efficiency

Two cultivars were used for transformation experiments. the transformation efficiency obtained in the present study ranged between 0.5 % and 1.2 %, with an average of 0.4 % for the whole 'HC' transformation experiments. When eliminating the experiments, which did not render any transgenic shoots, the efficiency became 0.64 %.

On the other hand, the transformation efficiency obtained for the whole 'Gala' transformation experiments ranged between 0.45 and 1.32, with an average of 0.6%, but when eliminating the experiments, which did not render any transgenic shoots, the efficiency became 0.84 %.

Szankowski *et al.* (2003) established a transformation system for the apple cultivars 'Holsteiner Cox' and 'Elstar', using the *bar* gene as selectable marker, and resulted in transformation efficiencies between (0.17 – 2.68%).

Degenhardt *et al.* (2006) recovered transgenic apple plants with efficiencies up to 24%, when they used a selection system based on the *phosphomannose-isomerase* gene (*pmi*) as a selectable marker and mannose as the selective agent was evaluated for the transformation of apple (*M. domestica)* cv. 'Holsteiner Cox'

6.1.2 Composition of regeneration media

In the present study, we used two different combinations of plant growth regulators for the respective cultivars. This is corresponding to the results of many previous studies on apple; Korban and chen (1992) demonstrated that, the optimum levels of plant growth regulator for shoot organogenesis could be influenced by cultivar. Yepes and Aldwinckle (1994) reported that the optimal media for regeneration of difference studied apple cultivars depend on the genotype. Puite and Schaart (1996) studied the effect of content of plant growth regulators in the media and the genotype on the regeneration of apple; they used in their study three apple cultivars 'Gala', 'Golden Delicious' and 'Elstar', they found the same result. Arinaitwe *et al.* (2000) demonstrated that cultivars differed significantly in their shoot proliferation responses to different TDZ concentrations.

MS medium was used as a basal medium in all the media as this medium was the most

commonly used for apple regeneration (Szankowski *et al.*, 2003; Belfanti *et al*., 2004a; 2004b; Li *et al*., 2007; Xu *et al*.,2009).

The concentration and the type of plant growth regulator added to the basal medium were depending on the cultivar used.

For 'HC' regeneration, TDZ at concentration of 3µM in combination with 1µM of IBA was used. Szankowski (2002) studied the effect of eight different concentrations of TDZ on the regeneration rates and the number of shoots per explant for apple cultivar 'HC'. They found that the regeneration rates and the number of shoots per explant did not differ significantly within the TDZ-series, the highest number of shoots per explant with a TDZ concentrations was reached with 3µM, this concentration was also used by (Li *et al*., 2007; Szankowski *et al*., 2009a) .

For 'Gala' regeneration, TDZ at concentration of 22.7 µM in combination with 2.6 µM of NAA was used. This combination was favorable and used by (Sansavini *et al*., 2003; 2004 and Belfanti *et al*., 2004a; 2004b). Other authors used different combinations of plant growth regulators for 'Gala' regeneration. For example (Yao *et al*., 1995) used a combination consisting of 22.2 µM BAP and 1.07 µM NAA, while (Puite and Schaart, 1996) found that, the best combination of plant growth regulators for 'Gala' regeneration consisting of 13.6 µM TDZ, 13.32 µM BAP, and 0.53 µM NAA.

Variability in differences in plant growth regulators necessary for regeneration of each genotype may be attributed to the difference in levels of endogenous hormones in these cultivars (Girish *et al.*, 2011).

6.1.3 Selectable markers

The selection of transgenic cells or tissues is a critical step in plant transformation, since it is the most important factor affecting the transformation efficiency (Zhang *et al*, 2005). Antibiotic or herbicide resistance genes are often used for selection of transgenic plants.

The most commonly used selectable marker gene for apple transformation was the neomycin phosphotransferase II (*npt*II) gene (De Bondt *et al*. 1996; Bolar *et al*. 2000; Liu *et al*., 2001; Sedira *et al*., 2001; Espley *et al*., 2007; Li *et al*., 2007; Szankowski, 2002, 2009a) which is responsible for the resistance against kanamycin.

Transformed plant cells of apple containing an *npt*II gene can theoretically survive under selection of 100 mg/l kanamycin (Szankowski, 2002).

In the first set of experiments in the present study, we used the *npt*II gene as an antibiotic resistance gene, we started with a concentration of 25mg/l kanamycin, and the concentration was increased to 50mg/l during the regeneration phase, a 75mg/l were applied when the

regenerated shoots were transferred to the micropropagation medium.

The most concentration used in previous studies was 50mg/l kanamycin as a selection agent for transgenic plants (Li *et al*., 2007; Xu *et al.*, 2009)

In a second set of experiments, we used the *bar* gene from *Streptomyces hygroscopicus*, thereby effecting resistance against the herbicide phosphinothricin/glufosinate ammonium. We started with 2.5mg/l PPT, and the concentration was increased until 5, 7.5 mg/l during the regeneration phase, 10mg/l PPT were applied when the regenerated shoots had to be transferred to the micropropagation medium.

De Bondt *et al*. (*1996*) also used the *bar* gene, to find out, whether or not the herbicide phosphinothricin is a better alternative to the antibiotic kanamycin for the selection of transgenic apple cv. 'Jonagold' shoots, they started directly after coculture with 10 mg/l ppt. but no transgenic ppt-resistant plants could be regenerated.

Szankowski *et al*. (2003) started with a concentration of 1 mg/l of the herbicide and they increased the concentration up to 10 mg/l during the regeneration phase. The immediate application of 10 mg/l at the beginning of regeneration may lead to massive cell death that probably also affects the viability of the transgenic cells. An initial low concentration of PPT might favour the transformed cells to reach a critical mass, which is supposed to be necessary for the organisation of *de novo* shoot meristems.

Li *et al*. (2007) also used the *bar* gene as a selectable marker gene, they started also with 1mg/l, then the concentration of applied PPT increased up to 5 mg/l in the micropropagation medium.

Although considerable improvement has been gained in the process of transformation in apple, the use of antibiotics and herbicides as selectable markers still imposes a limitation according to the consumer acceptance (Penna *et al*., 2002; Degenhardt and Szankowski, 2006). Thus, a major problem for genetically modified (GM) apple is the use of the *npt*II as a selection gene-marker for legal restrictions in Europe.

6.2 Phenotype

In the present study it was shown that the homologous overexpression of *MdMyb10* transcription factor gene enhances flavonoid biosynthesis in *M. domestica*.

'HC'*MdMyb10* transgenic plants resulted from transformation using both pJan-MdMyb10, pGIIMH35S- MdMyb10 constructs were highly phenotypically distinguishable from non-transgenic plants, red coloured calli, red shoots and red well growing plants were scored(Fig.9,10). Red and green shoots were both harvested from transgenic apple explants.

Espley *et al*, (2007) and Espley, (2009) reported that overexpression of *MdMyb10* in apple generated a strong phenotype, with highly pigmented plants due to enhanced levels of anthocyanin.

Li, 2007 show a similar result on phenotype change due the *LC* expression in 'HC' transgenic apple plants.

Recently, (Kortstee *et al*., 2011) suggested using of the *MdMyb10* as a marker gene in transformation of apple and other crops. Therefore, anthocyanin production as result of the apple *MdMYB10* gene can be used as a selectable marker for apple, strawberry and potato transformation, replacing kanamycin resistance.

The results showed that the red colour formed in the case of 'HC' transgenic plant using pGIIMH35S-MdMyb10 was deeper than'HC' transgenic plant using pJan-MdMyb10. The reason might be that in the first case the *MdMyb10* gene is under control of double 35S promoter.

On the other hand, 'Gala' transgenic plants resulted from transformation using pJan-MdMyb10 construct were highly phenotypically distinguishable from non-transgenic plants, only during the first 2-3 weeks from regeneration steps. This is contrary to the results obtained by (Espley, 2009). This type of variation can be explained by variation in expression of the introduced *MdMYB10* gene caused by copy number, position of integration or silencing of the transgene (Butaye *et al*., 2005).

Changes of the phenotype were detected in *MdMyb9* and *MdMyb11* transgenic plants for both cultivars used, represented an increase of some polyphenolic compounds and a decrease of others.

The results also showed that the homologous over expression of *MdMyb9* and *MdMyb11* transcription genes enhances flavonoid biosynthesis in *M.domestica*. However, no visible developmental phenotypes were observed during the deferent steps of transformation experiments, only some red colour was seen in the calli and parts of the explants in the first two weeks on the regeneration media.

In previous studies, the *MdMyb9, MdMyb11* were classified as genes that play a role in regulation of flavonoid and anthocyanine pathway. To explain its role as transcription factors involved in this pathway; Coffman *et al*. (1997) reported that regulation of gene expression is not mediated solely by activators, but also by the action of repressors; in some cases a transcription factor may perform both activities. Aharoni *et al*. (2001) found that flowers of transgenic tobacco lines overexpressing *FaMyb1* gene showed a severe reduction in pigmentation. A reduction in the level of cyanidin 3-rutinoside (an anthocyanin) and of

quercetin-glycosides (flavonols) was observed, suggesting that *FaMyb1* may play a key role in regulating the biosynthesis of anthocyanins and flavonols in strawberry. It may act to repress transcription in order to balance the levels of anthocyanin pigments produced at the latter stages of strawberry fruit maturation, and/or to regulate metabolite levels in various branches of the flavonoid biosynthetic pathway.

Recently, Brüggemann, 2011 studied the role of *MdMyb9, MdMyb11* in the regulation of the flavonoid pathway in apple. He found that *MdMyb9* and *MyMyb11* show high similarity to known proanthocyanidin-regulators, analysis of amino acid sequences of the two genes showed a close relationship to known regulators in the flavonoid biosynthetic pathway (*AtMyb123* from Arabidopsis and *OsMyb3* from Rice), in the branch that control the accumulation of proanthocyanidins.

In this study the results showed that the main branches of the flavonoid pathway were affected by overexpression of *MdMyb9, MdMyb11* genes were flavon-3-ols (catechins) and hydroxycinnamice acids. It is well known that flavon-3-ols are building blocks for proanthocyanidins. This is in corresponding with the results obtained by Brüggemann (2011).

Successful expression and functionality of the *bar* gene was confirmed by the leaf-painting assay. A high concentration of BASTA® was used (600 mg/l); this high concentration was used by (Kiesecker, 2000) on chickpea, (Hassan, 2006) used it on pea, (Briviba *et al.*, 2004; Rühmann *et al.*, 2006) on apple. Herbicide tolerance gives another advantage for the transformed plants as they to survive when the same herbicide is used to control weeds.

6.3 Molecular analysis

In the present study, we used endogenous genes for transformation, in a cis-genic approach. The integration of T-DNA into the genomic DNA of all transgenic lines was confirmed by PCR, for *MdMyb10* transgenic lines, we used specific primers For *MdMyb10* gene. Although, it was expected to find the amplicon in both non -transgenic and transgenic plants, it was found only in the transgenic lines. To explain why, we checked the database; we found that the *MdMyb10* endogenous gene has an intron with an approximately size 3Kb, this big size could not be amplified by the used primers, the only amplified fragments came from the *MdMyb10* inserted, which was amplified from cDNA and cloned into the binary vectors used.

On the other hand, when we used specific primers for the *MdMyb9* as well as *MdMyb11* gene, we got the same amplicon in non-transgenic and transgenic lines; with a size 200bp higher then the expected one. This guided us to use another strategy to confirm the integration of T-

DNA for each gene: one primer forward from the 35S promoter and the reverse primer was specific for the respective gene.

In Southern blot analysis, we used specific primers to generate the probes used for *MdMyb9*, *MdMyb10* genes, and we used one forward primer from the 35S promoter and one specific reverse primer to generate the probe for *MdMyb11* gene.

As it was expected, in all analysed transgenic lines as well as in non-transgenic plants, we obtained one gene copy for the endogenous genes. We also obtained a single copy in most of the transgenic lines analysed but two or four copies were also obtained.

Southern blot results showed it was difficult to distinguish between 'Gala' non-transgenic plants and *MdMyb9* 'Gala' transgenic plants (line1) (Fig.12), the reason could be that the insert gene was very closed or inside the endogenous gene

In reverse transcriptase PCR, when we use specific primers to confirm the transcript of transgenes, we obtained the expected amplicon in non- transgenic as well as in transgenic plants, because of transcription of endogenous genes. To be able to distinguish between non-transgenic and transgenic plants, we used one specific primer forward and the second from the binary vectors from the region after the stop codon and before the polyadenylation site.

6.4 Real Time analysis and phenotype

6.4.1 MdMyb9

In the present study, the expression of the *MdMyb9* gene transcript showed significant increases in 'HC' and 'Gala' transgenic plants in comparison to non-transgenic controls. There was 47- fold increase in relative transcript levels for the 'HC' transgenic plants, while 'Gala' transgenic plants showed increases ranging between 3.7- fold (line1) and 1451- fold (line2).

Although, the *MdMyb9* gene was considered as one of the transcription factor genes that, regulate the anthocyanin biosynthetic pathway (Chagné *et al.*, 2007), there was no change of phenotype correlating with the increases in relative transcript levels. The reason could be that the branches of flavonoid biosynthesis, and the structural genes affected by overexpression of *MdMyb9* gene do not lead to anthocyanicin accumulation, but to accumulation of other compounds (flavon-3-ols, hydroxycinnamic acids).

6.4.2 MdMyb10

The expression of *MdMyb10* gene led to transcript increases in 'HC' transgenic plants in comparison to the non-transgenic control (fig.22, 45). There was a 1261- fold increase in

relative transcript levels for the transformed plants with the binary vector pJan-Mdmyb10, and an 847-fold increase for the transformed plants with the binary vector pGIIMH35S-Mdmyb10. The increases in transcript levels correlate with the red colour appearance (change of phenotype) (Fig.9, 10).

Similar results were published from other species: there was always a correlation between the high transcript levels of *Myb10* and accumulation of anthocyanin. Expression of sweet cherry *PavMYB10* gene transcript was examined using qPCR analysis during fruit development in two cherry cultivars, 'Rainier' and 'Stella'. Transcript of *PavMYB10* accumulated in the fruit tissues is much higher in the fruit at the latter two stages of fruit development (Lin Wang *et al.*, 2010). This confirmed the correlation between the change of phenotype (red colour at the latter two stages of cherry fruit development) and the high transcript levels of *Myb10*.

On the other hand, the expression of *MdMyb10* gene transcript showed also an increase in 'Gala' transgenic plants in comparison to the non-transgenic control (Fig.23). There was a 150-800-fold increase in relative transcript levels for the transformed plants using the binary vector pJan-MdMyb10, but the change of colour was observed only in the first two weeks during the regeneration steps. This can be explained by the fact that different cultivars shows different transcript levels for the same gene which in correspond with (Lin-Wang *et al.,* 2010) results, who reported expression of the strawberry genes, *FvMYB10* and *FaMYB10*, was examined during a fruit development series of wild diploid strawberry *Fragaria vesca* and cultivated octaploid strawberry *Fragaria ananassa*. There was a high increase in the relative transcript levels of the *MYB10* transcription factor in the fruit tissues. In *F. ananassa*, transcript levels of *FaMYB10* were detectable but low until fruit were at full size. Upon ripening and colour change, there was an almost 40,000-fold increase in relative transcript level. Expression levels of *FvMYB10* in *F. vesca* also correlate with colour change .

Espley *et al*.(2007)also reported that qPCR analysis of the expression of the *MdMYB10* gene in both red- and white-fleshed cultivars of apple during fruit development revealed massive increases in the relative transcript levels of *MdMYB10* in the fruit tissues of 'Red Field' compared with 'Pacific Rose^{TM}'. In 'Pacific Rose^{TM}' cortex, transcript levels were barely detectable, whilst in 'Pacific Rose^{TM}' skin, transcript was detectable only by mid-season, and the level of transcript correlated with changes in the transcript levels of the biosynthetic genes, particularly at the 102 DAFB time point.

Ban *et al*. (2007) reported that, *MdMybA* expression was analyzed by northern blotting. The transcript of *MdMyb A* was detected only in red-colored skin at 116 DAFB, although high levels of anthocyanin accumulation were observed at both 16 and 116 DAFB when

anthocyanin concentrations in the skin samples were measured. Moreover, the expression levels of *MdMybA* in 'Jonathan' (a deep-red cultivar) was much higher than that in 'Tsugaru' (a pale-red cultivar).

Similar data were also reported by (Honda *et al.,* 2002) and (Ubi *et al.,* 2006), the final anthocyanin concentrations in 'Jonathan' were much higher than those in 'Tsugaru' (about 20 times higher in absorbance at 530 nm). These results indicate that the level of *MdMYBA* expression is positively correlated with the accumulation of anthocyanins in apple skin after a certain age (i.e. not in young fruit skin).

6.4.3 MdMyb11

In the present study, when 'HC' and 'Gala' cultivars transformed with the binary vector pJan-MdMyb11, the expression of the *MdMyb11* gene transcripts showed moderate increases in 'HC' and 'Gala' transgenic plants in comparison to non-transgenic ones. There was 6- fold increase in relative transcript levels for the 'HC' transgenic plants, while 'Gala' transgenic plants showed increases up to 10-fold.

On the other hand, when the construct pGIIMH35S was used to transform 'HC' cultivar, the expression of the *MdMyb11* gene transcript showed 4-fold increase in relative transcript levels for the 'HC' transgenic plants.

The results of this study showed that there was no change of phenotype correlate with the increases in relative transcript levels. The reason could be that, the branches of flavonoid biosynthetic pathway, and the structural genes were affected by overexpression of *MdMyb11* gene.

To explain this, the results of (Soglio *et al*., 2009) showed that, the expression level of two enzymes belonging to flavonoid biosynthetic pathway; chalcone synthase I, chalcone-flavone isomerase were decreased from May to September, when a remarkable decrease of the *MdMyb11* was seen by cDNA microarray analysis, during the same period. This can be explained that there is a correlation between the expression of *MdMyb11* and the genes encoding these two enzymes, which are placed at the beginning of flavonoid biosynthetic pathway, which mean accumulation of the phenolic compounds in this place.

This is correspond with the results obtained by Stracke *et al.* (2007) who determined the regulatory potential of three transcription factors *Myb11*, *Myb12* and *Myb111*. this group used a combination of genetic, functional genomics and metabolite analysis approaches to show a high degree of functional similarity and displayed very similar target gene specificities for several genes of flavonoid biosynthesis, including chalcone synthase, chalcone isomerase,

flavonone 3-Hydroxylase and flavonols synthase1.

The results of real time PCR confirmed that the three transcription factors used in this study overexpressed, the effect of this expression led to change of phenotype in the case of the *MdMyb10* gene, which mean accumulation of anthocyanins. While no changes of phenotype in the case of the *MdMyb9, MdMyb11* genes, which mean accumulation of others compounds in the flavonoid pathway.

6.5 Metabolites

In the present study, transformation of 'HC' and 'Gala' apple cultivars with the binary vector pJan- MdMyb9, led to an increase of the total contents of flavon-3-ols for 1.21 and 1.41 times in comparison to non –transgenic 'HC' and 'Gala' plants respectively.

Transformation of the 'HC' apple cultivar with the binary vector pJan-MdMyb11, led to an increase of the total contents of flavon-3-ols 1.53 times. Moreover, transformation of 'HC' with the vector pGIIMH35S-*MdMyb11*, led to an increase of the total contents of flavon-3-ols for 2.82 times.

The relation between flavon-3-ols levels and plant diseases resistance was the topic of many previous studies.

Treutter and Feucht (1990b) reported higher levels of flavan-3-o1s in apple leaf tissues of *V. inaequalis* resistant cultivars in relation to the susceptible ones. They explained the reason for the presence of catechins (flavan-3-o1s) in defence mechanisms of plants which is probably that flavan-3-ols may interact with proteins and inhibit the enzymes secreted by diverse pathogenic fungi.

Feucht *et al.* (1992) observed a dramatic increase of catechins and their polymers in the boundary zones around the infection of *V. inaequalis* in apple leaves. Resistant cultivars had higher concentrations of flavan-3-ols in leaf tissue and fruit skins than susceptible ones; levels were 6.5-fold higher in leaves and 3-fold higher in fruit skins. Approximately twice the number of different flavan-3-ols was found in leaves and fruit skin of the resistant group than in the susceptible one. They also indicated that epicatechin is the main flavanol synthesised during damage to fruit by the *V. inaequalis* fungus.

In cherry leaves infected by *Blumeriella jaapii*, higher concentrations of catechin, epicatechin were discovered, compared with healthy tissues (Niederleitner *et al*., 1994).

On the other hand , 'HC' and 'Gala' plants transformed with the pJan- *MdMyb9* binary vector showed a 2 and 1.92 times increases in the total content of hydroxycinnamic acids in comparison to 'HC' and 'Gala' controls, respectively. And also 'HC' plants transformed with

the pJan-MdMyb11 binary vector showed a 2 times increases in the total content of hydroxycinnamic acids in comparison to 'HC' controls . Moreover, 'HC' plants transformed with pGIIMH35S-MdMyb11 binary vector showed a 3.46 times increases in the total content of hydroxycinnamic acids in comparison to 'HC' controls. The relation between hydroxycinnamic acids levels and plant disease resistance was analysed in previous studies. Picinelli *et al*. (1995) reported that the content of chlorogenic acid and coumaric acid derivatives were generally higher in the leaves of resistant apple cultivars than in the sensitive ones. They found higher levels of two derivatives of p-coumaric acid, called p-coumaric1 and p-coumaric 2, in leaves of polygenic resistant varieties. In resistant cultivars, phenolics, such as chlorogenic, caffeic and ferulic acids accumulate at a faster rate than in the susceptible ones (Picinelli *et al*., 1995; Usenik *et al*., 2004; Treutter, 2005) .

Mikulic Petkovšek *et al*. (2003) studied the content of chlorogenic acid in three scab resistant apple cultivars ('Topaz', 'Gold Rush', 'Goldstar') and two scab susceptible ones ('Golden Delicious Weinsberg', 'Golden Delicious Clone B'). Leaves of the cultivar 'Goldrush' contained statistically significantly larger quantities of chlorogenic acid than leaves of the remaining scab resistant and scab susceptible cultivars. They reported that the scab infection of the leaves of scab susceptible cultivars affected the accumulation of chlorogenic acid in the way that its content increased with the degree of infection. Greater amount of chlorogenic acid was found in the leaves of the cultivar 'Golden Delicious Weinsberg' than in the leaves of the cultivar 'Golden Delicious Clone B'.

Mikulic Petkovšek *et al*. (2007) found that the values of the scab resistant cultivars for chlorogenic acid were at a higher level, compared to susceptible cultivars.

Mikulic Petkovšek *et al*. (2009) reported that tissue infected with *Venturia inaequalis* showed in comparison to the healthy tissue up to 7.6 times more hydroxycinnamic acids and up to 2.6 times more flavan-3-ols.

Schovánková and Opatová. (2011) studied the defensive reaction of the apple (*M. domestica* Borkh.) cultivar 'Idared' after inoculation with three different pathogens (*Penicillium expansum*, *Monilinia fructigena*, and *Gloeosporium* spp.). Changes in phenolic content and activity of phenylalanine-ammonia lyase were determined after 7, 14, and 21 days after the inoculation. The increase in phenols concentration and in phenylalanine-ammonia lyase activity varied in the place of fungal attack, in the tissues around rotten zone and in the healthy part. Increases of the concentration of chlorogenic acid in the peel were obtained after the inoculation with *Penicillium expansum.*

In the present study 'HC' and 'Gala' plants transformed using the binary vector pJan-

MdMyb10 showed increases in the total contents of polyphenol compounds analysed up to 1.1 and 1.96 times in comparison to non –transgenic 'HC' and 'Gala' plants, respectively.

The relation between polyphenol compounds levels and plant disease resistance was mentioned in many previous studies.

Mikulic Petkovšek *et al*. (2008, 2009) reported that the content level of total phenolics in the infected tissue was 1.3–2.4 times higher than in the healthy leaves and fruit.

In details, we can see that 'HC' and 'Gala' apple plants transformed with the binary vector pJan-MdMyb10, showed increases in the total content of dihydrochalcons up to 1.1 and 1.7 times in comparison to non–transgenic 'HC' and 'Gala' plants, respectively.

It was suggested by Hamauzu (2006) that phloridzin (dihydrochalcon) is hydrolysed *in vivo* by various fungi (*V. inaequalis* included) to create phloretin, which, in turn, is degraded to phloroglucinol, phloretic acid and p-hydroxybenzoic acid, which inhibit the development of *V. inaequalis* .

Mikulic Petkovšek *et al*. (2009) also found that the infected leaves contained statistically more phloridzin in comparison with the healthy ones – i.e. from 1.2 to 2.8 times more. It is evident that infection with the *V. inaequalis* fungus increased higher phloridzin synthesis. Leser and Treutter (2005) also confirmed this effect.

On the other hand, 'HC' and 'Gala' apple plants transformed with the binary vector pJan - MdMyb10, showed increases in the total content of flavonols up to 1.6 and 1.7 times in comparison to 'HC' and 'Gala' non-transgenic plants, respectively.

Mikulic Petkovšek *et al*. (2009) reported that tissue infected with *Venturia inaequalis* showed in comparison to the healthy tissue up to 2.9 times higher values of flavanols.

Feucht (1994) gave the same conclusion, that leaves infected with the *V. inaequalis* fungus accumulated flavonols. In contrast, Picinelli *et al.* (1995) found no relation between flavonol levels and scab resistance in apples.

The role of flavonoids in plant disease resistance is well studied, and confirmed in numbers of previous researches, The present study provides a way to investigate and take advantage of this role through changes and the resulting increase in these compounds.

6.6 Perspectives

The results of the present study exhibit that overexpression of the transcription factor genes *MdMyb9* and *MdMyb11* led to increases of some polyphenolic compounds and the decrease of others. The reason could be that the branches of flavonoid biosynthetic pathways and the structural genes were affected by this overexpression.

In addition, the reason might be that regulation of the structural genes involved in anthocyanin biosynthesis is directly controlled by a combination of two distinct transcription factor families with homology to the protein encoded by the vertebrate proto-oncogene c-Myb, and the vertebrate basic-helix-loop-helix (bHLH) protein encoded by the proto-oncogene c-MYC, respectively (Mol *et al.,* 1998). Based on this, it can be assumed that the *MdMyb9*, *MdMyb11* genes influence the anthocyanins biosynthetic pathway with a combination of one of basic helix–loop–helix (bHLH) transcription factors, which is missing here.

8 OUTLOOK AND FURTHER EXPERIMENTS

In the present study, different transgenic apple lines from two cultivars were obtained. Those lines are still *in vitro,* more Real Time PCR analysis can be done to know the effect of overexpression of the different transcription factors used on the structural genes involved in the flavonoid pathway, and to detect which genes were affected.

It is necessary to make more morphological evaluation of greenhouse transgenic plants and evaluation for plant disease resistance by infection with one bacterial disease, such as *Erwinia amylovora* which caused fire blight on apple, or infection with the *Venturia inaequalis* fungi which caused the apple scab.

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Appendix I. Content of individual phenolic compounds (mean ± SD, mg /100 g dry. wt., n=5) total phenolic compounds (mg /100g dry .wt.) in leaves were taken from 'HC' non-transgenic Plants and 'HC' transgenic plants (For all constructs used in this study)

	HC control	HC-Pjan-Myb9-line1	HC-Pjan-Myb10-line1	HC-Pjan-Myb11-line1	HC-Pjan-Myb11-line2	HC-PGIIMH35S-Myb10-line1	HC-PGIIMH35S-Myb11-line1
phenolic compounds							
Catechin	0.61±0.43	1.86±0.87	0.47±0.29	0.09±0.11	2.98±0.78	0.79±0.31	0.44±0.4
Epicatechin	1.28±0.61	2.99±0.6	1.35±0.46	0.42±0.81	3.69±1.36	1.37±0.59	1.49±1.17
(epi)catechin-hexoside	19.52±6.48	21.07±3.97	28.1±6.71	32.33±12.89	21.26±19.58	24.82±9.29	58.48±29.75
Total flavan-3-ols	**21.41**	**25.92**	**29.92**	**32.84**	**27.93**	**26.98**	**60.41**
Caffeic acid-glucose-ester	0.36±0.33	1.41±0.6	0.16±0.05	1.42±1.46	1.46±1.18	0.83±0.45	3.56±1.9
Chlorogenic acid	3.56±1.09	6.44±1.18	4.35±1.06	5.09±2.52	9.3±6.74	8.79±3.64	25.35±11.96
Coumaric acid-glucose-ester	4.3±2.96	7.94±4.28	2.04±1.02	2.65±1.98	7.86±4.86	4.53±1.63	6.6±1.4
Ferulic acid-glucose-ester	1.45±0.78	2.16±0.71	0.78±0.23	1.16±0.88	1.63±076	1.6±0.41	1.24±0.43
Coumaric acid-glucoside	1.04±0.78	2.5±1.53	0.76±0.61	0.45±0.38	1.47±0.92	1.32±1.34	0.25±0.09
Total hydroxycinnamic acids	**10.71**	**20.46**	**8.1**	**10.77**	**21.73**	**17.08**	**37.0**
Cyanidin-galactoside	**1.5±0.94**	**0.63±0.27**	**1.46±0.82**	**1.24±0.68**	**0.84±0.58**	**17.3±21.16**	**3.9±2.62**
Phloridzin	127.8±57.99	92.21±17.05	126.7±43.07	63.34±34.64	100.7±47.7	82.32±16.82	140.1±56.14
Phloretin-2'-O-xyloglucoside	64.84±25.9	45.29±7.57	56.07±23.77	23.07±13.63	39.16±21.71	40.7±10.72	34.31±2.16
Phloretin	55.43±34.86	39.74±12.55	74.14±37.41	10.66±1.68	63.53±61.42	19.37±7.11	14.91±7.2
Total dihydrochalcones	**248.1**	**177.2**	**255.9**	**97.07**	**234.0**	**142.4**	**189.4**
Q-arabinoside	11.29±5.37	9.62±1.77	15.51±5.22	9.16±1.53	11.12±5.26	8.5±1.99	5.32±0.25
Q-rutinoside	1.17±0.67	1.66±0.24	1.63±1.14	1.02±0.4	07.±0.73	1.1±0.53	0.29±0.24
Q-xyloside	0.44±0.7	0.04±0.09	0.23±0.27	1.31±1.21	0.11±0.13	0.09±0.06	0.05±0.08
Q-galactoside	11.55±9.44	9.81±2.76	18.17±7.81	6.8±2.84	5.48±3.67	9.83±4.17	1.87±3.39
Q-rhamnoside	5.46±2.38	5.57±3.46	14.65±6.39	9.24±5.16	11.58±10.65	3.06±0.75	6.27±3.7
Total flavonols	**29.91**	**26.7**	**50.2**	**27.53**	**29**	**22.58**	**13.8**
Total Phonolic content	**311.63**	**250.91**	**345.58**	**169.45**	**282.9**	**226.34**	**345.1**

Appendix 2. Content of individual phenolic compounds (mean ± SD, mg /100 g dry. wt., n=5) total phenolic compounds (mg /100 g dry .wt.) in leaves were taken from 'Gala' non-transgenic Plants and 'Gala' transgenic plants (For all constructs used in this study)

	ala control	ala-Pjan-yb9-line1	ala-Pjan-yb9-line2	ala-Pjan-yb9-line3	ala-Pjan-yb10-line1	ala-Pjan-yb10-line2	ala-Pjan-yb10-line3	ala-Pjan-yb10-line4	ala-Pjan-yb11-line1
enolic compounds									
atechin	14±0.5	52±1.3	35±0.1	75±0.6	26±0.58	35±0.38	03±0.7	46±0.1	31±0.16
Epicatechin	1.58±0.5	5.64±4.2	1.48±0.6	10.95±3.7	3.01±1.76	2.69±1.09	3.65±0.5	1.78±0.5	1.95±0.33
(epi)catechin-hexoside	24.82±14	18.95±15.4	11.74±6.7	25.34±15.7	60.93±47.17	67.91±29.22	45.28±4.8	2.523±1.22	14.37±7.04
Total flavan-3-ols	**27.55**	**28.11**	**13.56**	**39.04**	**65.2**	**71.45**	**50.96**	**27.48**	**16.63**
Caffeic acid-glucose-ester	1.25±0.3	3.62±2.5	0.15±0	3.57±2.6	2.01±0.98	0.74±0.37	1.87±0.7	0.43±0.2	0.26±0.14
Chlorogenic acid	4.1±4.2	13.83±5.3	2.1±0.3	17.91±78	2.62±1.5	5.47±3.04	3.38±1.2	3.75±1.9	6.12±3.05
Coumaric acid-glucose-ester	9.32±2.5	11.34±4.5	1.88±0.6	14.13±5.1	9.94±4.74	5.57±1.7	8.48±1	5.56±2	5.36±2.05
Ferulic acid-glucose-ester	4.28±1.3	2.16±0.7	0.95±0.4	2.63±0.8	4.76±3.76	2.77±1.25	7.67±2.3	2.36±1	0.8±0.35
Coumaric acid-glucoside	2.61±1.2	1.23±0.7	0.33±0	1.23±1	5.64±4.23	2.24±0.82	4.16±1.4	2±0.8	0.66±0.12
Total hydroxycinnamic acids	**20.56**	**32.18**	**5.41**	**39.47**	**24.97**	**16.8**	**25.56**	**14.1**	**12.31**
Cyanidin-galactoside	**0.98±0.7**	**0.95±0.6**	**0.7±0.5**	**2.21±0.9**	**4.31±4.31**	**1.66±04**	**1.9±0.8**	**0.93±0.5**	**0.56±0.28**
Phloridzin	102.5±30.2	80.87±27.4	78.06±18	99.41±28.3	148.1±61.56	148.9±4215	157.3±13.9	107±33.5	64.33±21.55
Phloretin-2'-O-xyloglucoside	44.19±13.3	23.91±12.1	28.51±7.5	34.13±10.8	50.08±21.04	52.51±17.46	61.52±7.4	42.35±15	17.42±7
Phloretin	48.83±30.6	43.83±40.7	33.41±24.9	81.64±39	133.0±698	125.6±5454	120.4±241	45.82±23.7	17.67±12.09
Total dihydrochalcones	**194.5**	**148.6**	**140**	**215.2**	**331.1**	**327**	**339.2**	**195.2**	**99.42**
Q-arabinoside	15.53±5.3	8.54±1.9	12.47±2.8	10.73±3.4	20.72±7.67	25.23±7.92	19.47±2.5	14.94±4.3	10.42±4.35
Q-rutinoside	1.4±0.8	0.2±0.1	0.14±0.1	1.06±0.7	2.55±1.65	2.73±1	3.08±0.6	1.34±0.6	0.15±0.09
Q-xyloside	0.91±0.7	0.39±0.5	0.12±0.1	0.21±0.1	1.7±1.56	1.96±1.08	1.1±0.7	0.31±0.4	0.21±0.29
Q-galactoside	22.71±9.1	7.79±9.5	10.96±3.8	14.19±7.6	29.75±16.28	41.14±12.71	4.127±4.8	21.57±6.8	11.49±7.67
Q-rhamnoside	13.02±5.7	9.32±5.7	7.02±.32	16.45±6.5	21.56±8.23	21.46±7.59	16.8±2.5	12.97±3.5	6.36±2.5
Total flavonols	**53.56**	**26.24**	**30.72**	**42.64**	**76.28**	**91.51**	**71.71**	**51.13**	**28.63**
Total Phonolic content	**297.15**	**236.08**	**190.39**	**338.56**	**518.6**	**585.2**	**489.33**	**188.84**	**157.55**

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1995-2000: B. sc. degree, Hort. Sci., Faculty of Agriculture, Damascus Univ., Syria. (Average: 65.35%)

2000-2001: Post-graduate studies, Hort. Sci., Faculty of Agriculture, Damascus Univ., Syria. (Average: 74.16%).

2003-2006: MS.c degree On Micropropagation of some important Apple rootstocks . Horticulture Science Dep., Faculty of Agriculture, Aleppo Univ., Syria. (Average: 89 %)

Training courses:

31/8- 4/9/ 2003: Workshop on "Biosafety in Agricultural Biotechnology", GCSAR and ICARDA, Syria, in cooperation with FAO.

14-21/3/2004: Short-Course on "DNA Molecular Marker Techniques for Crop Improvement", ICARDA -Aleppo, Syria.

22/3-1/4/2004: Short-Course on "Using of Biological and molecular Techniques for Crop Improvement in the dry area" AESC-Damascus, Syria.

18-22/12/2004: The 5th ISTA/FAO Workshop on Electrophoretic and PCR-Techniques for Variety Verification and GMO Detection, Giza –Egypt.

19/6-23/6/2004: Short-Course on "DNA Molecular Marker Techniques for Crop Improvement" GCSAR cooperation with ICARDA-Damascus, Syria

5-15/9/ 2005: The Use of Advanced Molecular Techniques in Plant-Microbe Interactions, ICGEB cooperation Atomic Energy Commission of Syria, Damascus, Syria

15/2-15/3/2006: Training course on : "Wheat transformation using Biolistic Gun" held at Agricultural Genetic Engineering Research Institute (AGERI), Giza- Egypt.

17-28/12/2006: Training course entitled "Genetic Transformation of Plants and Role of Molecular Markers in Crops Improvement" held at GCSAR-Biotechnology Department, Damascus -Syria.

Professional Societies:
Arab Agricultural Engineering Syndicate, Damascus, Syria.

Publications :

1.**Al-Rihani, K**., Dayoub, A, Khalhout, A, Abdul-Kader, A. (2005) *In Vitro* propagation of the apple rootstock M27. Res J Aleppo Univ Agric Sci Ser. 53: 47-66.

2. **Al-Rihani, K**., Khalhout, A, Abdul-Kader, A. (2008) A study on the *in Vitro* propagation of the apple rootstock MM111, Jordan Journal For Agricultural Sciences. 2:191-206.

3. Ali Bacha,N., **Al-Rihani K.,** Abdul-Kader, A. (2009) Development of *in vitro* propagation method for the local apple variety " Sukari". Bassel al-Assad J Engin Sci Agric Food Chem Biotech.26: 213-234.

4. Al-Tinawi, I., Ali Bacha,N., **Al-Rihani, K.,** and Abdul-Kader, A. (2009) *In vitro* propagation of the McIntosh and Freedom apple varieties. Bassel al-Assad J Engin Sci Agric Food Chem Biotech. 26: 47-64.

12 STATEMENT

Herewith I declare that I wrote my Ph-D Thesis without external support and that I did not use other than quoted sources and auxiliary means.
All statements which are literally or analogously taken from other publications, have been identified as quotations.

Hannover, 20.06. 2006