Transcript analysis of apple scab susceptible and resistant *Malus domestica* Borkh. cultivars and establishment of a mannose selection transformation system for apple

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ABSTRACT

In order to compare transcription profiles of *Malus domestica* cultivars that are differentially sensitive to apple scab (caused by the fungus *Venturia inaequalis*), two cDNA libraries were generated. Subtraction hybridization was performed between cDNA populations from uninfected young leaves of the resistant cultivar 'Remo' and the susceptible 'Elstar'. In total, 480 EST clones were obtained: 218 preferentially expressed in 'Elstar' and 262 clones in 'Remo'. The putative functions of about 50% of the cloned sequences could be identified by sequencing and subsequent homology searches in databases or by dot-blot hybridization. In 'Remo' the levels of transcripts encoding proteins related to plant defense (such as cysteine protease inhibitor, endochitinase, ferrochelatase, and ADP-ribosylation factor) were highly up-regulated compared to 'Elstar'. A large number of clones coding for metallothioneins type 3 (91 out of 262) were isolated in 'Remo' cDNA population. The corresponding transcripts were only present in small amounts in young uninfected leaves of 'Elstar', but were up-regulated in this cultivar after infection with *V. inaequalis*. The constitutively high-level expression of PR proteins may protect 'Remo' from infection by different plant pathogens.

Some of the identified genes could be further utilized in apple transformation, in order to obtain transgenic plants resistant against diseases. Transformation technologies rely on the use of selectable marker genes, which are co-introduced, with the gene of interest to distinguish transformed from non-transformed cells. However, public concern is claiming for alternative selection systems that avoid the antibiotic and herbicide resistance genes. For this reason, a positive selection system basing on the use of a phosphomannose-isomerase gene (*pmi*) as a selectable marker gene and mannose as the selective agent was established for transformation of apple and compared with herbicide selection. The *pmi* gene along with a *gus* gene was transferred into apple cv. 'Holsteiner Cox' *via Agrobacterium tumefaciens*-mediated transformation. Leaf explants were selected on medium supplemented with different concentrations and combinations of mannose and sorbitol to establish an optimized mannose selection protocol. Several transgenic lines were regenerated with efficiencies up to 24%. Integration of transgenes in selected plants was confirmed by PCR and southern blot analysis. Histochemical GUS staining and chlorophenol red assays confirmed transgene activity in transgenic plants. The PMI/mannose selection system proved to be superior to herbicide or antibiotic resistance genes.

Keywords: suppression subtractive hybridization, metallothionein, pathogenesis-related proteins, *Agrobacterium tumefaciens*, mannose, chlorophenol red assay, GUS assay, positive selection system.

ZUSAMMENFASSUNG

Zur Identifizierung von Genen, die in die Apfelschorf-Resistenz involviert sind, wurden Transkriptomanalysen unterschiedlich anfälliger Apfelsorten (*Malus domestica*) durchgeführt. Aus unintifizierten jungen Blättern der resistenten Sorte 'Remo' und der anfälligen Sorte 'Elstar' wurden cDNA Bibliotheken erstellt. Mit Hilfe der Suppression Subtractive Hybridization (SSH) wurden die Populationen voneinander subtrahiert. Insgesamt wurden 480 EST (expressed sequence tags) Klone erhalten, wovon 218 vornehmlich in 'Elstar' und 262 vornehmlich in 'Remo' exprimiert wurden. Die putative Funktion von ca. 50% der klonierten Sequenzen wurde über Sequenzierungen und anschließenden Homologievergleich mit Sequenzen aus Datenbanken oder durch dot-blot Hybridisierungen identifiziert. Im Vergleich zu 'Elstar' waren in 'Remo' die Transkripte von Genen, die für Proteine kodieren, die in die Pathogenabwehr involviert sind (z.B. Cystein-Protease Inhibitoren, Endochitinasen, Ferrochelatasen, ADP-Rybosylierungsfaktor), stark hochreguliert. In der 'Remo' cDNA Population wurde außerdem eine hohe Anzahl von Klonen identifiziert (91 von 262), die für Metallothionein Typ 3 kodieren. Die jeweiligen Transkripte waren in jungen Blättern von 'Elstar' nur in geringem Maße nachzuweisen, nach einer Infektion mit *Venturia inaequalis* stieg der Gehalt jedoch deutlich an. Der konstitutiv hohe Expressionslevel der PR-Proteine in 'Remo' dient möglicherweise den Schutz von Infektionen durch verschiedene Pathogene.

Differentiell exprimierte Gene stellen potentielle Kandidaten dar, um mit Hilfe der Transformation Resistenz in anfälligen Sorten zu etablieren. Bei dem Transfer von Genen werden zusätzlich Markergene eingesetzt, um die wenigen Zellen eines Gewebes, die die fremde DNA aufgenommen haben, zu selektieren. Bei der Transformation von Apfel wurden bisher Herbizid-und Antibiotikaresistenzgene verwendet, deren Einstz aber kritisch diskutiert wird. Daher wurde im Rahmen der Arbeit ein sog. positives Selektionssystem für die Apfeltransformation etabliert, welches auf der Verwendung einer Phosphomannose-Isomerase (*pmi*) als selektivem Markergen und Mannose als selectivem Agens beruht. Mittels des *Agrobacterium tumefaciens* vermittelten Gentransfers wurde das *pmi* Gen zusammen mit dem Reportergen für eine ß-Glucuronidase in die Apfelsorte 'Holsteiner Cox' übertragen. Die Selektionsbedingungen für Apfel wurden durch Variation der Konzentrationen und Kombinationen von Sorbitol (metabolisierbarer Zucker) und Mannose. Mit Transformationseffizienzen bis zu 24% wurden mehrere transgene Linien regeneriert. Die

Integration des *pmi*-Gens wurde mittels Southern Blot Analysen von DNA der regenerierten Pflanzen bestätigt. Die Funktionalität des *pmi*-Gens sowie des *gus* Gens wurden in einem Chlorophenol-Rot Assay bzw. einem histochemischen GUS-Assay nachgewiesen. Das PMI/Mannose System erwies sich als effektiver als die Selektion mit Antibiotika oder Herbiziden.

Schlagwörter: Suppressive Subtraktionshybridisierung, metallothionein, pathogenesis-related proteins, *Agrobacterium tumefaciens*, Mannose, Chlorophenol-Rot Assay, GUS Assay.

Abbreviations

Chemicals

BSA	bovine serum albumin
BPB	bromophenol blue
CPR	chlorophenol red
CTAB	hexadecyl trimethyl ammonium bromide
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetra acetic acid
EtBr	ethidium bromide
FDA	Fluoresceindiacetat
MOPS	3-(N-morpholino) propane sulfonic acid
MS	Murashige & Skoog medium (1962)
NaOAc	sodium acetate
NBT	nitroblue tetrazolium
PPT	phosphinothricin
PVP- 40	polyvinylpyrrolidone
SDS	sodiumdodecylsulfate
SSC	sodiumchloride -sodiumcitrate
Tris	Tris-hydroxymethyl-aminomethane
x-GlucA	5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid

Molecular biology

cDNA	copy DNA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ds	doble stranded
ESTs	expressed sequence tags
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease (RNAse free)
PCR	polymerase chain reaction
RNA	ribonucleic acid
mRNA	messenger RNA

Units

°C	degree Celsius
µmol	micromole
µg	microgram
µL	microliter
µM	micromolar
bp	base pair
Ci	Curie
g	gram

h	hour
Kb	kilobase
L	liter
m	meter
M	molar
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mol	mole
Nm	nanometer
nM	nanomolar
nmol	nanomole
Pa	Pascal
Psi	pound per square inch
s	second
V	volt
vol	Volume
x *g*	gravitational force

Other abbreviations

Fig.	figure
LB	Luria-Bertani Broth
n.s.	not significant
PDA	Potato dextrose agar
RT	Room temperature
Tab.	table
UV	ultra violet
YEP	yeast extract peptone

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1 GENERAL INTRODUCTION

1.1 Apple (*Malus domestica* Borkh.*)*: origin, distribution and economic importance

Apples belong to the Pomoideae subfamily Rosaceae, along with pear (*Pyrus communis*), prune (*Prunus domestica*) and cherry (*Prunus avium*). The common domesticated apple is believed to have originated in the Heavenly Mountains on the border of Western China, in the former USSR and in Central Asia, and is putatively an interspecific hybrid complex, designated *Malus domestica* Borkh. (KORBAN and SKIRVIN, 1984, PHIPPS et al., 1990). In medieval times, monasteries were responsible for selection, propagation and perpetuation of hundreds of different cultivated types. These plantings later became major sources of breeding stock for horticulturists in the 1800's that were developing new techniques for making deliberate crosses between desirable selections (MAC HARDY, 1996).

During the late 19th and 20th centuries, *M. domestica* cultivars bred in Europe, Russia, North America, New Zealand, Japan and Australia were introduced throughout the world and form the basis for most current commercial apple production (WAY et al., 1991, JANICK et al., 1996). Nowadays more than 7.000 varieties have been described (http://www.whfoods.com/genpage.php) and breeders worldwide create new selections annually; nevertheless, only a few dozen are widely produced in commerce today (JANICK et al., 1996).

In 2004 apple was the third most cultivated fruit crop in the world (5,280 M Ha) and the third fruit crop in production (59,059 Mt), after citrus and banana. The most important producers in this same year were China, USA and Poland (http://faostat.fao.org/faostat/servlet/). In Germany, it corresponded to 80% of the fruit production (http://news.agrar.de/archiv/20050222-00000/).

Beside the economic importance, apple has become a model woody perennial angiosperm for genomic research due to the relatively small genome size (769 Mb/haploid) (PATOCCHI et al., 1999) distributed into 17 chromosomes. Most cultivated apples are diploids ($2n = 34$) and self-incompatible.

1.2 Apple diseases

Apples are host of a large number of diseases, including apple scab, fire blight, mildew, and bacterial cancer. The diseases are caused by different pathogens, such as fungi, bacteria, nematodes, viruses and phytoplasmas, or can be physiological, genetical or have an unidentified basis (WAY et al., 1991). Economic losses are variable since some can kill trees, while others may infect fruits, making them unmarketable.

Apple scab, caused by the fungus *Venturia inaequalis* (Cke.) Wint, is the most important disease of this culture. It presents a wide spread distribution, occurring nowadays in all regions where apple is cultivated and can cause losses of about 80% where the summer presents high humidity and mild temperatures (BONETI et al., 1999). The control of this disease is normally chemical, and over 20 fungicide applications can be necessary per season (KOLLAR, 1997).

V. inaequalis overwinters as a pseudothecia in the apple leaf litter. The sexual stage begins after a period of saprophytic activity and vegetative growth following leaf abscission (MAC HARDY, 1996) (Fig. 1). In spring the ascospores discharged from the pseudothecia are dispersed by wind to young leaves and sepals where infection occurs. The second inoculum is marked by conidia production in the scab lesions that develop from infections by primary inoculum. The growth of hyphae between the cuticle and epidermal cell wall results in the development of conidiophores and conidia that rupture the cuticle, when a scab lesion is clearly visible macroscopically. Conidia are produced over approximately four weeks in a lesion. The conidia can cause secondary infections on leaves, fruits and shoots during wet weather throughout the remainder of the growing season (MAC HARDY, 1996).

The apple scab symptoms appear as light, olive-coloured spots on the abaxial surface of sepals of young leaves of the flower buds. The lesions then become olive grey with velvety surface and finally acquire a metallic black color. Occasionally, small scab spots are produced on twigs and blossoms. Infected fruits are scabby and sometimes cracked. If the fruits are infected early, they become misshapen and frequently drop prematurely. When fruits are infected approaching maturity, the disease causes only small lesions (AGRIOS, 1997) (Fig. 2).

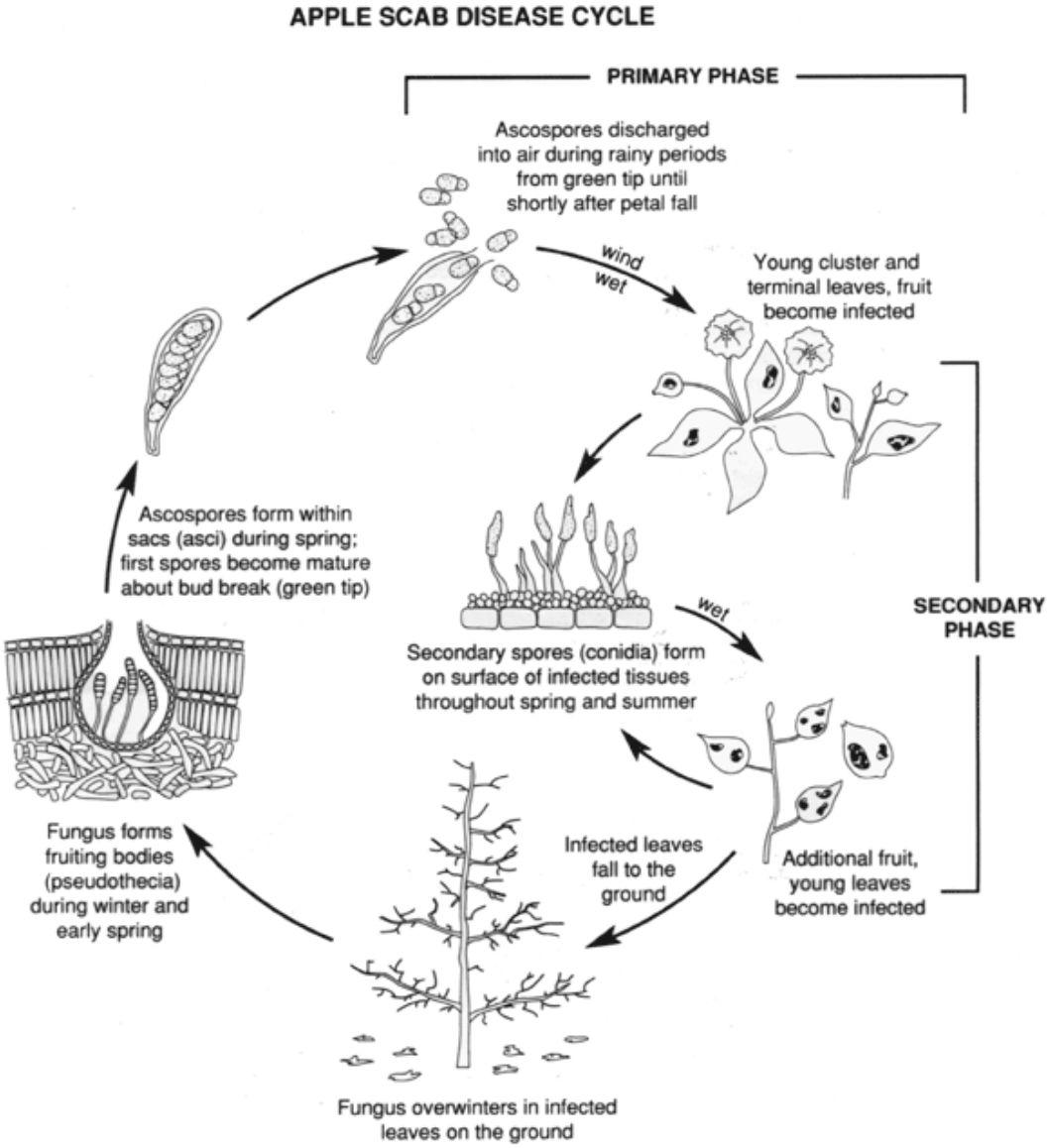

Fig. 1: Life cycle of *Venturia inaequalis.* Source: http://plantclinic.cornell.edu/FactSheets/apple_scab_factsheets/apple_scab_disease_cycle.htm

Fig. 2: Symptoms of apple scab on a. fruits and b. leaves. Source: http://hflp.sdstate.edu/IMAGE.jpg

1.3 Classical genetic breeding

Although the high genetic diversity in the genus *Malus*, much of the world's production consists of a narrow genetic base of the varieties 'Delicious' and 'Golden Delicious' (WAY et al., 1991). The most important commercial cultivars are included in a large scale in breeding programs to improve fruit quality. The cultivar 'Golden Delicious' and its derivatives, such as 'Gala', are the parents most often used in the crosses. Due to limitations of conventional breeding, the potential of *Malus* germoplasm remains under-exploited (HAMMERSCHLAG, 2000).

Although conventional apple breeding programs have generated new cultivars with improved qualities, a large number of commercially grown apple cultivars have been derived either as chance seedlings, e.g. 'Delicious', 'McIntosh' and 'Granny Smith' or from natural spontaneous mutations that have occurred in somatic tissues of some of the important apple cultivars (KORBAN and CHEN, 1992).

Nowadays, the new established agriculture techniques allied to the high market demand apple cultivars showing high productivity, uniformity, and long-term storage. In addition, resistance to diseases, pests and storage disorders are also desired for large-scale fruit production.

Because of the long juvenile period, which can take from six to ten years, high levels of heterozygosis and time necessary to evaluate hybrids, traditional apple breeding programs are slow (KORBAN and CHEN, 1992, BROWN, 1992). Normally after specific crosses, backcrosses are necessary to eliminate undesired characteristics from the wild species and a new cultivar can take over 40 years to be established on the market (ANDREAS PEIL, Dresden Pilnitz, Germany, personal communication).

1.4 Advances of plant biotechnology

In the last few years, considerable knowledge about molecular biology of plants has been gained by application of new genomic technologies. The complete genome of several organisms has been sequenced. Genome sequencing has provided the foundation for systematic analyses of gene expression, and for the spatial location of proteins and nucleic acids.

However, less than 10% of *Arabidopsis thaliana* genes have any experimentally defined function (The *Arabidopsis* Genome Initiative 2000). About 30% of the genes of this plant have no significant similarity at the protein level to genes found in any other organism. The sequencing of chromosome 1 of *A. thaliana* predicts 6,848 proteins. Around one-third of these are 'hypothetical', without matching to any EST (expressed sequence tags) in the databases.

Seventy per cent of the annotated proteins have some similarity to other 'hypothetical', 'unknown' or 'putative function' proteins already sequenced from plants and other eukaryotic genomes (THEOLOGIS et al., 2000).

Since the completion of the *A. thaliana* genome sequencing and the first-pass analysis and prediction of gene function, different approaches are in course to determine the function of genes. Chemical mutagenesis, gene mapping and, increasingly, RNA interference (RNAi) are the most used. The latter, a knocking out system, uses constructs encoding self-complementary 'hairpin' RNAs, homologous to the mRNA from the target gene, and a complex system of post-transcriptional gene silencing takes place (MATTHEW, 2004).

Another challenge is to define the functions of genes in a cellular and whole-organism context, once the catalogue of all genes and predicted proteins generated by whole-genome sequencing alone can not provide a complete understanding of the interactions among organisms. Which genes are expressed under different circumstances, how plants grow and develop as well as how they respond to abiotic and biotic stresses are all important questions that the genome programs are not able to answer so promptly and are now under investigation based on different methods (CÁNOVAS et al., 2004). The establishment of expressed sequence tags (EST) databases from plant tissues has enabled the development of global analysis methods to study changes in gene expression associated with important processes in plants.

Moreover, complex regulatory routes, from post-translational modifications to protein turnover cannot be studied at the cDNA level (PANDEY and MANN, 2000). To overcome this problem, several strategies are evolving. The proteome approach is helping to answer questions of functional analysis and to complement data derived from transcriptome analysis (THIELLEMENT et al., 1999). Proteomics, the systematic analyses of protein expression, involves the separation, identification and characterization of proteins in an organism. The new techniques involved enable the simultaneous characterization and analysis of the expression profiles of a large set of proteins (DAFNY-YELIN et al., 2005).

A holistic view of a cell as a self-replicating entity is preserved by studying the functions of all genes and proteins within a cell, while the extensive and precise nature of genome-based information permits the isolation and characterization of components. Genome-based information also provides direct comparison of related and divergent cellular functions in different sequenced organisms, adding depth to our understanding of cellular functions. Future insights into basic plant biology and the ability to manipulate plants through genetic engineering for agronomic improvement will depend on whether we can identify the genes that control fundamental developmental and metabolic processes.

1.5 Isolation of differentially expressed genes

In the past decades, techniques for the evaluation of gene expression have progressed from methods developed for the analysis of single, specific genes (e.g. Northern, slot and dot blotting; semi-quantitative and quantitative reverse transcription and PCR; and nuclease protection assays) to techniques focused on identifying all genes that differ in expression among experimental samples. A variety of methods has been developed to identify the differentially expressed genes associated with a particular phenotype. These methods include differential display, representational difference analysis (RDA), subtractive hybridization, suppression subtractive hybridization (SSH), differential screening, conventional cDNA array hybridization, serial analysis of gene expression (SAGE) (reviewed by NAKATA and McCONN 2002 and YANG et al., 1999).

SSH is a PCR-based cDNA subtraction method, which can be used to compare two populations and obtain ESTs that are either overexpressed or exclusively expressed in one population compared to another. This method is used to selectively amplify target ESTs and simultaneously suppress nontarget DNA amplification. The method allows the detection of low-abundance differentially expressed transcripts such as many of those likely to be involved in signaling and signal transduction, and might thus identify essential regulatory components in several biological processes. The mRNA differential display and RNA fingerprinting by arbitrary primed PCR are potentially faster methods for identifying differentially expressed genes. However, both have a high level of false positives, biased for high copy number mRNA and might be inappropriate in experiments in which only a few genes are expected to vary (DIATCHENKO et al., 1996, DIATCHENKO et al., 1999).

The SSH method has been used to isolate genes from all kingdoms, including nematodes, fungi and plants. One of the most important aims is the isolation of genes expressed after pathogen infection and have been applied for several plant species like barley (HEIN et al., 2004), potato (BIRCH et al., 1999, BEYER et al., 2001, MONTESANO et al., 2001), tomato (MYSORE et al., 2002) and rice (XIONG et al., 2001). The molecular pattern carried out in abiotic stress process has also been studied in species like sugarcane (WATT, 2003), *A. thaliana* (KANG et al., 2003), rice (WANG et al., 2002a, WANG et al., 2002b), *Sesbania rostrata* (CATURLA et al., 2002), *Medicago truncatula* (NAKATA and CONN, 2002) and *Aegicieras corniculatum* (FU et al., 2005). Genes involved in some physiological stages of development, like flower development and taproot in sugar beet (KLOOS et al., 2002) have also been isolated. The symbiosis process has also been extensively studied in *Medicago*

truncatula (VOIBLET et al., 2001, BRECHENMACHER et al., 2004, WULF et al., 2003, TAYLOR and HARRIER, 2003).

Less common is the application of this technique to isolate genes from different cultivars of the same species. But also in this case the method proved to be efficient. The gene pools of two strains of *Xylella fastidiosa* were subtracted in order to identify genes responsible for the disease process in citrus and grape (HARAKAVA and GABRIEL, 2003). The isolation of cDNA clones differentially accumulated in the placenta of pungent pepper was also carried out between two different cultivars (KIM et al., 2001) and genes from two *Globodera* nematode species were isolated in order to investigate which genes were involved in the parasitic process of tomato and potato plants (GRENIER et al., 2002).

SSH allows the construction of EST libraries containing hundreds of differentially expressed genes. Genes of all classes have already been isolated, and not only have the most common, but also rare transcripts been identified.

1.6 Cloning and isolation of genes from *M. domestica*

The isolation of genes responsible for resistance against different pathogens e.g. *Venturia inaequalis*, *Podosphaera leucotricha* and *Erwinia amylovora* from different species of the *Malus* genus is in course (SCHMIDT et al*.,* 1999, FISHER and FISHER, 1999, ZIMMER, 1999, MANTINGER, 1999, FISHER, 1999). Several genes have already been identified, which are responsible for resistance against apple scab, the most important disease of this culture (Tab. 1). Among them, the *Vf* has been the most widely used in classical breeding all over the world.

Molecular markers linked to important phenotypic traits are an important tool in shortening the length of the selection process, once they can reduce time and costs of breeding programs (TARTARINI et al, 1999). A number of random amplified polymorphic DNA (RAPDs) markers tightly linked to the already identified apple scab resistance genes have been found (KOLLER et al., 1994, YANG and KRÜGER, 1994, HEMMAT et al., 1995, GARDINER et al., 1996, TARTARINI 1996, YANG et al., 1997a,b). Some of them have been transformed into SCAR, SSR and cleaved amplified polymorphic sequence (CAPS) markers (GIANFRANCESCHI et al., 1996, YANG and KORBAN, 1996, YANG et al., 1997a, b, TARTARINI et al., 1999, BUS et al., 2000 and 2005).

The *Vf* locus is focus of a large number of investigations. Based on several molecular markers the genetic map has been constructed (VINATZER et al., 1998, PATOCCHI et al., 1999, TARTARINI et al., 1999, VINATZER et al., 2001, XU and KORBAN, 2002, VINATZER et al.,

2004) and the results allowed the identification of a cluster of genes, which were cloned and isolated. A high number of hybridizing bands indicated that a gene cluster of many *Cf* (from tomato) resistance gene homologues is present in the *Vf* region. The authors called the members of the cluster '*HcrVf*' genes (homologous to *Cladosporium fulvum* resistance genes of the *Vf* region) (VINATZER et al., 2001). Transgenic plants carrying one gene of this cluster (*HcrVf2*) showed resitance against *V. inaequalis* (BELFANTI et al., 2004). The efficiency of different promoter lengths of these genes was also investigated using transgenic research (SILFVERBERG-DILWORTH et al., 2005). Other genes also responsible for resistance against *V. inaequalis* have been studied and isolated based on molecular markers and genetic maps, like *Vh2* and *Vh4* (BUS et al., 2005), *Vr2* (PATOCCHI et al., 2004), *Vb* and *Va* (HEMMAT et al., 2003), *Vm* (CHENG et al., 1998), *Vx* (HEMMAT et al. 2002), *Vbj* (GYGAX et al., 2004) and the recently identified *Vr2* (PATOCCHI et al., 2004). .

Beside these genes directly correlated to apple scab, another genes involved in the interaction pathogen x host are also under investigation. Genes belonging to the pathogenesis-related class (PR) are focus of a large number of studies. The genomic cloning and linkage mapping of the *Mal d 1* (pathogenesis related PR-10) (GAO et al., 2005a) and *Mal d 3* (non-specific lipid transfer protein) genes (GAO et al., 2005b) have been done. The linkage map position of two *Mal d 3* genes was constructed based on PCR cloning of ten cultivars. Two distinct *Mal d 3* genes were localized (GAO et al., 2005b). In addition to *Mal d 3*, three more apple allergens genes have been identified by gene cloning and sequencing: *Mal d 1*(VANEK-KREBITZ et al., 1995), *Mal d 2* (KREBITZ et al., 2003) and *Mal d 4* (VAN REE et al., 1995). Leucine-rich repeat sequences, sharing homology with known resistance genes have also been identified and cloned (KOMJANC et al., 1999, BALDI et al., 2004).

Organ specific genes have also been identified using differential display. cDNA fragments of two transcripts preferentially expressed in flowers (WATILLON et al., 1998) and genes expressed early in fruit development were also isolated by this method (DONG et al., 2000). A gene encoding a polygalacturonase inhibitor in apple fruit was shown to be developmentally regulated and activated by wounding and fungal infection (YAO et al., 1999). Transcripts of a thaumatin-like gene (PR protein) were highly expressed in the fruit, but were rarely found in other tissue types (KIM et al., 2003). Bacterial expression of a 3-hydroxy-3-methylglutaryl-CoA reductase was used to elucidate the mechanism of superficial scald in apple fruit (PECHOUS and WHITAKER 2002).

Tab. 1: Specific apple scab resistance genes and source (MAC HARDY, 1996, HEMMAT et al., 2002, PATHOCCHI et al., 2004).

Gene	Source
Vf	*M. floribunda* # 821
Vc	*Cathay crab*
Vm	*M. micromalus*
Vb	*Hansen's baccata* # 2
Vbj	*M. baccata jackii*
Vr	*M. pumila* R12740-7A
Vj	*Jonsib Crab*
Va	*Antonovka* PI 172612

1.7 Apple transformation

The genetic transformation of plants is the process where a defined fragment of DNA is introduced and integrated into the genome of the plant, without fecundation. The 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 for plants (BRASILEIRO and DUSI, 1999). The most widely used method for introducing foreign genes into dicotyledoneous plants is the *Agrobacterium tumefaciens*-mediated transformation.

Transformation of *Malus* is nowadays a common practice in several laboratories and the protocols have been constantly improved to enhance the efficiency (JAMES et al., 1993, YEPES and ALDWINCKLE, 1994, DE BONDT et al., 1994, YAO et al., 1995, DE BONDT et al., 1996, NORELLI et al., 1996, PUITE and SCHAART, 1996, HAMMERSCHLAG et al., 1997, LIU et al., 1998, SRISKANDARAJAH and GOODWIN, 1998, BOLAR et al., 1999).

Because of the susceptibility to diseases of the most important commercially apple cultivars and rootstocks, genetic transformation is emphasizing the development of improved forms of varieties with diseases resistance. The transformations are mostly based on traditional cultivars and transformation have been carried out using genes isolated from apple (BELFANTI et al., 2004) or from other organisms (NORELLI et al., 1994, WONG et al., 1999, NORELLI et al., 2000, BOLAR et al., 2000, HANKE et al., 2000, BOLAR et al., 2001, LIU et al., 2001, SZANKOWSKI et al., 2003, MARKWICK et al., 2003). Genes affecting some physiological or morphological characters have also been introgressed in transgenic apples like growth (HOLEFORS et al., 2000), flowering (YAO et al., 1999) and self-fertility (VAN NERUM et al., 2000). Rootstock scions have also been used in transgenic assays to influence rooting rates

and growth (HOLEFORS et al., 1998, WELANDER et al., 1998, ZHU and WELANDER, 1999, SEDIRA et al., 2001, PAWLICKI-JULLIAN et al., 2002, IGARASHI et al., 2002). The study of gene function of some genes like sorbitol-6-phosphate (KANAMARU et al., 2004, CHENG et al., 2005), polygalacturonase (ATKINSON et al., 2002) and from several promoters (KO et al., 2000, GITTINS et al., 2001 and 2003) have also been carried out using apple.
Although considerable improvement have been gained, the use of antibiotics and herbicides as selectable marker still impose limits (PENNA et al., 2002).

1.8 Selection marker genes

Selective marker genes are introduced into plant genome to express a protein with, generally, an enzymatic activity, allowing to distinguish transformed from non-transformed cells. To obtain transgenic plants the delivery of a foreign gene of interest and a selectable marker gene that enables the selection of transformed cells are necessary. The use of a marker gene in a transformation process aims to give a selective advantage to the transformed cells, allowing them to grow faster and better, and to kill the non-transformed cells (BRASILEIRO and DUSI 1999). Over the past several years, consumer and environmental groups have expressed concern about the use of antibiotic- and herbicide-resistance genes from an ecological and food safety perspective (PENNA et al., 2002).

1.8.1 Negative selectable marker genes

In the negative selection system, the entire cell population is subjected to a negative (toxic) selection pressure that only transformants can bear. There are two main categories of genes rising negative selection: the antibiotic and herbicide resistance genes. The most widely used selectable marker gene is the neomycin phosphotransferase (*nptII*), which confers resistance to amino glycoside type antibiotics such as kanamycin, neomycin and geneticin. The great majority of transgenic apple plants have been obtained using this gene (DE BONDT et al., 1996, PUITE and SHAART, 1996, HOLEFORS et al., 1998 and 2000, MAXIMOVA et al., 1998, WELANDER et al., 1998, WONG et al., 1999, BOLAR et al., 1999, 2000 and 2001, KO et al., 2000 and 2002, SEDIRA et al., 2001, LIU et al., 2001, FAIZE et al., 2003, BELFANTI et al., 2004, RADCHUK and KORKHOVOY, 2005, CHENG et al., 2005).
Among the latter category, the *pat* and *bar* genes, isolated from *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus* respectively, code for the phosphinothricin-N-acetyltransferase enzyme (PAT; EC 2.3.1.-) (MURAKAMI et al., 1986). The PAT enzyme inactivates herbicides with phosphinothricin (PPT) through the acetylation

of the PPT free amino group using acetyl coenzyme A as a cofactor. It prevents PPT from binding to the glutamine synthetase enzyme (GS). Normally PPT acts as a competitive inhibitor of GS, once it is similar to glutamate, the substrate of this enzyme. The GS plays an essential role in the nitrogen metabolism and ammonia assimilation regulation and catalyses the conversion of glutamate to glutamine, removing the toxic ammonia from the cell. When GS is inhibited, ammonia accumulates and chloroplast structure disrupts, leading to photosynthesis inhibition and plant cell death (LINDSEY, 1992). This gene has also already been used to generate transgenic apple plants (SZANKOWSKI et al., 2003), although it is much less common.

Regardless the use, most cells transformed with negative selectable marker genes will not regenerate into plants because dying untransformed cells release growth inhibitors and toxic substances, compromising the uptake of essential minerals and vitamins from the culture medium (PENNA et al., 2002).

1.8.2 Positive selectable marker genes

In this category of marker genes the identification and selection of transgenic cells without the injury or death of the non-transformed cells is possible, once the transformed cells acquire the characteristic to metabolize some compounds, which the plant normally can not metabolize (ARAGÃO and BRASILEIRO, 2002). In the positive selection, the transgenic cells enjoy a metabolic advantage over the untransformed cells that are starved rather than killed. This is achieved by using a physiologically inert substance as selective agent, which is than converted due to the enzyme in transgenic cells into a compound exerting positive effects (PENNA et al., 2002). Genes that code for enzymes able to metabolize different sugar sources like phosphomannose isomerase (*manA*), xylose isomerase *xylA* (HALDRUP et al., 1998a; 1998b) and *DOG ^{R}1* (KUNZE et al., 2001) are successfully being used.

1.8.2.1 Phosphomannose isomerase

Normally plant species cannot metabolize mannose. After uptake, mannose is actively transported into the cell and phosphorylated by endogenous hexokinases to mannose-6-phosphate (FERGUSON et al., 1958, MALCA et al., 1967). Mannose-6-phosphate is no further utilized due to a deficiency of phophomannose-isomerase (PMI). The synthesis of mannose-6-phosphate depletes cells of orthophosphate that is required for ATP production. The ATP as well as phosphate starvation deplete cells of energy for critical functions such as cell division and elongation, resulting in severe growth inhibition (GOLDSWORTHY and STREET, 1965,

LOUGHMAN, 1966, JOERSBO et al., 1998). The accumulation of mannose-6-phosphate also inhibits phosphoglucose isomerase, causing a block in glycolysis and induces an endonuclease to degrade DNA, responsible for apoptosis and programmed cell death (STEIN and HANSEN, 1999).

The *pmi* gene (*pmi*: *manA* from *Escherichia coli*) (MILES and GUEST, 1984) codes for the phosphomannose isomerase (PMI) enzyme (PMI; EC 5.3.1.8), commonly found across kingdom. However, this enzyme is absent in many plants, and have been reported only rarely, like in soyabeans (GOLDSWORTHY and STREET, 1965, LEE and MATHESON, 1984). PMI catalyzes the reversible isomerization of mannose-6-phosphate to fructose-6-phosphate, which serves as precursor for the glycolytic pathway (Fig. 3 and 4).. In addition, this enzyme is also involved in the synthesis of GDP-mannose, a major constituent of glycosylation reaction products as well as a precursor for the ascorbate biosynthetic pathway in plants (WHEELER et al., 1998).

Since mannose cannot normally be metabolized by plant cells, when mannose is added to the culture medium, plant growth may be minimized due to mannose-6-phosphate accumulation. Cells transformed with the *manA* gene are able to utilize mannose as a carbon source and grow either in the presence of or with the addition of only small amounts of other carbon sources such as glucose or sucrose. As a result, transformed cells acquire a metabolic advantage, compared with the non-transgenic cells that remain unable to metabolize mannose-6-phosphate (HANSEN and WRIGHT, 1999).

ManA has been successfully used as a selection gene in a variety of crops such as sugar beet (JOERSBO et al., 1998 and 2000), maize (NEGROTTO et al., 2000), rice (LUCCA et al., 2001 and HE et al., 2004), sweet orange (BOSCARIOL et al., 2003), pepper (KIM et al., 2002) and wheat (WRIGHT et al., 2001). As advantages, this hexose is i. soluble in plant culture media, ii. absorbed by plant cells, iii. cheap, iv. easily available, v. safe and vi. the transformation efficiencies are normally higher when compared to herbicide or antibiotic resistance genes (ARAGÃO and BRASILEIRO, 2002).

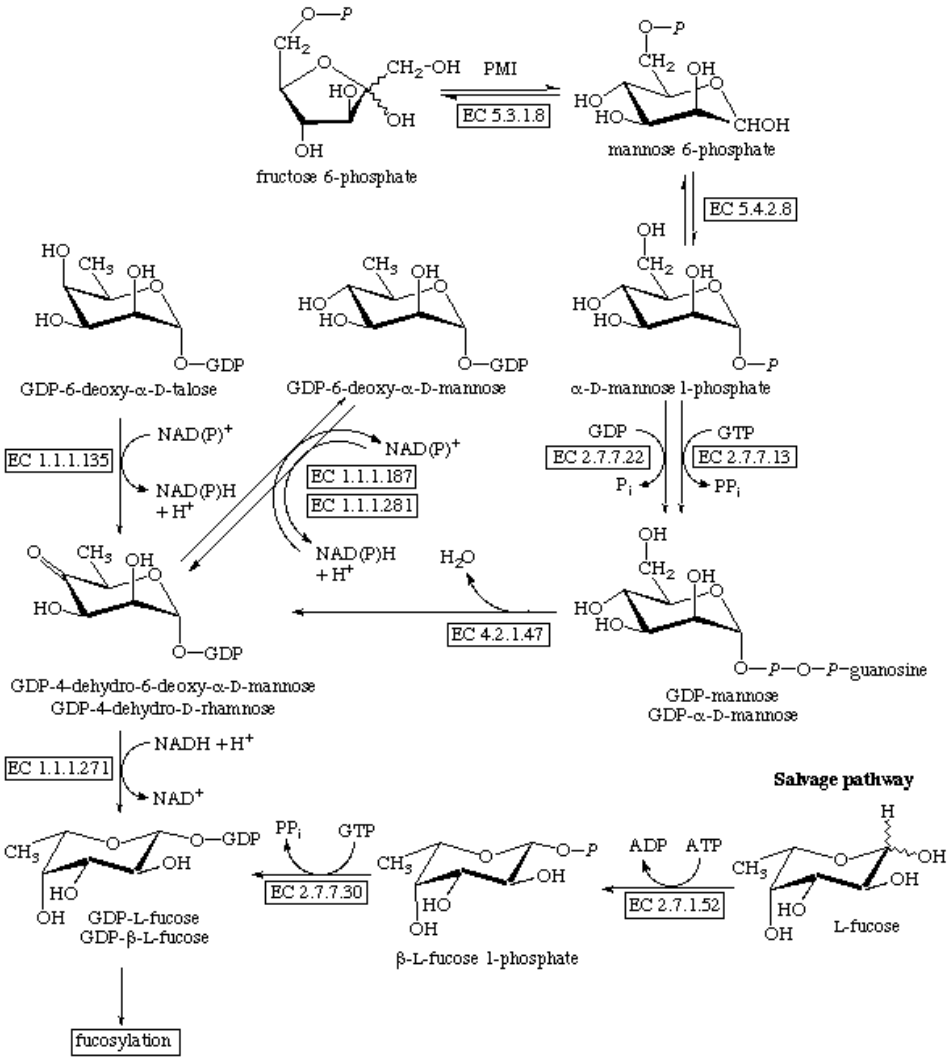

Fig. 3: Phosphomannose-isomerase pathway.
Source: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC5/3/1/8.html

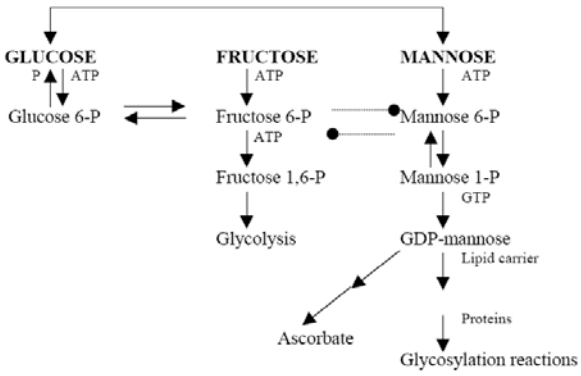

Fig. 4: Basic intermediary metabolism involving mannose in nonleguminous plant cells not transformed with PMI. The reaction catalyzed by PMI is indicated by the dashed lines and rounded arrows (PRIVALLE et al., 2002).

2 SCOPE OF THE THESIS

Apple scab, caused by the fungus *Venturia inaequalis* is the most important disease of *Malus domestica.* Over 15 treatments with fungicide per season can be required for its control.
Combining resistance genes is one of the most important aims in classical breeding programs. However, apple presents a long juvenile period and is self-incompatible and is, therefore, highly heterozygous. In classical breeding, all genes will be newly recombined in each generation and several selections and crosses need to be done until individuals carrying the resistance genes as well as other requested quality traits can be selected.
As an alternative to the introduction of resistance by classical breeding, genetic transformation is a promising approach to establish resistance in well established commercial cultivars. The most common and less cost intensive method for transformation of plants is the *Agrobacterium*-mediated. Several transgenic apple plants have been developed using the *npt*II gene as selectable marker. However, in recent years, the use of antibiotics and herbicide resistance genes for selecting transformed cells has generated widespread public concern because of inadequate evidence of the transformed gene's impact on human health and the environment. Especially antibiotic and herbicide resistance genes have provoked concerns about whether those marker genes could be transferred into microorganisms by horizontal gene transfer resulting in an increased number of resistant pathogens or that genes cross out into wild relatives transforming them into weedy pests. Thus it is important to evaluate the efficiency of alternative selection systems, such as those based on non-metabolizable agents.
Another public claim is the fact that transformations events are mainly based on the use of genes isolated from other species (inter-specific). For these reasons, the use of genes from the same specie as well as the development of alternative selection strategies are highly desirable and would certainly contribute to the acceptance of transgenic plants.
Recently, positive selection systems have been developed, in which the transgenic cells enjoy a metabolic advantage over the non-transformed cells that are starved rather than killed. The *manA* gene/mannose as selection agent is one of these systems. The supporting principle in this approach is the inability of some plants to use mannose as a carbon source.

The isolation of genes can be carried out by different methods, in order to further use them to obtain transgenic plants with improved characteristics. The characterization of expression

profile of cultivars showing different response to pathogens attack is an important tool to isolate genes involved in the defence mechanisms. Several pathogenesis related proteins (PR), such as chitinase, β-1,3-glucanase, osmotin, PR1 protein and thaumatin like protein were showed to be constitutively expressed in the apoplast of *M. domestica* cv. 'Remo' (GAU et al., 2004). This cultivar is resistant against several diseases, including apple scab, mildew and fire blight and contains the *Vf* resistance locus (FISHER and FISHER 1999). Different methods have been used to isolate genes expressed under specific circumstances. Among them the suppression subtractive hybridization (SSH) has been used with success in several species but up to now the isolation of apple genes by this method has not been described.

The isolation of pathogenesis related genes conferring quantitative or partial resistance against pathogens and their pyramidizing in one cultivar by transformation is important as a strategy to reduce the risk of resistance breakdown and to achieve durable resistance so that the pathogen has to simultaneously circumvent different resistance mechanisms to be able to infect the plant.

The work presented here is divided in two chapters:
In the first chapter the objectives are to explore the differences in transcript profile of closely related *M. domestica* cultivars 'Remo' and 'Elstar' (resistant and susceptible against *Venturia inaequalis*, respectively). In order to obtain further information about the differences between the mRNA expression profiles of the non-inoculated leaves of apple scab-resistant cv. 'Remo' and the susceptible cv. 'Elstar', the suppression subtractive hybridization (SSH) method was chosen.
In the second chapter, the objectives are to obtain genetically modified plants of *M. domestica* in order to evaluate the viability of the positive selection system Phosphomannose isomerase-mannose in the transformation of apple, to avoid the use of antibiotic and herbicide resistance genes in this system and enhance the transformation efficiencies.

3 MATERIAL AND METHODS

3.1 Materials

All media, buffer, stocks/and working solutions were prepared using double-distilled water. According to different requirements, solutions and media were autoclaved (20 min, 120°C, 2 x 10^5 Pa) or filter sterilized with 0.2 µm filters.

Cellophan membrane: C 325 P (disks of ∅ 8 cm), Pütz GmbH+Co Folien KG

Columns: MoBiTec GmbH

Filters (0.2 µm): Sartorius, Millipore

Filter miracloth: Calbiochem – 129 µm

Parafilm: Nescofilm

Plastic material: Greiner bio-one, Huhmaki, Sarstedt, Kitzel

X-ray film: Kodak

TINA software package: Raytest Isotopenmeßgeräte GmbH

3.2 Reagents

- **Amersham Pharmacia** (Freiburg, Germany): [^{32}P] CTP, Sephadex-G-50
- **Applichem** (Darmstadt, Germany):, Dimethyl sulfoxide, Formaldehyd, Formamide, Glacial acetic acid, Glycerin, Isopropanol, 2-propanol, Litium chloride, Magnesium chloride, Maleic acid, MOPS, NBT, SDS, Sodium chloride, Sodium citrate, Sodium hydroxide, Sodium phosphate, Tris ultrapure, Tween 20
- **BD Biosciences Clontech** (Palo Alto, USA): Difco Potato dextrose agar, Smart cDNA Synthesis kit, PCR select cDNA subtraction kit
- **Biozym** (Rockland, USA): Seakem LE Agarose
- **Carl Roth GmbH CO** (Karlsruhe, Germany): Ethanol, Mannose
- **Duchefa** (Haarlem, Netherlands): Antibiotics, Gelrite, Murashige and Skoog (MS) basal salts including vitamins, Sucrose, Sorbitol, Myo-inositol, Plant growth regulators, Plant agar, xGlcA cyclohexylammonium
- **Fluka** (Ulm, Germany): 2-Butanol
- **Gibco BRL** (Karlsruhe, Germany): Agar, Yeast extract

- **J.T. Baker** (B.-V.-Deventer, The Netherland): Ammonium acetate, Sodium acetate trihydrate, Sodium citrate dehydrate, Phenol, Potassium acetate
- **MBI Fermentas GmbH** (St. Leon-Rot, Germany): restriction endonucleases, dNTPs, DecaLabel DNA Labeling kit, RNAse, RNAse inhibitor, DNA leader markers
- **Merck** (Darmstadt, Germany): Bromophenol blue, Isoamyl alcohol, Potassium ferricyanide (II), Pepton, Potassium ferricyanide (III)
- **Qiagen** (Hilden, Germany): QuantiTect SYBR Green PCR kit
- **Promega** (Mannheim, Germany): pGem T Vector kit
- **Riedel-de-Häen** (Seelze, Germany): Gluphosinate ammonium –Pestanal, Potassium nitrate, Potassium dihydrogen phosphate, Sodium hydroxide.
- **Roche** (Mannheim, Germany): Anti-digoxigenin AP-Fab fragments, Blocking reagent, CDP-star kit, DIG DNA Labeling mix, DIG High Prime DNA labeling kit
- **Serva** (Heidelberg, Germany): Ethidium bromide, N-Laurylsarcosyl, 2-β-mercaptoethanol
- **Sigma** (St. Louis, USA): Chlorophenol red, Ficoll 400, Hexadecyl trimethyl ammonium bromide, PVP 40, *Taq* polymerase, FDA
- **Stratagene** (La Jolla, CA, USA): XL1-blue *Escherichia coli*
- **Syngenta** (Basel, Switzerland): pNOV2819
- **Tentenal Photowerk GmbH** (Norderstedt, Germany): Tentenal superfix solution, Tentenal rapid developer
- **ThermoHybaid** (Ulm, Germany): primers

3.3 List of devices

Apparatus Manufacturer

Autoclave: Varioklav H+P

Centrifuges: Juoan CR3i; Eppendorf centrifuge 5415R

Cleanbanch: Biowizard -Kojair

Dot blot system: Gibco

Electrophoresis apparatus: Bio Rad Sub-cell GC

Electroporation device: BTX Electronic Genetics – PEP Personal Electroporation Pak

Gel documentation: Bio Rad Gel Doc 1000, Intas

Incubator: Memmert

Magnetic stirrer with heating plate: IKAMAG, RH, Janke & Kunkel /IKA Labortechnick

PCR-device: MJ Research – PTC-200 /Peltier Thermal Cycler /Biometra

pH- Meter: Beckman pHI31 pH meter

Real time PCR device: ABI PRISM 7000 Sequence Detection System /Applied Biosystems

Spectrophotometer: Beckman DU7500

Power supply: Pharmacia Electrophoresis power supply – EPS 600, Elite 300 Plus

Vortexing device: MS2,Minishaker IKA Werk

Waterbath: GFL 1092

Weighing device: Kern KB, Kern 770, Sartorius BP2100S

3.4 Plant material

3.4.1 Apple scab resistant Malus domestica cv. 'Remo'

This cultivar was introduced by the Pillnitz breeding institutions, and belongs to the denominated Re-cultivars®. The cultivars 'Elstar', 'Golden delicious' and 'Cox Orange' were used as donors in cross combinations to guarantee the fruit quality. As sources for scab resistance, 'Steinantonovka', carrying the resistance gene VA, *Malus* x *floribunda* (*Vf*), *M.* x *micromalus* (*Vm*), *M.* x *atrosanguinea Vf*?, *M.* x *pumila* (*Vr*), the cultivars 'James Grieve', 'Cox Orange' and 'Oldenbung', among others were also used. Other cultivars were also used as sources of mildew and fire blight resistance. The 'Remo' cultivar proved in field experiments to be resistant against apple scab, fire blight and mildew, and is considered a special cultivar for processing (FISCHER and FISCHER, 1999).

3.4.2 Apple scab susceptible cv. 'Elstar'

Originated in the Netherlands about 1955, 'Elstar' is a cross between the cultivars 'Golden delicious' and 'Ingrid Marie'. This cultivar is characterized by fine quality and the fruits are suitable for all-purposes (http://www.parkfruitfarm.co.uk/fruit%20varieties.htm).

3.4.3 Greenhouse-grown plants

The two years old plants of the cultivars 'Remo', 'Elstar', 'Holsteiner Cox' and 'Gloster' were grown in a greenhouse at 22ºC under daylight with additional illumination (~250 µmol per $m^2.s^{-1}$) under a 12h photoperiod.

3.4.4 In vitro plants

Sterile plants of the *M. domestica* Borkh. cv. 'Holsteiner Cox' and cv. 'Elstar' were introduced *in vitro* by Dr. Iris Szankowski (University of Hanover). The *in vitro* plants of the cv 'Remo' were kindly provided by Susanne Rühmann and Prof. D. Treutter (Institute of Fruit Science – Fruit Tree Physiology, TU Munich-Freising Weihenstephan, Germany). 'Remo' and 'Elstar' were cultivated on medium according to PERALES and SCHIEDER (1993) while 'Holsteiner Cox' was cultivated on C1 medium (Tab. 2). The cultures were maintained at 25°C under 16/8h light conditions and subcultured every four weeks.

Tab. 2: Composition of medium for *in vitro* culture of the cv. 'Remo' and 'Elstar'

Components	***PERALES & SCHIEDER (1993)***	***C1***
MS salts incl. Vitamins (g/L)	4.4	4.4
Sucrose (%)	3	3
Myoinositol (g/L)	0.1	0.1
BAP (µM)	3.1	4.4
IBA (µM)	-	0.5
NAA (µM)	0.5	-
GA_3 (µM)	2.8	-
Plant Agar (%)	0.8	0.8
pH	5.6 – 5.8	5.6 – 5.8

3.5 Cultivation of *Venturia inaequalis* and inoculation of *Malus domestica*

3.5.1 Mycelium production

The strain (designated as no. 15) of *V. inaequalis* used was obtained from the cv. 'Elstar' at the Biologische Bundesanstalt (Dossenheim, Germany) and kindly provided by Dr. Katja Schulze (Institute of Vegetable and Fruit Science, Fruit Science Division, University of Hanover).

From plates containing mycelium of *V. inaequalis,* four pieces of the border of grown mycelium were transferred to a new PDA plate. The plates were cultivated at 19°C under light for four weeks and were used as source for conidia production.

PDA Medium pH 5.6 ± 0.2

Potato Dextrose Agar 39 g/L

Bidest. water 1.000ml

After autoclaving media were poured in Petri dishes and cellophane membrane disks were placed above the medium, in Petri dishes

3.5.2 Cultivation and harvesting of conidia

From the mycelium plates 4 – 5 pieces of the mycelium were cut and placed into 50 mL tubes. After addition of 10 mL bidest. water it was mixed. Around 0.75 mL of the solution were placed on each PDA plate containing cellophane membrane, according to PARKER et al. (1994). The conidia plates were cultivated for 5 – 8 days under light condition at 19°C.

The cellophane membrane containing the conidia was collected and placed in tubes containing 20 mL bidest. water. After shaking for 5 min, conidia were washed from the cellophane by agitating in sterilized deionized water and the liquid was filtered through Miracloth®. Spore density was quantified and adjusted to a final concentration of 0.5 - 0.8 x 10^4 conidia/mL.

3.5.3 Vitality staining of conidia

On a microscope plate 300 µL 130 mM Tris-HCl (pH 7.4) and 5 µL FDA buffer was placed. After addition of 5 µL of conidia suspension, it was analysed under the microscope using blue light (450 – 490 nm). The percentage of alive conidia was represented as the number of conidia with green color.

FDA solution
5 mg FDA
1 mL aceton

3.5.4 Determination of the germination of conidia

On a microscope plate containing drops of 2% water agar, 5 µL of conidia suspension in the final concentration was placed. The microscope plate was placed in a petri dish containing water-saturated tissue paper and incubated it at 21°C under 100% humidity. After 24h the conidia germination was observed under the microscope as described above.

3.5.5 Inoculation of in vitro plants

To inoculate the plants, conidia were harvested after propagation for seven days on PDA plates. Steril *in vitro* plants of the cvs ‘Remo’ and ‘Elstar’ were inoculated by spraying 1 mL of a suspension containing 1x10^6 conidia in water. The inoculated plants were incubated according to the descriptions above. Control plants were sprayed with water. Leaves were harvested after 24 and 48h.

3.6 RNA isolation

The youngest leaves from three healthy plants (5 g) from greenhouse as well as *in vitro* plants were collected and immediately frozen in liquid nitrogen. Total RNA was isolated with some modifications according to MENNHAJ et al. (1999). The leaves were ground in liquid nitrogen and 15 mL of lysis buffer and 15 ml PCI mix were added. After shaking for 20 min, samples were centrifuged at 11500 x *g* for 20 min at 4ºC. Then, another 15 mL of PCI mix were added to the supernatant and centrifuged for 15 min in the same conditions. The RNA was allowed to precipitate in the presence of 3.5 M LiCl overnight at 4°C. After centrifugation the pellet obtained was dissolved in 1 ml DEPC treated water and precipitated by incubation for 1 hour at -20°C in the presence of 500 µl 3M Na–acetate (pH 5.2) and 5 mL of cold 96% ethanol. After washing in 70% cold ethanol, the pellet was air dried, dissolved in 500 µL DEPC treated water and stored at -70°C. All solutions, except lysis buffer and PCI, were treated with DEPC water.

Lysis buffer
100 mM Tris-Cl pH 8.0
600 mM NaCl, 20 mM EDTA
4% SDS

PCI mix (24:23:1)
24 mL phenol
23 mL chlorophorm
1 mL isoamylalcohol

0,1% DEPC treated water
Add DEPC to the water.
Shake vigorously and incubate at 37ºC for 12 hours.
The solution was finally autoclaved.

3.7 DNA isolation

DNA was isolated from different apple cultivars from *in vitro* plants or leaves of greenhouse-grown plants using the CTAB method (DOYLE and DOYLE, 1990). Around 2 g plant material were immediately frozen and macerated in liquid nitrogen. Pre heated (60ºC) CTAB buffer was added and after 30 min incubation at 60ºC, 10 mL CI-mix were added. After centrifugation for 10 min at 3500 x *g* at 4ºC, 2/3 vol isopropanol (-20ºC) were added and a pellet was obtained by centrifugation for 10 min. After washing in 10 mL washing buffer and centrifuging for 10 min, the pellet was dissolved in 3 mL TE buffer containing RNAse and incubate for 30 min at 37ºC. The precipitation was carried out by addition of 1 mL of 7.5 M NH_4-acetate and 8 mL ethanol absolute (-20ºC) and centrifugation for 20 min at 3500 x *g*. The pellet was dissolved in 1 mL bidest water and stored at -20ºC.

CTAB buffer
3% CTAB
1.4 M NaCl
0.2 % β-mercaptoethanol
20 mM EDTA
0.5% PVP-40
100 mM Tris-HCl pH 8.0

TE buffer
10 mM Tris-HCl, pH 8.0
1 mM EDTA
10 µg/mL RnaseA

CI-mix
23 mL clorophorm
1 mL isoamylalcohol

Washing buffer
76% ethanol
10 mM ammonium acetate

3.8 Determination of DNA and RNA concentrations by spectrophotometer

The concentrations of DNA and RNA were determined by spectrophotometer at an extinction of λ = 260 nm, by using the formula:

$$\text{DNA concentration} = (OD_{260} \cdot D \cdot 50\ \mu g/mL)$$

$$\text{RNA concentration} = (OD_{260} \cdot D \cdot 40\ \mu g/mL)$$

D = Dilution factor

The purity of DNA and RNA samples were determined by the ratio between the OD_{260}/OD_{280}. For DNA a pure sample has a ratio of 1.8 and for RNA 2.0.

3.9 RNA gel electrophoresis

The electrophoretic separation of RNA was carried out under denaturation conditions. To 2 µg RNA, 18 µl *Northern-mix* were added and the probes were incubated for 15 min at 65°C on water bath. After cooling on ice for two min, the samples were loaded on a 1.5% agarose gel, and separated at 80 V, for 45 min. RNA bands were visualized under UV light.

10x MOPS buffer pH 7.0
0.2 M MOPS
50 mM Na-acetate
10 mM EDTA

Northern mix
5 mL sample buffer
1 mL loading buffer
40 µL ethidium bromide

RNA formaldehyde Gel
1.5 % agarose (w/v)
1x MOPS
1.26% formaldehyde

Sample buffer
1x MOPS
6.5% formaldehyde
50% formamide

Loading buffer
0.25% bromophenol blue
0.25% xylenecyanol FF
50% Glycerin
1 mM EDTA

3.10 DNA gel electrophoresis

Separation of DNA was carried out in a 0.8 – 1.5% agarose gel (w/v in 1x TAE buffer). Samples were mixed with loading buffer in different concentrations, according to the purposes, and were loaded in the gel. The electrophoresis was carried out at 4.5 – 6 V/cm. Different DNA markers were used to determine the band sizes.

After staining for 15 min in TAE buffer containing EtBr, gel images were recorded under the UV-light.

TAE-buffer (50x)
40 mM Tris-Ac pH 7.5
20 mM glacial acetic acid
1 mM EDTA

Loading buffer
100 µL bromophenol blue solution
50% glycerin
20 µL TAE buffer

3.11 Suppression subtractive hybridization (SSH)

The subtractive hybridization is an efficient method for isolation of cDNAs from different expressed genes. The PCR-Select^{TM} cDNA subtraction kit (Clontech) is based on the method developed by DIATCHENKO et al. (1996). The combination of subtractive hybridization and PCR allows the isolation of several cDNAs, expressed only or in a higher amount in one of the RNA-populations. The scheme of this method is presented in the Fig. 5.

Two subtractions were carried out, in order to generate two different libraries:

1. "Forward subtraction"- Isolation of genes only or highly expressed in the cv. '**Remo'** (tester 1) using the cDNA population of **'Elstar'** as 'driver 1';
2. "Reverse subtraction"- Isolation of genes only or highly expressed in the cv. '**Elstar'** (tester 2), using the cDNA population of '**Remo'** as 'driver 2';

The tester is designated as the cultivar from which the genes should be isolated, which should be not expressed, or should present a lower expression as the driver cultivar. The driver is designated as the cultivar used to subtract the genes differentially expressed.

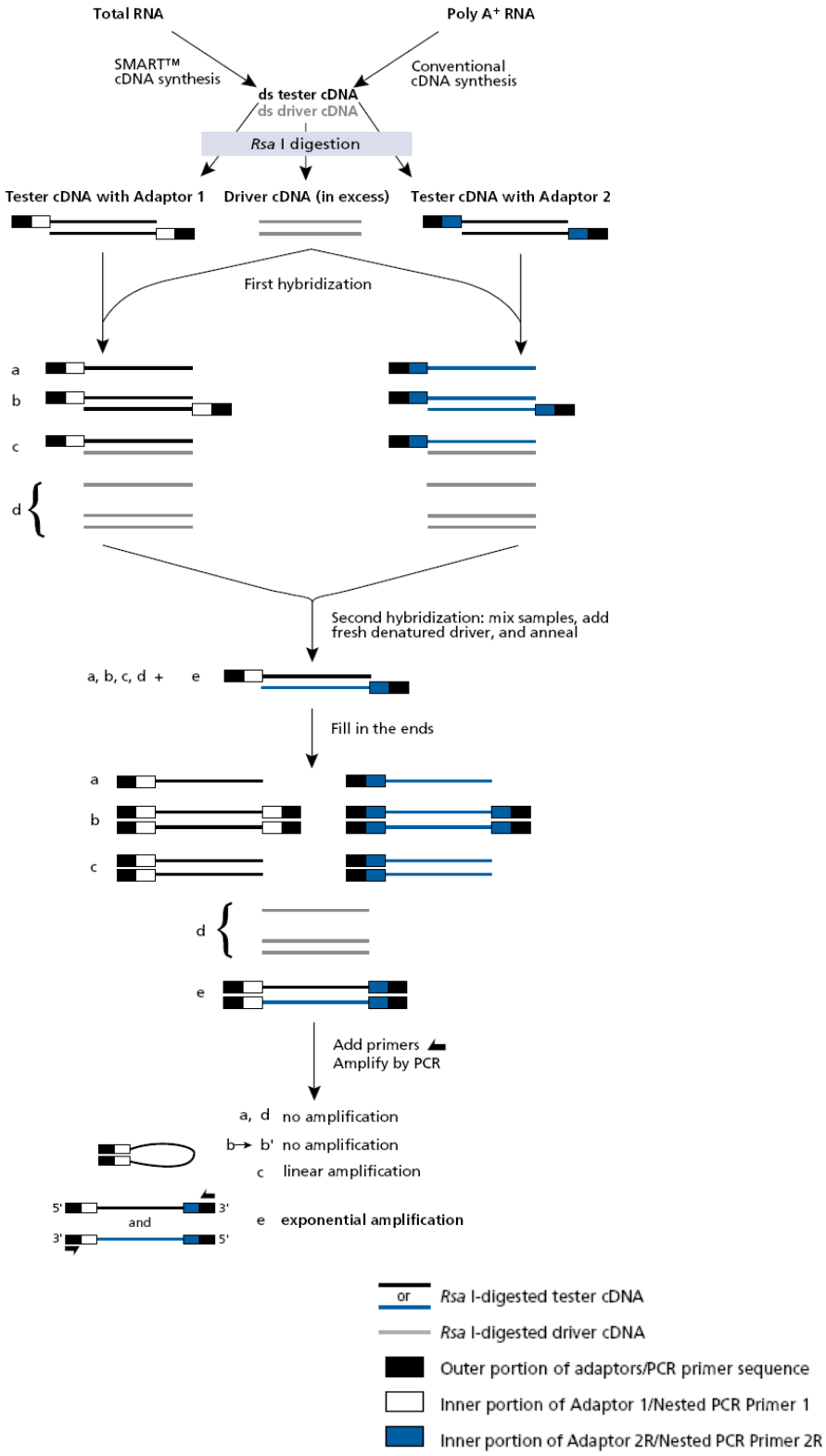

Fig. 5: Scheme of the suppression subtractive hybridization method
http://www.clontech.com/clontech/archive/OCT01UPD/pdf/PCR-SelectProducts.pdf

3.11.1 cDNA synthesis

cDNA from the samples were synthesized from total RNA using the SMART PCR cDNA Synthesis kit according to the procedures described in the manual (Clontech, Palo Alto, CA, USA).

3.11.2 SSH - PCR Select^{TM} cDNA Subtraction kit (BD Clontech)

In a 0.5 ml tube 43.5 µL of ds cDNA was mixed with 1x *RsaI* reaction buffer and 15 U of *RsaI* restriction enzyme and incubated for 1.5 h at 37^{o}C, in order to obtain short blunt-ended fragments. After stopping the reaction by adding 2.5 µL 20x EDTA/Glycogen mix, cDNAs were precipitated. The precipitation step was carried out by adding 50 µL phenol:chloroform:isoamylalcohol (25:21:1) and centrifuging at RT for 10 min at 10000 x *g*. The aqueous layer was than mixed with 50 µL chloroform:isoamylalcohol (24:1) and again precipitated at the same conditions. Twenty five µL of 4 M NH_4OAc and 187.5 µL of 95% ethanol were added and after another centrifugation step at 10000 x *g* for 10 min at RT, the pellet was washed in 200 µL 80% ethanol and centrifuged at 10000 x *g* for 5 min. The pellet was than dissolved in 5.5 µL water.

After dilution of 1.5 µL of the tester cDNA with 7.5 µL water, 2 µL were mixed with 3 µL water, 1x ligase buffer, 400 u T4 DNA ligase and 2 µL adapter 1 (5'- cta ata cga ctc act ata ggg ctc gag cgg ccg ccc ggg cag gt-3') or 2 µL adapter 2R (10 µM) (5'-gta ata cga ctc act ata ggg cag cgt ggt cgc ggc cga ggt-3'), respectively in two reactions. The reactions were incubated overnight at 16^{o}C and stopped by adding 1 µL 20x EDTA/glycogen mix. The ligase was inactivated by heating for 5 min at 72^{o}C.

3.11.3 Subtractive Hybridization

Both tester cDNA populations, ligated to the different adapters were mixed with the driver-cDNA in separated reactions containing 1.5 µL driver-cDNA, 1.5 µL tester-cDNA (ligated to adapter 1 and adapter 2R, respectively) and 0.4x hybridization buffer in a final volume of 4 µL, and allowed to hybridize. After 1.5 min at 98^{o}C, the reactions were incubated for 8 h at 68^{o}C. Samples were than purified and 1 µL was submitted to the second hybridization step. After denaturation for 1.5 min at 98^{o}C, 1 µL of the driver cDNA was mixed with 0.4x hybridization buffer and 2 µL sterile water. The second hybridization step was carried out overnight at 68^{o}C and was completed after addition of 200 µL dilution buffer and heating at 75^{o}C for 7 min.

3.11.4 PCR Amplification of subtracted cDNAs

The PCR reaction allowed the selective amplification of differentially expressed cDNAs. One µL cDNA was mixed with 2.5 µL 10x reaction buffer, 0.5 µL dNTP mix (10 mM), 1 µL 10 mM primer 1 (5'-cta ata cga ctc cat ata ggg c-3'), 0.5 µL 50x Advantage Klen Taq polymerase mix (Clontech) and 19.5 µL water. The adapters were then extended by incubation for 5 min at 75°C. The first PCR was carried out as presented in Tab. 3.

For the second PCR reaction, the first PCR product was diluted 1:10 and 1 µL was used. In a reaction containing 2.5 µL 10x PCR reaction buffer, 1 µL Nested primer 1 (10 µM, 5'-tcg agc ggc cgc ccg ggc agg t-3'), 1 µL Nested primer 2 (10 µM, 5'-agc gtg gtc gcg gcc gag gt-3'), 0.5 µL dNTP mix (10 mM), 0.5 µL 50x Advantage Klen Taq polymerase mix and 18.5 µL water the PCR was carried out according to instructions contained in Tab. 3.

Tab. 3: Conditions of the first and second PCR amplification of fragments subtracted by SSH

1[a]. PCR	**Temperature (°C)**	**Time (s)**	**Cycles**
Denaturation	94	30	
Annealing	66	30	27
Elongation	68	90	
2[a]. PCR	Temperature (°C)	Time (s)	Cycles
Denaturation	94	30	
Annealing	68	30	12
Elongation	72	60	

3.12 Ligation of the ESTs to pGEM-T Vector System I (Promega)

The pGEMT Vector (Fig. 6) contains a cloning site inserted into the coding region of the *lacZ* gene, which codes the β-galactosidase enzyme. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates. This vector also contains an *amp* gene, which confers resistance against the ampicillin, allowing the growth of transformed bacteria on medium containing this antibiotic.

The ligation of the ESTs was done according to the manufacture's protocol. The enzymes and buffers were provided with the kit. For ligation, 50 ng of plasmid were mixed with 0.5 µg of the subtracted cDNA in the presence of 2x rapid ligation buffer, 1 µL T4 DNA ligase and deionized water to a final volume of 10 µL. After incubation for 1h at RT the ligation product was transferred to *Escherichia coli* XL1-blue.

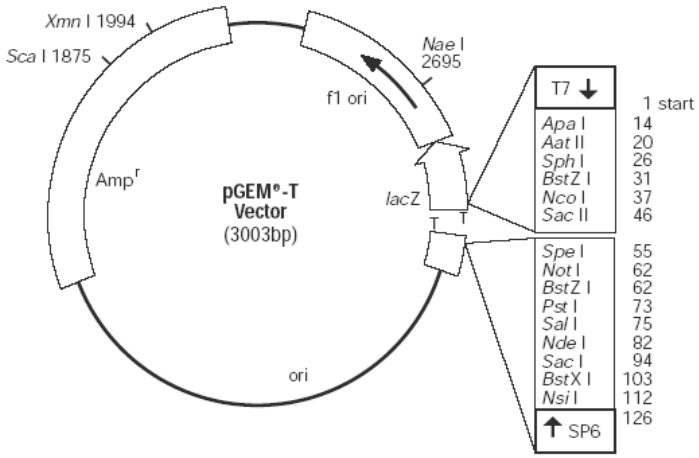

Fig. 6: Map of the binary plasmid vector pGEM-T Vector (Promega).
http://www.tcd.ie/Genetics/staff/Noel.Murphy/recombinant%20dna%20ge4021/pgem.pdf

3.13 Transformation of E. coli XL-1Blue by heat shock

The ligation product (5 µL) was mixed with 200 µL bacteria. The mixture was incubated on ice for 30 min, then at 42 ºC for 90 s and again for 2 min on ice. After adding 600 µL LB medium, the bacteria were recovered and allowed to grow for 1h at 37ºC. The bacteria were plated on LB medium containing 100 µg/mL ampicillin, 0.5 mM IPTG and 80 µg/mL X-Gal, since the plasmid contain a blue/white selection system. After incubation overnight at 37°C, the white colonies were picked up and incubated on 10 mL LB containing 100 µg/mL ampicillin.

LB medium
0.5% (w/v) yeast extract
1% (w/v) pepton or trypton
1% (w/v) NaCl
pH 7.2

To prepare a long-storage stock culture, 10 mL LB medium were inoculated with 5 µL bacteria culture and incubated overnight at 37ºC. From this culture, 850 µL were mixed with 250 µL autoclaved 87% glycerin. The cultures were stored at -80°C.

3.14 Plasmids Isolation and Purification

3.14.1 Isolation using the Qiagen kit

The colonies with the bacteria containing the plasmids of interest were incubated overnight under shaking at 37°C in 125 mL LB Medium, containing the appropriate antibiotic. The plasmids were isolated according to the kit manuals (Qiagen). The pellets were dissolved in TE buffer.

TE buffer
10 mM Tris HCl pH 8.0
1 mM EDTA

3.14.2 Isolation using the HB-Lyses method

For plasmid isolation, the colonies were pre-cultured overnight at 37°C under shaking, in micro centrifuge tubes of 1.5 mL, and centrifuged at 11500 x *g* for 2 min. The pellet obtained was dissolved in 300 µL of buffer 1 and the bacteria were lysed by applying 300 µL of buffer 2, mixing and incubating at RT for 5 min. After addition of 300 µL of buffer 3, it was incubated on ice for 10 min and centrifuged at RT for another 10 min at 11500 x *g*. The supernatant containing the plasmids was centrifuged again at the same conditions and 800 µL was centrifuged with 700 µL isopropanol, at 11500 x *g* for 30 min to elute the DNA. The pellet was washed with 500 µL ice cold 70% ethanol, incubated for 2 min, centrifuged for 10 min at 11500 x *g* and dried by incubation in water bath for 10 min at 60°C. The plasmid DNA was dissolved in 50 µL distilled water and stored at -20°C.

Buffer 1
50 mM Tris-HCl pH 8.0
10 mM EDTA
400 µg/ml RNase

Buffer 2
200 mM NaOH
1% SDS

Buffer 3
2.55 M K-Acetate pH 4.8

3.15 Polymerase Chain Reaction (PCR)

PCRs were carried out in a 25 µL final volume. Between 500 ng and 1 µg cDNA or DNA were used as template, according to the different experiments. The reactions, except somewhere else described, contained 1 µL of 10 µM dNTPs, 1 µL of 10 pM of each primer, 1 U *Taq* polymerase and 1x buffer provided by the supplier of the enzyme. The primers used varied according to the experiment, and are described in the Tab. 4.

The reaction conditions of the PCR are shown in Tab. 5. The annealing temperature was dependent of the primer. The PCR reaction conditions for the specific primers for the *pat* and *pmi* genes are shown in the Tab. 6.

3.16 Colony PCR

The fragments cloned into pGEM-T Vector and transformed into *E. coli* X-blue 1 were amplified by colony PCR. Therefore, using a pipette tip, a small amount of the colonies grown on LB plates were transferred to 0.2 mL tubes. The primers T7 and M13, with homology to the sequence of pGEM-T Vector were used. The conditions were the same as previously described.

3.17 Reverse Transcription (RT) – PCR

For semi quantitative RT-PCR, total RNA from cv. 'Remo' and cv. 'Elstar' were first treated with DNAse to prevent amplification of fragments from genome. To 1µg RNA, 1x reaction buffer and 1 U DNAse were added, and filled to 9 µl with DEPC treated water. After incubation for 30 min at 37ºC, 1 µL of 25 mM EDTA was added and incubated for 10 min at 65ºC to stop the reaction.

After treatment, cDNA was synthesized from RNA in a two-step reaction. In the first step, 500 ng RNA were mixed with 10 mM dNTPs and 10 pmol/µL Oligo(dT)23 primer in a final volume of 10 µL. After incubation for 10 min at 70ºC and cooling on ice for 2 min, the RT-Mix, containing 1x buffer, 1 µL MMuLV polymerase and 50 Units RNAse inhibitor, were added. The first strand synthesis was completed by incubation for 1h at 50ºC.

The cDNA (1 µg) was directly used for PCR reaction. Amplification products were directly separated in a 1% (w/v) agarose gel. The quantification of expression was analyzed by the TINA software package (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

Tab. 4: Primer sequence for specific gene fragments.

Primer	Sequence
thaumatine-like protein, *pr5* fwd	5'-atg tcg atg atg aag agc ca-3'
thaumatine-like protein, *pr5* rev	5'-tta tgg gca gaa agt aat gac g-3'
rubisco small subunit, *ssu* fwd	5'-cgg cac cgt ggc tac agt at-3'
rubisco small subunit, *ssu* rev	5'-cac gaa tcc atg ctc caa ct-3'
elongation factor 1a, *ef1a* fwd	5'-caa tgt gag agg tgt ggc aat c-3'
elongation factor 1a, *ef1a* rev	5'-gga gtg aag cag atg atc tgt tg-3'
metallothionein-like protein, *mt3* fwd	5'-aag tgc gac aac tgc gac tg-3'
metallothionein-like protein, *mt3* fwd b	5'-gga att aag aga gcc aaa gc-3'
metallothionein-like protein, *mt3* rev	5'-tga cca cag gtg cag ctc aca-3'
ribonuclease-like protein, *pr10b* fwd	5'-ctc ttg atg aca gaa ccg ct-3'
ribonuclease-like protein, *pr10b* rev	5'-cct gct agg ttg ttc aat gc- 3'
ADP-ribosylation factor, *arf* fwd	5'-aga aca tca gct tca ccg tc-3'
ADP-ribosylation factor, *arf* rev	5'-gag tgg agg cca agc tta tc-3'
ferrochelatase, *hemH* fwd	5'-agt ctc tat gtg ctc gct ca-3'
ferrochelatase, *hemH* rev	5'-cag tta tgt gga gga tgc tg-3'
cysteine protease inhibitor, *cpi* fwd	5'-atc gag gtc aca gat ggt gg-3'
cysteine protease inhibitor, *cpi* rev	5'-aac ctc tct tcc ttg cct cc-3'
metallothionein-like protein, *mt2* fwd	5'-cat gga ggc atc tga gat gg-3'
metallothionein-like protein, *mt2* rev	5'-gac gtg cag ctc aga aga ag-3'
glutathione-S-transferase, *gst* fwd	5'-cag gta caa ggc gag tga tt-3'
glutathione-S-transferase, *gst* rev	5'-gga atg tgg tga aga tca gc-3'
T7	5'-taa tac gac tca cta tag gg-3'
M13 forward	5'-gtt ttc ccc agt cac gac-3'
Pmi-forward	5`-aca gcc act ctc cat tca-3`
Pmi-reverse	5`-gtt tgc cat cac ttc cag-3`
pat-forward	5`-tcg aag tcg cgc tgc cag aa-3`
pat-reverse	5`-gca cgg tca act tcc gta c-3`

Tab. 5: PCR conditions for amplification of specific gene fragments.

	Temperature (°C)	***Time (s)***	***Cycles***
Denaturation	94	180	
Denaturation	94	30	
Annealing	*	60	35
Elongation	72	90	
Elongation	72	150	

* 50°C, unless described in the text

Tab. 6: PCR conditions for amplification of fragments of the *pat* and *pmi* genes.

	Temperature (°C)	*Time (s)*	*Cycles*
Denaturation	94	60	
Denaturation	94	45	
Annealing	60	45	30
Elongation	72	45	
Elongation	72	300	

3.18 Real Time PCR

Expression level of selected genes was determined by using the two step QuantiTect ™ SYBR® Green PCR kit. The PCR amplifications were performed according to the manufactures user manual with small modifications. The volume of each PCR reaction was reduced to 25 µL instead of 50 µL.

Total RNA was isolated from young and old leaves of the cultivars ‘Remo’ and ‘Elstar’ and was treated with DNAse and subsequently converted into cDNA, as previously described. The PCR transcript quantification was performed with six genes (metallothionein type 2, metallothionein type 3, ribulose 1-5-bisphosphate carboxylase small subunit, ADP-ribosylation factor, PR10b and glutathione-S-transferase) and the housekeeping gene β-Actin as endogenous control. Amplification was carried out with 50 ng cDNA per PCR reaction under the following conditions: one initial activation step of the HotStart Taq DNA polymerase followed by 35 cycles according to the Tab. 7 in the presence of 0.4 µM of the respective primers. The results were analyzed using the delta-delta CT-method.

Tab. 7: PCR conditions for amplification of fragments by real time PCR.

	Temperature (°C)	*Time (s)*	*Cycles*
Denaturation	94	15	
Annealing	50	30	35
Elongation	72	30	

3.19 Labeling of probes

3.19.1 Radioactivity labeling of probes using ^{32}P

The cDNA populations obtained using the Smart kit following the manufacture's manual, for both 'Remo' and 'Elstar' cDNA populations, were used to synthesize ^{32}P labeled cDNA probes of both cultivars. Therefore, 2 µL of cDNA from each cultivar were added to a reaction containing 2 µL of the polymerase provided with the kit, 2 µL of 5'PCR primer II A (10 µM), 2 µL dNTPs (10 µM from dATP, dTTP and dGTP, and 0,05 µM dCTP) and 5 µL (α-^{32}P) dCTP (activity > 3.000 Ci/mol, 10 µC/µl). The labeled cDNA was obtained by PCR (Tab. 5). The purification was done on Sephadex G50 columns. The columns were prepared by addition of 1 mL Sephadex G50 and centrifugation for 5 min at 1500 x *g*. After washing with 100 mL TNE buffer and centrifuging for 5 min at 1500 x *g*, the labeled cDNA was placed into the columns and purified by centrifugation for 5 min at 1500 x *g*.

TNE buffer
10 mM Tris-HCl pH 7.5
100 mM NaCl
1 mM EDTA

3.19.2 Digoxigenin labelling of probes

PCR fragments of the genes of interest were amplified by PCR (Tab. 5 and 6) using the specific primers. The products of these amplifications were labeled by digoxigenin-dUTP labelling kit, according to the manufacture's protocol (DIG High Prime DNA Labeling, Roche diagnostics, Germany).

For labelling of the probe, 2 µL of PCR product were mixed with 5 µL of 10x buffer, 10 pmoL/µL from forward and reverse primers, 5 µL of PCR DIG labelling mix and 0,75 µL of the enzyme mix according to the instructions of the manual (PCR DIG Probe Synthesis kit, Roche). The probe was denaturated by heating at 95°C for 10 min and immediately cooled on ice.

3.20 Fragment sequencing and homology search on databases

ESTs were sequenced using the M13 forward primer in the Innovation Technology Transfer Centre (University of Bielefeld, Germany). After sequencing, the fragments were kindly compared to the sequences in the NCBI databases using the BLAST algorithms by Dr. Abdul Al-Nasser.

3.21 Reverse Northern blot analysis

The cDNA fragments from both libraries were amplified from pGEM-T Vector by colony PCR. Aliquots of 1 µL of PCR reaction were transferred to two nylon membranes in replicates, according to the dot blot Gibco system. The cDNAs were cross-linked to the membrane by exposure to short-wavelength UV irradiation for three min (SAMBROOK et al., 1989). The membranes were pre-hybridized for two hours with pre-hybridization buffer and subsequently incubated overnight at 60°C in hybridization buffer (pre-hybridization buffer containing the labeled cDNA). The membranes were washed twice in low stringency buffer for 15 min at RT and twice in high stringency buffer for 15 min at 60°C. Hybridization signals were detected using Phosphoimager plates (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany) and quantified by the TINA 2.09 software package.

The fragments that hybridized only with the tester labeled cDNA or showed at least three fold higher signals on these membranes, compared to the signals on the membrane hybridized with the driver labeled cDNA were sent for sequencing.

Pre-hybridization buffer	100x Denhard
20x Denhardt	*2% polyvynilpirrolodone (PVP) 10*
5x SSPE	*2% serum albumine bovine protein*
0.2% SDS	*2% Ficoll 400*
0.2 mg/mL salmon sperm DNA	
20x SSPE pH 7.4	*Low stringency buffer*
3.6 M NaCl	2x SSC
0.2 M NaH_2PO_4	0.2% SDS
20 mM EDTA	
High stringency buffer	*20x SSC*
1x SSC	0.3 M Na-Citrat pH 7.0
0.2% SDS	3 M NaCl

3.22 Dot Blot

Aliquots of 1 µL of PCR product of all colonies (amplified with the T7 and the M13Rev primers) were diluted to a final volume of 100 µL and were blotted onto nylon membranes (Dot blot system, Gibco) and fixed by exposure to UV light for three min. Membranes were pre-hybridized for 6h at 60°C and hybridized overnight at 68°C with pre-hybridization buffer

containing the digoxigenin labeled cDNA. Subsequently, the membranes were washed twice for 10 min in low stringency buffer and twice for 10 min with high stringency buffer and again once with buffer 1 for one min. Afterwards the membranes were incubated for 30 min at 68°C with low stringency buffer containing 1% blocking solution and then in washing buffer 1 for 10 min. For detection, 4 µL of anti-digoxigenin AP (alkaline phosphatase) were added to 20 mL buffer 1 and the membranes were incubated for 30 min at RT. After washing the membranes twice with buffer 1 for 15 min, the development was performed by addition of developing buffer. Membranes were incubated until signals were visible.

Pre-hybridization buffer
5x SSC
0.1% n-laurylsarcosyl
1% blocking solution

Low stringency buffer
2x SSC
0.1x SDS

High stringency buffer
0.1x SSC
0.1% SDS

Buffer 1
100 mM Tris HCl pH 7.5
150 mM NaCl

Developing buffer
337.5 µg/mL nitroblue tetrazolium (75 mg/mL) and 175 µg/mL X-phosphate (50 mg/mL) in 10 mL 100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$

3.23 Southern Blot

Approximatelly 20 µg DNA was used from each sample. The DNA was first cleaved by adding 60 units of the enzyme *BamH*I and 1x specific buffer to a final volume of 300 µL. The mixtures were incubated overnight at 37ºC (temperature required for the enzyme). After addition of another 20 units of enzyme, the probes were incubated for 2h to complete the digestion. The precipitation was carried out by addition of 1 M NH_4-acetate and 70% cold ethanol and then centrifuged for 20 min. The pellets were washed in 70% cold ethanol and dissolved in bidest. water.

The samples were separated in 0.8% (w/v) agarose gel overnight at 20 V. The gel was denaturated for 40 min (denaturation buffer) and neutralized for 40 min (neutralization buffer). The DNA was transferred to nylon membranes overnight by capillarity in the presence of 20x SSC buffer. After transfer, the DNA was fixed to the membrane by incubation at 80ºC for 2h.

The pre-hybridization was done by incubation at 40ºC for 30 min (pre-hybridization buffer) and the hybridization on the same buffer, containing the labeled probe, at 42ºC overnight. The membrane was then washed twice for 5 min in low stringency buffer at RT and twice for 15 min at 65°C in high stringency buffer.

To avoid unspecific hybridization, the membrane was incubated for 30 min in a blocking solution and then incubated for 30 min in blocking solution containing anti-digoxigenin AP, (alkaline phosphatase). After that, the membrane was washed twice for 15 min in washing buffer.

To visualize the bands, the membrane was first incubated for 5 min in detection buffer and then CDP-Star was spread over the membrane. After incubation for 5 min at RT, the membranes were exposed to X-ray film (Kodak) for 8 – 15 min. The films were immersed in Tentenal developing solution until the bands were visible, and then rinsed in water and immersed in Tentenal fixation solution for 2 min.

Denaturation buffer
1.5 M NaCl
0.5 N NaOH

Neutralization buffer pH 5,5
1 M Tris-HCl
3 M NaCl

Pre-hybridisation buffer
7% SDS
50% formamide, deionized
5x SSC
0,1% N-laurilsarcosil
2% blocking reagent (Roche)
50 mM sodium phosphate, pH7.0

Low stringency buffer
2x SSC
0.1% SDS

High stringency buffer
0.1x SSC
0.1% SDS

Maleic acid buffer pH 7,5
0.1 M maleic acid
0.15 M NaCl

Blocking solution
1% blocking reagent (Roche)
maleic acid buffer

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

Washing buffer
Maleic acid buffer
0.3% Tween 20

Detection buffer pH 9,5
100 mM Tris HCl
100 mM NaCl

3.24 Northern Blot

Around 10 µg of total RNA from each sample were loaded on a denaturating gel and separated for 1h at 20 V and one additional hour at 60 V. The RNAs were then directly transferred to nylon membranes by capillarity overnight in the presence of 20x SSC buffer. The conditions for hybridization and developing of the membranes, as well as the labeling of the probe with digoxigenin were the same as used for southern blot.

3.25 Stripping membranes

After hybridization, the membranes were stripped for further rehybridization with other probes, according to the instructions of DIG application manual. Membranes used for Southern blot were stripped by rinsing for 1 min in water, washing twice for 15 min at 37ºC in solution A and finally rinsing in 2x SSC buffer for 5 min.

The Northern blot membranes were stripped by two times incubation for 60 min in solution B at 80ºC and rinsing in 2x SSC for 5 min. Membranes were stored in 2x SSC.

Solution A	*Solution B*
0.1 M NaOH	50% formamide
0.1% SDS	5% SDS
	50 mM Tris-HCl, pH 7.5

3.26 Plant tissue culture

3.26.1 Organogenesis from apple leaf explants

Leaf explants were cut of the four youngest leaves of four-week non-rooted *in vitro* plants. The tip apex and the petiole were removed, in order to originate wounding places, from where the regeneration should occur. The explants were then placed with the upper surface touching the medium and incubated for two weeks in dark by 25ºC. After that, they were incubated in a 16/8h (light/dark) rhythm. The medium was changed after two weeks. After a period of six weeks, the regenerated shoots were placed on elongation medium for another three weeks and finally in the cultivation medium, proper for each cultivar. Depending of the objectives, the plants were rooted on the proper medium (Tab. 8).

3.26.2 Explant sensitivity to mannose

Explants sensitivity to mannose was tested by transferring segments of the four youngest leaves of four weeks old *in vitro* apple shoots onto regeneration medium supplemented with

various concentrations of mannose (0, 1, 2, 2.5, 5 and 10 g/L) each in combination with different sorbitol concentrations (5, 10, 15 and 30 g/L). Evaluation was done after 7 weeks by determination of regeneration rate, percentage of callus bearing and necrotic explants as well as number of shoots per explant.

Tab. 8: Culture medium for *in vitro* culture of *M. domestica*.

	Regeneration medium	***Elongation medium***	***Rooting medium***
MS salts including vitamins (g/L)	4.4	4.4	4.4
Sorbitol (%)	3	-	-
Sucrose (%)	-	3	3
Myoinositol (g/L)	0.1	0.1	0.1
TDZ (µM)	3	-	-
IBA (µM)	1	-	1-2
BAP (µM)	-	4.4	-
GA_3 (µM)	-	0.28	-
Plant agar (%)	-	0.8	0.7
Gelrite (%)	0.3	-	-
pH	5.6 – 5.8	5.6 – 5.8	5.6 – 5.8

3.27 Transformation of *Malus domestica via Agrobacterium tumefaciens*

3.27.1Agrobacterium tumefaciens strain

For all transformations of the *M. domestica* cv. 'Holsteiner Cox', the *A. tumefaciens* strain EHA105 (HOOD et al., 1993) was used.

3.27.2 Bacterial cultures

For transformation, 250 µL of a bacteria glycerol stock culture (stored at -80°C) were inoculated in 25 mL YEP medium in the presence of the appropriate antibiotic and allowed to grow under shaking overnight at 28ºC. The purification of the bacteria was done by centrifugation for 10 min at 3600 x *g*. The pellet was then resuspended in MS-Medium (4.4 g/L MS salt including vitamins, pH 5.7) and adjusted to an OD_{600} of 0.8. This suspension was used for transformation of the plants.

The glycerin stock culture of bacteria (850 µL bacteria + 150 µL glycerin 100%) were stored at -80ºC.

3.27.3 Electro-competent cells of Agrobacterium tumefaciens

A single bacteria colony was incubated overnight at 28°C in LB Medium with vigorous shaking. The bacteria were then washed three times by spinning at 1250 x *g* for 5 min at 4°C, and re-suspending the pellet in 50 mL of ice-cold 10% glycerol. Finally, the bacteria were re-suspended in 1 mL ice-cold 10% glycerol and aliquots of 40 µL were stored at –80°C.

3.27.4 Electroporation

Plasmids were introduced into *A. tumefaciens* by electroporation. Plasmid DNA (1 µg) and the agrobacteria were gently mixed on ice and placed in a 0.2 cm precooled cuvette. Conditions were as follow: set capacitor: 25 µF; resistor 200 – 400 Ω; voltage 1.25 – 2.5 kV; pulse: 6.25 – 12 kV/cm for 4 – 8 msec. Immediately after transformation, 500 µL precooled SOC medium was added to the sample. After inoculation for 30 min on ice, the sample was shaked for 2h at 28ºC and plated on YEP medium.

3.28 Transformation vectors

3.28.1 pNOV2819

The plasmid pNOV2819 (Fig. 7), used in the transformation experiment was kindly provided by Syngenta. This vector confers spectinomycin resistance in bacteria and carries between T-DNA left and right border the *pmi*-gene under the control of CMPS promoter (cestrium yellow leaf curling virus promoter). The vector was modified by the introduction of a *gus* gene (with intron) by using the *Hind*III restriction site (Dr. M. Wallbraun, Centrum Grüne Gentechnik, Germany.

3.28.2 pIBGUS

The vector pIBGUS (Fig. 7, DE KATHEN and JACOBSEN, 1995) carries on its T-DNA a GUS gene and both the *npt*II gene coding for neomycin phosphotransferase II and the *pat* gene, encoding the phosphinotricin-acetyltransferase. The *pat* gene is controlled by the CaMV35S promoter. pIBGUS was used to select transgenic cells with the herbicide glufosinate-ammonium/ppt. The plasmids were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation.

3.29 Agrobacterium-mediated transformation

For transformation the four youngest leaves of four-weeks old *in vitro* plants were used. The plants were cut as described above and incubated within an *Agrobacterium* suspension for 1h under shaking.

After drying on filter paper, the explants were placed with the adaxial side to the medium and co-cultivated in the dark for three days at 25ºC on regeneration medium in absence of selection agent. To eliminate the bacteria, the explants were washed twice for 15 min in bidest water and 15 min in MS medium containing the antibiotic 200 mg/L ticarcillin under shaking.

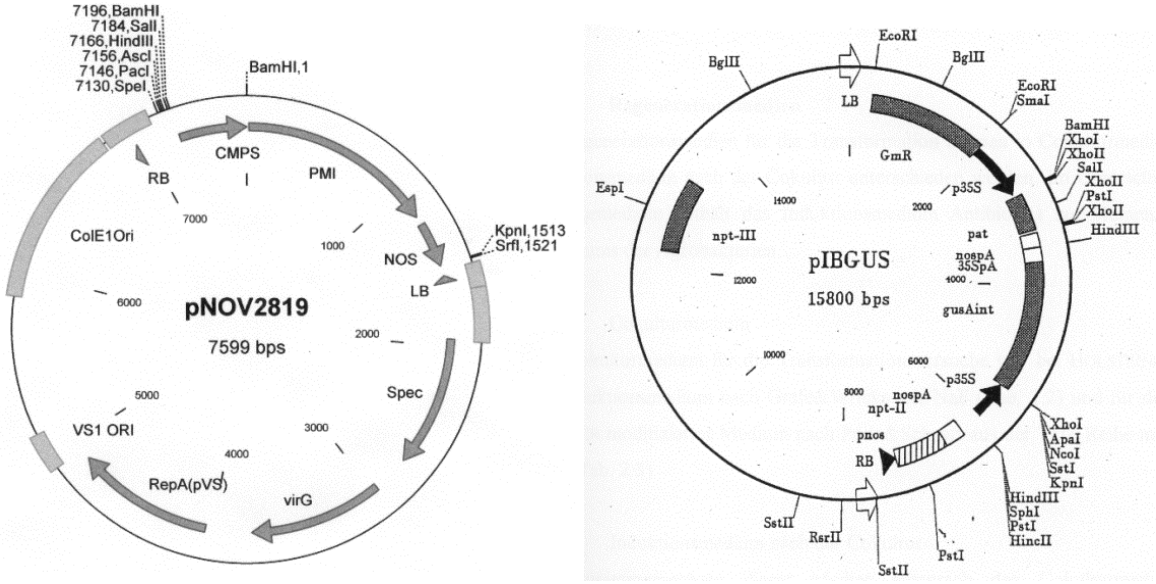

Fig. 7: Map of binary plasmid vectors pIBGUS and pNOV2819.

3.30 Selection agent and regeneration of transgenic plants

3.30.1 Selection with mannose

Adventitious shoot development was induced on regeneration medium (Tab. 8). To completely eliminate the agrobacteria, 150 mg/L ticarcillin was added to the medium. The selection of explants transformed with the pNOVGUS construct, under selection of the *pmi* gene was done on medium supplemented with various combinations and concentrations of sorbitol/mannose (Tab. 9).

3.30.2 Selection with phosphinotricin

The selection of explants transformed with the pIBGUS construct, was carried out using the herbicide phosphinotricin. The selection started directly after co-culture with 1 mg/L glufosinate ammonium/PPT in the regeneration medium. The contents of glufosinate

ammonium were enhanced during the regeneration up to 10 mg/L, according to SZANKOWSKI, (2002).

Tab. 9: Mannose selection conditions after transformation with pNOVGUS.

Sorbitol/mannose concentration (g/L) for initial or subsequent culture	Sorbitol/mannose concentration (g/L) for further culture							
	0S/5 M	0S/10M	0S/15M	10S/5M	15S/ 2.5M	15S/5M	15S /10M	30S/5M
30S/0M					x	x	x	x
30S/1M	x	x	x	x	x	x	x	x
30S/2M	x	x	x	x	x	x	x	x
30S/2.5M	x	x	x	x	x	x	x	x
30S/5M*								
30S/10M*								
15S/0M					x	x		
15S/2M								
15S/5M*								
15S/10M*								
10S/0M*								
10S/2M*								
10S/5M*								
10S/10M*								
5S/0M*								
5S/2M*								
5S/5M*								
5S/10M*								

*Explants were cultivated subsequently on the same medium.

3.31 Gene expression assays

3.31.1 Histochemical GUS assay

Expression of the GUS gene was assayed using a modified protocol of JEFFERSON et al. (1989). For histochemical staining, entire plants or tissue sections were incubated in a equilibration buffer. After 1h 0.5 mg/mL x-GlucA, dissolved in DMSO was added to the buffer. The tissues were incubated at 37ºC for 16-20h. Tissues were finally bleached in 70% ethanol. The influence of the different selection conditions on proliferation of transformed cells were determined three weeks after transformation by quantification of GUS expression. Each explant was scanned (CanoScan 1250 U2; Canon), blue stained areas were measured and related to the total size of the explant. Experiments were repeated three times (in case of 30 g/L Sor/2 g/L Man five times) with a minimum of 30 explants per experiment. Statistical analysis were done using the softwares SAS8.1 and SigmaPlot 8.0.

Equilibration buffer

0.2 M sodium phosphate buffer

10 mM EDTA

0.5 mM $K_4[Fe(CN)_6]$

0.5 mM $K_3Fe(CN)_6$)

3.31.2 Chlorophenol red assay

To verify *pmi* activity in transgenic plants a chlorophenol red (CPR) assay was carried out using mannose as a screen for transgenic plants (KRAMER et al., 1993). Leaves of three plants of four transgenic lines were incubated in 15-well plates (one leaf per well) for 5 days in the dark at 27°C in MS liquid medium supplemented with mannose (5 g/L) and chlorophenol red (50 mg/L). The pH was adjusted to 6.0, a pH at which CPR has a deep red colour. As a control, leaves of non-transformed 'Holsteiner Cox' were used. The evaluation was based on a colour change. Tissue able to metabolize the mannose acidifies the medium and turnes it from red to yellow. Media containing non-transgenic tissue remain red (basic) since explants are unable to metabolize mannose.

3.33 Statistical analyses

GUS assays were statistically analysed using the method 'comparison with the best' for one-sided hypothesis according to HSU (1996). Each sample once represented the control group and the simultaneous one-sided upper confidence limits were estimated at the α-level (α = 0.05) using Dunnett's many-to-one multiple comparison procedure. Tests were performed with SAS 8.01 statistical software (SAS Institute, Cary, NC, USA) using the 'proc glm' procedure. Data are presented as box plots (SigmaPlot 8.02, SPSS Inc. Chicago, IL, USA).

4 CHAPTER 1

4.1 RESULTS

4.1.2 Construction of two subtractive cDNA libraries

The pathogen x host interaction is a very complex process, involving several genes and pathways until now not completely elucidated. Several genes should be constitutively expressed in plants that are involved in the first contact between both organisms that can trigger compatible or incompatible processes. The genes that are constitutively expressed in resistant and susceptible cultivars are therefore probably not the same, or should be expressed at different levels. In order to isolate genes only or highly expressed in *Malus domestica* cv. 'Remo' (bearing the *Vf* locus and resistant against *Venturia inaequalis*) and 'Elstar' (susceptible against this fungus) the suppression subtractive hybridization (SSH) method was chosen. This technique overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics. It can achieve greater than 1,000-fold enrichment for differentially expressed cDNAs (DIATCHENKO et al., 1996).

Total RNA from young and healthy plants of the cvs. 'Remo' and 'Elstar', maintained under greenhouse conditions, was isolated from the four youngest leaves, and used to synthesize cDNA. The cDNA from both cultivars was further subtracted by SSH and the ESTs obtained represented clones differentially expressed in 'Remo' and/or 'Elstar'. The fragments obtained were amplified by PCR (Fig. 8) and cloned into the pGEM-T vector and the plasmids were transferred into *Escherichia coli* XL-1blue by heat shock transformation. Around 480 transformed colonies were isolated, and two libraries were generated, representing ESTs from both cultivars. These were cultured on LB medium (stock culture) and stored at -80^{o}C until further analysis.

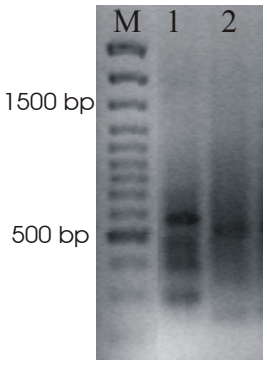

Fig. 8: Fragments amplified after first (lane 1) and second (lane 2) PCR of SSH in the subtraction reaction using cv. 'Remo' as 'tester'. M – DNA marker (100 bp, Fermentas).

4.1.3 REMO library construction

From the transformed colonies of *E. coli* XL-1blue, 262 contained ESTs only or highly expressed in 'Remo'. The fragment size after PCR amplification with T7 and M13 rev primers (which flank in the plasmid pGEM TEasy Vector) varied between 300 and 1,000 bp (Fig. 9) This library is described later on as REMO and the fragments by the letter R followed by the number of the clone.

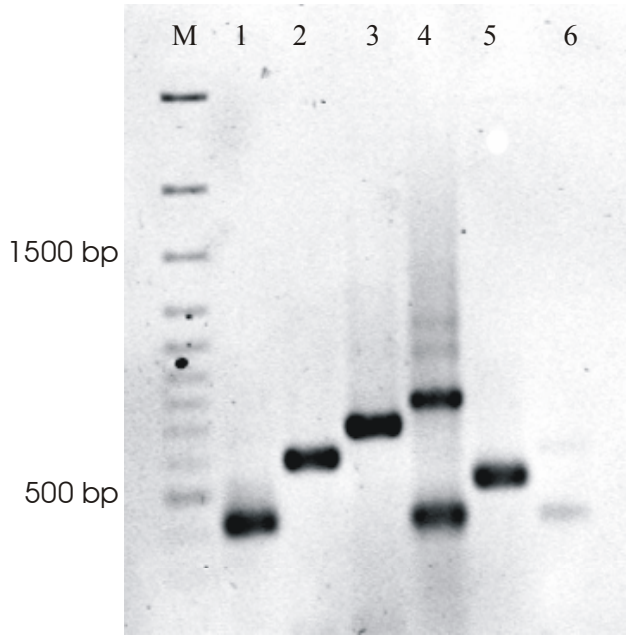

Fig. 9: Amplitude of variation in fragment size of ESTs from REMO library. M – DNA marker (100 bp, Fermentas); lanes 1- 6- Different EST clones were loaded with PCR-products from different EST clones.

A subtracted cDNA screening was performed by reverse Northern blot by hybridization with total cDNA from both cultivars radioactive labeled in order to eliminate false positive clones isolated in the SSH libraries (Fig. 10). Therefore, the fragments of all colonies were amplified

by PCR reaction using the primers T7 and M13 rev, and were transferred to two nylon membranes, in duplicates, by dot blot system. Less than 10% of the fragments were false positives in the REMO library. These clones were discarded.

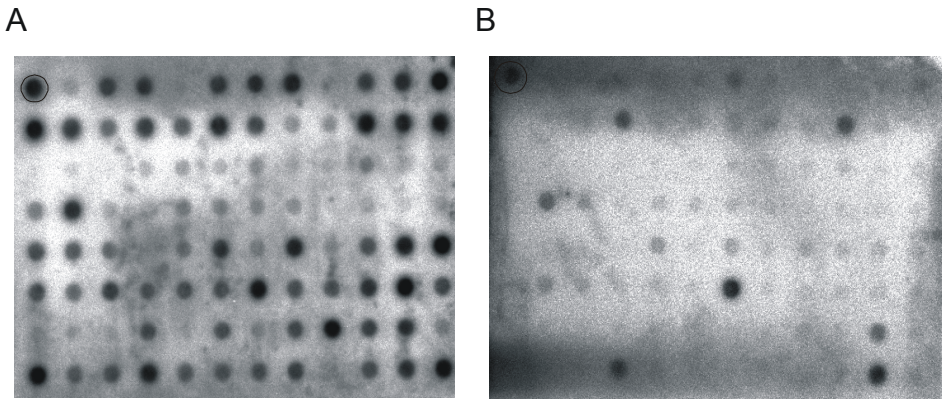

Fig. 10: Reverse northern blot of ESTs isolated in REMO library, hybridized A. with 'Remo' cDNA and B. with 'Elstar' cDNA labeled with ^{32}P. Positive control: Fragments amplified with elongation factor (ef1a) primers from cv. 'Remo' – Up left position.

4.1.3.1 Sequencing and identification of ESTs from REMO library

In a first set, 10 clones were sequenced (Innovation Technology Transfer Centre, University of Bielefeld, Germany), to get an overview of the library. The sequences were kindly compared to the databases by Dr. Abdul Al-Nasser. As the sequencing of these first clones of REMO library raised to the identification of four copies of a metallothionein, a dot blot was carried out (Fig. 11), aiming to determine the number of colonies containing metallothionein fragments in order to avoid the sequencing of clones representing the same gene. Therefore, PCR amplified fragments (amplified with T7 and M13 rev primers) of all colonies from both libraries were transferred to nylon membranes in duplicates, which where allowed to hybridize with a digoxigenin labeled fragment of metallothionein (clone R75), amplified from the REMO library. By this method, it was possible to identify at least 90 metallothionein ESTs in the REMO library and four in ELSTAR, which were no further sent for sequencing. The sequencing of the other ESTs was carried out, and the comparison to the NCBI database gave rise to the Tab. 10.

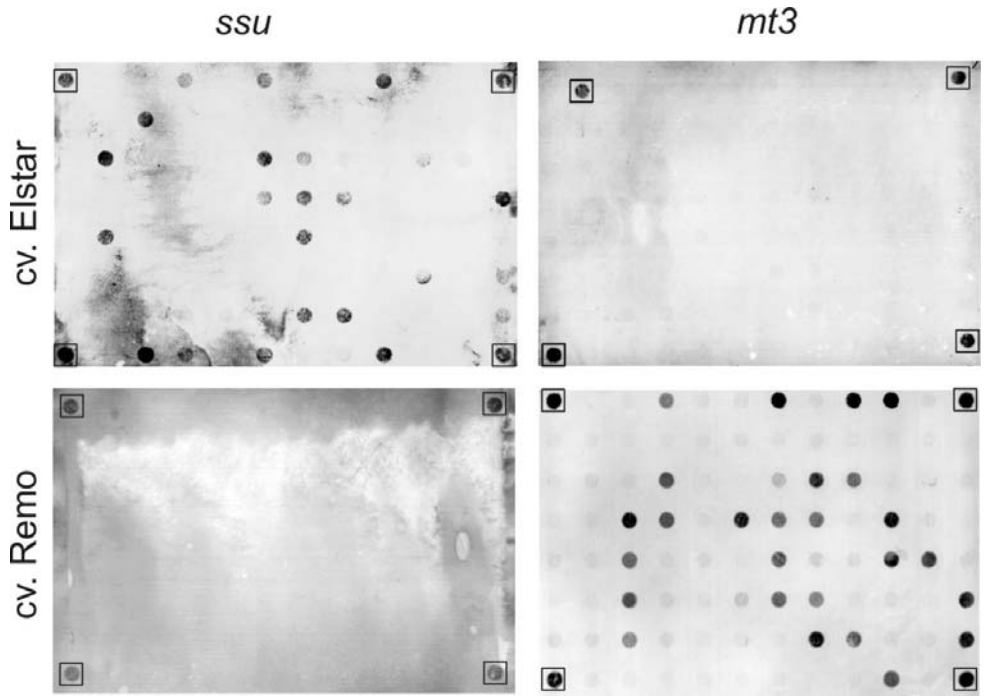

Fig. 11: Dot blot hybridization of EST clones from the ELSTAR and REMO cDNA libraries hybridized with a probe for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*ssu*; NCBI Accession No. L24497) and a probe for a metallothionein-like protein (*mt3*; U61974).

4.1.3.2 Characterization of the REMO library

Several disease resistance genes were found to be constitutively expressed in REMO library (Tab. 10). A larger number of genes belonging to this class was found in this library, in comparison to the ELSTAR library. Considering all clones of the library, 9% belonged to the disease resistance genes class. The most representative class was metallothionein, with 35% of the fragments isolated. cDNAs related to photosynthesis represented 1% and 8% of the clones belonged to the oxidative stress class (Fig. 12).

Even after discarding by dot blot screening, a large number of metallothionein ESTs were sequenced in REMO library. Almost all of these fragments showed high homology to the metallothionein like protein type 3 (NCBI database), isolated from *M. domestica* cv. Granny Smith. One of these fragments (R77) represented small variations in the sequence, showing a high homology to a metallothionein isolated from *Pyrus pyrifolia*, another member of the *Malus* genus.

Two genes were classified as belonging to the oxidative stress response genes. Some genes related to photosynthesis were also isolated. A large number of genes of unknown function and 'hypothetical proteins' were also isolated.

Tab. 10: Identification of EST clones from the apple scab resistant cultivar 'Remo'.

Clone no.	Accesssion number of EST from cv. 'Remo'	Number of identical clones	Accesion number of matching sequence	Best e-value (Blastx)	Origin of matching sequence	Matching sequence from database
			Pathogenesis-related (PR) and Disease resistance genes			
R27	CO729291	3	AAB71505	1e-18	*Pyrus communis*	Cysteine protease inhibitor
R64	CO729292	3	AAS79333	8e-25	*Malus domestica*	Endochitinase class III PR3
R74	CO729293	1	NP_567802	1e-09	*Arabidopsis thaliana*	Wound-responsive protein-related
R155	CO729294	1	P42044	3e-88	*Cucumis sativus*	Ferrochelatase II
R178	CO729295	6	AAK13027	2e-42	*Malus domestica*	Ribonuclease-like PR-10b
R185	CO729296	1	AAF79587	2e-49	*Arabidopsis thaliana*	ADP-ribosylation factor
R111	CO729328	1	NP_568651	5e-39	*Arabidopsis thaliana*	Senescence-specific cysteine proteinase
R112	CO729329	3	AAR92154	6e-31	*Iris hollandica*	Putative cysteine protease
R25	CO729323	1	AAS79332	2e-44	*Malus domestica*	ß-1,3 glucanase PR2
R36	CO729326	2	AAD26552	3e-14	*Malus domestica*	Major allergen Mal d 1 (Mal d I)
			Oxidative stress			
R4	CO729297	1	T07182	1e-24	*Lycopersicon esculentum*	SENU5, senescence up-regulated
R31	CO729298	20	CAD42938	0.001	*Antrodia camphorata*	Manganese superoxide dismutase
			Metallothionein			
R8	CO729299	1	O24059	5e-24	*Malus domestica*	Metallothionein-like protein type 3
R75	CO729300	83	O24059	3e-29	*Malus domestica*	Metallothionein-like protein type 3
R76	CO729301	1	U61974	1e-13	*Malus domestica*	Metallothionein-like protein type 3
R77	CO729302	1	AAF78526	3e-20	*Pyrus pyrifolia*	Metallothionein-like protein
R87	CO729303	1	O24059	3e-29	*Malus domestica*	Metallothionein-like protein type 3
R92	CO729304	1	O24059	7e-24	*Malus domestica*	Metallothionein-like protein type 3
R119	CO729333	1	O24059	3e-29	*Malus domestica*	Metallothionein-like protein type 3
R142	CO729305	1	O24059	5e-24	*Malus domestica*	Metallothionein-like protein type 3
R143	CO729306	1	O24059	2e-28	*Malus domestica*	Metallothionein-like protein type 3
			Photosynthesis			
R172	CO729307	1	NP_051088	8e-59	*Arabidopsis thaliana*	Cytochrome b6
R117	CO729332	1	AAO69667	3e-32	*Phaseolus acutifolius*	Vacuolar ATPase subunit E
R102		1	AAF66242	8e-15	*Lycopersicon esculentum*	Dicyanin
			Others			
R161	CO729308	1	O65759	9e-33	*Cicer arietinum*	Histone H2A
R176	CO729309	1	AAQ24632	9e-05	*Oryza sativa*	Glycine- and proline-rich protein
R105	CO729327	1	NP_174450	1e-16	*Arabidopsis thaliana*	Copper amine oxidase
R122	CO729335	1	NP_195485	1e-05	*Arabidopsis thaliana*	VQ motif-containing protein
			Hypothetical proteins			
R93	CO729347	1	BAD07869	4e-18	*Oryza sativa*	Hypothetical protein
R26	CO729324	1	D75542	9e-08	*Deinococcus radiodurans*	Hypothetical protein

No matching sequences were found in NCBI database for the following EST clones: R1 (CO729310*), R2 (CO729319*), R6 (CO729311*), R12 (CO729315*), R13 (CO729316*), R18 (CO729318*), R20 (CO729320*), R22 (CO729321*), R24 (CO729322*), R30 (CO729325*), R45 (CO729405*), R52 (CO729338*) R55 (CO729312*), R62 (CO729339*), R86 (CO729341*), R95 (CO729342*), R97 (CO729313*), R115 (CO729330*), R123 (CO729336*), *GenBank accession number.

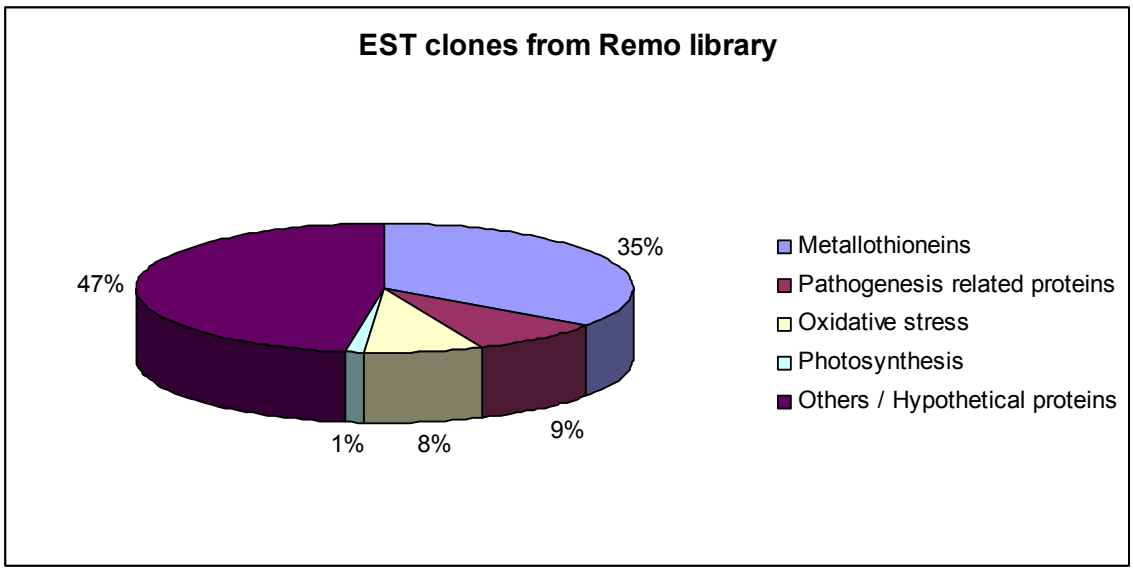

Fig. 12: Classification and quantification of all up-regulated EST sequences isolated by SSH from the *M. domestica* cv. 'Remo' after subtraction with the *M. domestica* cv. 'Elstar'.

4.1.4 ELSTAR library

Using the subtraction method, 218 transformed colonies corresponding to ESTs exclusively or highly expressed in the apple scab susceptible cv. 'Elstar' were isolated. After PCR amplification using the T7 and M13 rev primers, the size of fragments varied between 300 and 1,200 bp in the agarose gel (Fig. 13). The subtraction cDNA screening by reverse northern blot from these fragments revealed than less then 10% were false positives. These were not further analyzed. The other fragments were sent for sequencing or analyzed by dot blot or PCR reaction.

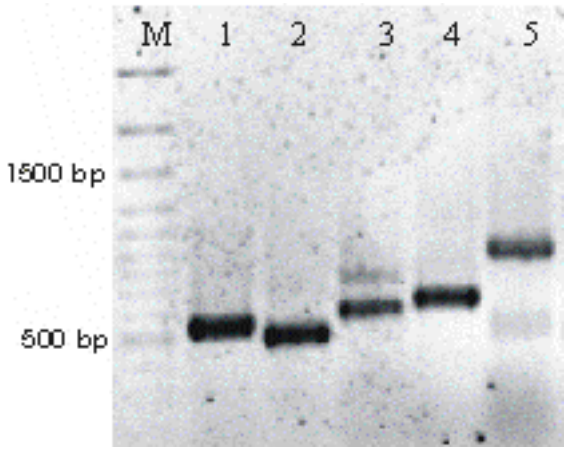

Fig. 13: Amplitude of variation in fragment size of ESTs from ELSTAR library. M - DNA marker (100 bp, Fermentas); lanes 1- 6- Different EST clones were loaded with PCR-products from different EST clones.

4.1.4.1 Sequencing and identification of ESTs from ELSTAR library

The sequencing of the first ESTs of the ELSTAR library, allowed the identification of two clones corresponding to the ribulose-1,5-bisphosphate carboxylase. Therefore a dot blot hybridization was performed, using a labeled fragment of this gene, isolated in this library (E55). The hybridization with all ESTs from both libraries, amplified by PCR (T7 and M13 rev primers) and fixed in nylon membranes by dot blot, allowed the isolation of at least 57 fragments corresponding to ribulose-1,5-bisphosphate carboxylase in the ELSTAR library and no one in the REMO library (Fig. 11).

4.1.4.2 Characterization of the ELSTAR library

From the sequenced fragments, three belong to the disease resistance genes class (5%), which were not found to be constitutively expressed in 'Remo', where ten different clones were isolated. Two genes related to oxidative stress were isolated (4%) and no metallothionein fragments were sequenced (Tab. 11). The most representative class of isolated genes was 'photosynthesis' (34%), suggesting a higher activity in this cultivar in comparison to 'Remo', were fragments representing only three different genes were isolated (Fig. 14). As in REMO library, a large number of fragments with unknown function, as well as 'hypothetical proteins' were identified (57%).

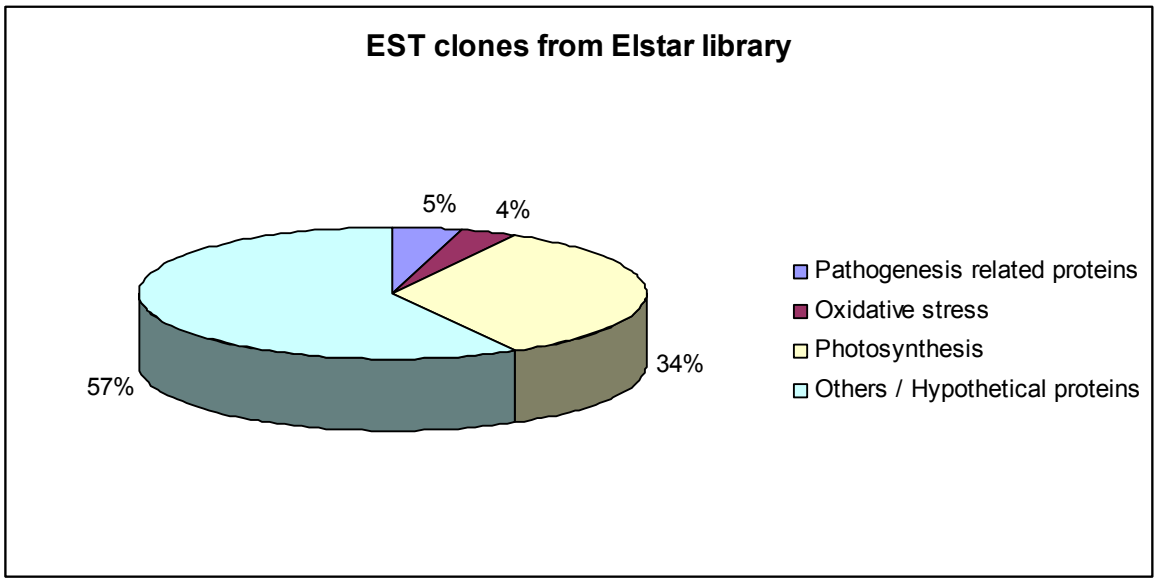

Fig. 14: Classification and quantification of all up-regulated EST sequences isolated by SSH from the *M. domestica* cv. 'Elstar' after subtraction with the *M. domestica* cv. 'Remo'.

Tab. 11: Identification of EST clones from the apple scab susceptible cultivar ʻElstar'.

Clone no.	GenBank accession number of EST clone	Number of identical clones	Accession number of matching sequence	Best e-value (Blastx)	Origin of matching sequence	Matching sequence from database
			Pathogenesis-related and Disease resistance genes			
E35	CO729261	5	NP_187079	5e-40	*Arabidopsis thaliana*	GDSL-motif lipase/hydrolase family protein
E73	CO729262	1	AAM90651	9e-34	*Rubus idaeus*	Chalcone synthase 11
E103	CO729263	4	NM_102249	9e-26	*Arabidopsis thaliana*	Bet v I allergen family protein
			Oxidative stress			
E109	CO729264	1	Q59296	2e-09	*Campylobacter jejuni*	Catalase
E125	CO729265	7	CAD42938	1e-4	*Antrodia camphorata*	Manganese superoxide dismutase
			Photosynthesis			
E40	CO729266	2	P12222	2e-13	*Arabidopsis thaliana*	Hypothetical 226 kDa protein ycf1
E50	CO729267	3	T12416	2e-49	*Mesembryanthemum crystallinum*	Fructose-bisphosphate aldolase
E55	CO729268	57	AAA33866	2e-47	*Malus domestica x Pyrus communis*	Ribulose 1,5-bisphosphate carboxylase, ssu
E81	CO729269	1	AAQ21121	4e-34	*Trifolium pratense*	Photosystem I psaH protein
E105	CO729270	2	AAS46120	2e-10	*Oryza sativa*	P700 apoprotein A2; psaB
E106	CO729404	1	AAB93776	9e-40	*Aponogeton elongatus*	Ribulose 1,5-bisphosphate carboxylase/oxygenase lsu
E130	CO729271	1	NP_176347	2e-29	*Arabidopsis thaliana*	Chlorophyll A-B binding protein
E135	CO729272	1	AAF78510	5e-19	*Pyrus pyrifolia*	Ferredoxin
E153	CO729273	5	NP_084801	8e-09	*Lotus corniculatus var. Japonicus*	ATP synthase CF0 C chain
E160	CO729288	1	T17373	2e-38	*Rubus rigidus*	NADH2 dehydrogenase (ubiquinone)
			Others			
E82	CO729289	1	S68805	2e-41	*Vigna radiata*	Pectin acetylesterase
E83	CO729290	6	T07086	3e-25	*Glycine max*	Acid phosphatase
E113	CO729275	1	BAA96072	6e-25	*Panax ginseng*	Ribosomal protein L29
E122	CO729276	1	O65759	8e-33	*Cicer arietinum*	Histone H2A
E131	CO729285	1	AAM53276	2e-38	*Rubus rigidus*	Quinone oxidoreductase-like protein
E166	CO729274	1	AAC49989	8e-06	*Sambucus nigra*	SNAIf precursor, type 2 ribosome-inactivating protein
E200	CO729343	1	AAA33056	7e-15	*Gossypium hirsutum*	Cotton fiber E6 protein.

Tab. 11: Identification of EST clones from the apple scab susceptible cultivar ʻElstar' (continuation).

Clone no.	GenBank accession number of EST clone	Number of identical clones	Accession number of matching sequence	Best e-value (Blastx)	Origin of matching sequence	Matching sequence from database
			Hypothetical protein			
E69	CO729277	1	NP_799510	6e-15	*Bacillus megaterium*	Hypothetical protein
E148	CO729278	1	NP_084698	7e-09	*Oenothera elata subsp. Hookeri*	Hypothetical protein
E163	CO729279	1	CAB61744	5e-37	*Cicer arietinum*	Hypothetical protein

No matching sequences were found in NCBI database for the following EST clones: E62 (CO729280*); E116 (CO729284*), E132 (CO729281*), E156 (CO729282*) * GeneBank accession number.

4.1.5 Transcriptional characterization of selected clones

To analyze the expression pattern of some of the ESTs isolated in both libraries, semi quantitative and quantitative methods were chosen.

4.1.5.1 Semi quantitative determination of difference in expression level by RT-PCR

The difference in expression level of some genes corresponding to ESTs isolated from the SSH libraries REMO and ELSTAR was analyzed by the semi quantitative RT-PCR method. For that, total RNA from the youngest leaves of greenhouse-grown plants of both cultivars were used. After generation of cDNA, PCR reactions were carried out using specific primers (Tab. 4). By using this method, metallothionein, ribulose-1,5, bisphosphate carboxylase, ribonuclease-like protein - pathogenesis related protein (PR) 10b, ADP rybosilation factor, ferrochelatase and cysteine protease inhibitor genes were analyzed. The elongation factor gene and thaumatin-like protein gene were used as controls.

Using the TINA 2.09 software package (Raytest, Straubenhardt; Germany) to analyze the results from PCR, it was possible to determine differences in the expression level of some of the genes between both cultivars. From the tested genes, metallothionein type 3, ADP ribosylation factor, ferrochelatase and cysteine protease inhibitor showed at least three fold increase in the expression level in 'Remo', confirming the results obtained by the SSH (Fig. 15). These genes were isolated from the REMO library. The ribulose-1,5-bisphosphate carboxylase gene, isolated from the ELSTAR library, proved to be highly expressed in 'Elstar' young leaves. The elongation factor and thaumatin-like protein genes were used as controls, once the former should be expressed in the same pattern in both cultivars, and the latter, a pathogenesis-related protein, was not isolated after the subtractions, suggesting that it should be expressed in the same amount in uninfected leaves of both cultivars. These genes did not show difference in expression between both cultivars. It was not possible to detect any difference in the expression pattern for PR10b. This result indicates that this gene was isolated as a false positive by the SSH method, or the expression pattern diverged between the initial RNA populations of both cultivars, but could not be further detected in new RNA samples isolated, which were used for the RT-PCR.

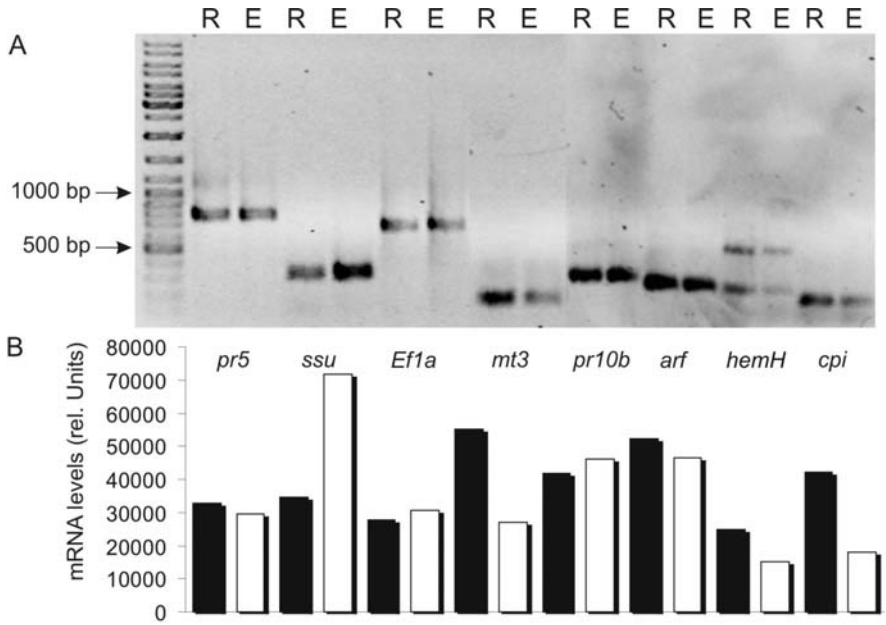

Fig. 15: Semi-quantitative assay of RNA transcripts by RT-PCR. The RT-PCR was performed with 1 µg aliquots of total RNA after treatment with DNAse. a. PCR products were separated by eletrophoresis in a 1% agarose gel. R products obtained from cv. 'Remo', E products amplified from the cv. 'Elstar'. b. Quantification of selected transcripts. *pr5* thaumatin-like protein, *ssu* Rubisco small subunit, *ef1a* elongation factor 1a, *mt3* metallothionein-like protein, *pr10b* ribonuclease-like protein, *arf* ADP ribosylation factor, *hemH* ferrochelatase, *cpi* cysteine protease inhibitor

4.1.5.2 Quantitative determination by Real time PCR - Expression level at different leaf stages

Several genes are differently expressed in young and old leaves of the same plant. As apple presents ontogenetic resistance against *V. inaequalis*, the expression level of some genes was analyzed, in order to investigate the possibility of some of them to be involved in this process. The quantitative analysis of expression level was carried out by real time PCR. Beside four genes isolated from REMO or ELSTAR libraries, metallothionein type 2 and glutathione S-transferase were also analysed. The cDNA from young and old leaves of both cultivars was amplified with primers specific for the genes (Tab. 4). The results were analysed using the ΔΔ Ct method (LIVAK and SCHMITTGEN, 2001). All samples were compared to 'Remo' young leaves (Tab. 12). As a control, the elongation factor gene was used.

The expression of Metallothionein type 3 was explicitly increased in old leaves of both cultivars. This pattern was not observed in the metallothionein type 2, which did not showed difference between 'Remo' and 'Elstar' and between young and old leaves. The Gluthatione S-transferase gene, although not isolated in the libraries was analyzed, and showed a

difference in the expression level between cultivars and a considerable difference between ʻRemo' young and old leaves. The differences observed for all genes were clearly lower than that observed in the Northern blot, Reverse northern blot, dot blot and RT-PCR analysis, and can be attributed to the method, which was not completely well established for these probes or to the new RNA samples isolated.

Tab. 12: ΔΔCt values obtained after Real time PCR analysis of different genes.

	ΔΔ Ct Value		
Gene	ʻRemo' young leaves vs. ʻRemo' old leaves	ʻRemo' young leaves vs. ʻElstar' new leaves	ʻRemo' young leaves vs. ʻElstar' old leaves
Metallothionein 3	22.9	1.5	22.6
Metallothionein 2	1.9	1	-1.2
Rubisco small sub.	2	-1.4	1
ADP ribosylation factor	3.1	3.3	1.4
PR 10b	2.5	1.1	1.4
Gluthatione S-transferase	9.1	4.5	5.3

4.1.6 Analysis of the Metallothionein type 3 (mt3)

Since more than 30% of the fragments isolated in the REMO library were identified as metallothionein type 3, this gene was more intensively investigated.

4.1.6.1 Cloning of a metallothionein gene

A *mt3* clone, named R75 (NCBI number: O24059), was identified in the REMO library (Tab.10). Using metallothionein specific primers (*mt3* fwd and *mt3* rev), a genomic DNA fragment of the same size of the cDNA fragment was amplified (151 bp).

In order to further investigate the genomic structure of this gene, another primer (*mt3* fwd b), flanking 37 bp upstream the cDNA was used to amplify a larger fragment of this gene in the genomic DNA from ʻRemo'. A fragment of about 1,200 bp was cloned, demonstrating the presence of at least one intron in this gene, which should be placed near to the N-terminal of this gene (Fig. 16). The entire sequence of the *mt3* cDNA is presented in the Fig. 17.

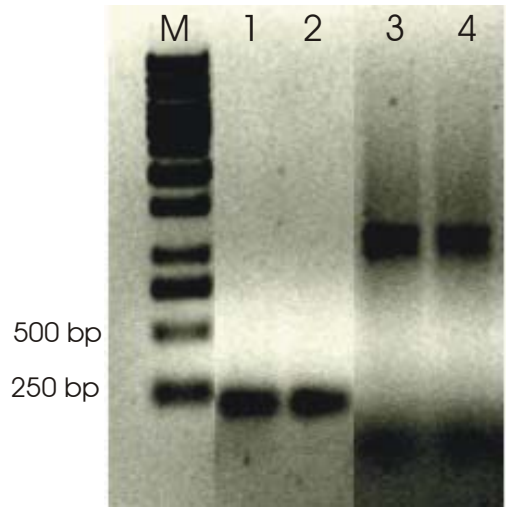

Fig. 16: Metallothionein small (lanes 1 and 2) and large (lanes 3 and 4) fragments cloned from 'Remo' (R) and 'Elstar' (E) genomic DNA. M- DNA marker (1 kb, Fermentas).

```
  1 TTCGAGCGGC CGCCCGGGCA GGTACACGGG GACAGCAAGC AAAATACCAT TCAAGCGAAA
 61 ACCCTAATTT AAACACATCT TCAGCTCCAA GTTCTTAAGT TTATCTTCAA CATGTCGGGC
121 AAGTGCGGCA ACTGCGATTG TGCTGACAGC TCCCAGTGCT TGAAGAAGGG AAACGGCTAC
181 GACTTGGTGA TCGTGGAGAC TGAGAACCGC TCCATGGACA CCGTCGTCGT GGACGCTCCT
241 GCAGCCGAGA ACGACGGAAA GTGCAAGTGT GGCACAACCT GCCCATGTGT GAACTGCACC
301 TGTGGTCAGT AAGCCCAGAT AACCAAATTA AAGATGTGAT TAATAGAAGT GTCATATTAA
361 TTAAGGGATT ATAGACCCTT AATTAATGAA AAGTGTTTGT GGGATAAAAA TAACGTTGTG
421 GCTTTGTCTT TTGTTTGCTT ATAGTATTTG AGTCTGTCGA GTGGCATGTT GTACCTCGGC
481 CGCGACCACG CT
```

Fig. 17: *M. domestica* metallothionein type 3 cDNA entire sequence (NCBI accession number: U61974).

4.1.6.2 Southern blot analysis

The genomic DNA from different cultivars ('Remo', 'Rewena', 'Reglindis', 'Topaz', 'Elstar', 'Holsteiner Cox, 'Golden Delicious') was digested using *BamHI*. Southern blot hybridization was performed using the small fragment of *mt3* amplified by PCR and labeled with digoxigenin to investigate the number of copies of metallothionein present in the genome of these cultivars (Fig. 18). A high number of copies was observed in all cultivars. There were differences in the copy number among cultivars, and the position of the genes in the genome was also probably not the same in all cases.

4.1.6.3 Northern blot

4.1.6.3.1 Expression of mt3 in young and old leaves of *Malus cultivars*

As the copy number proved to be different among cultivars, a northern blot analysis was carried out to compare the expression profile of this gene among different cultivars. The pattern of expression in young and old leaves was also compared, once it showed a great

difference in the real time PCR analysis (Fig.19). In all cases the expression was higher in old leaves. Differences in the expression of young leaves among different cultivars was also evident.

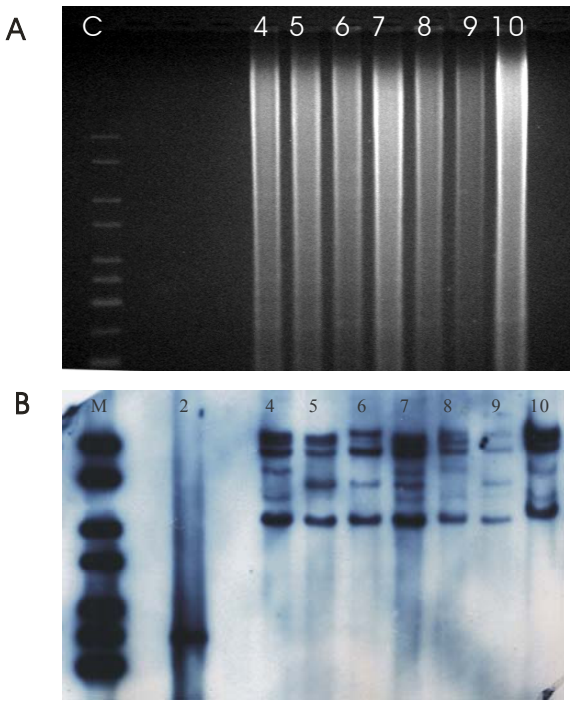

Fig. 18: A- Agarose Gel of genomic DNA from seven apple cultivars digested with the *BamH*1 restriction enzyme; B- Southern blot analysis of genomic DNA from seven apple cultivars hybridized with a digoxigenin labeled probe of *mt3*. M – molecular weight markers (1kb), marker; 2 – positive control – *mt3* PCR amplified cDNA of 'Remo'; 4 – 'Remo'; 5 – 'Rewena'; 6 – 'Reglindis'; 7 – 'Topaz'; 8 – 'Elstar'; 9 – 'Holsteiner Cox'; 10 – 'Golden Delicious'.

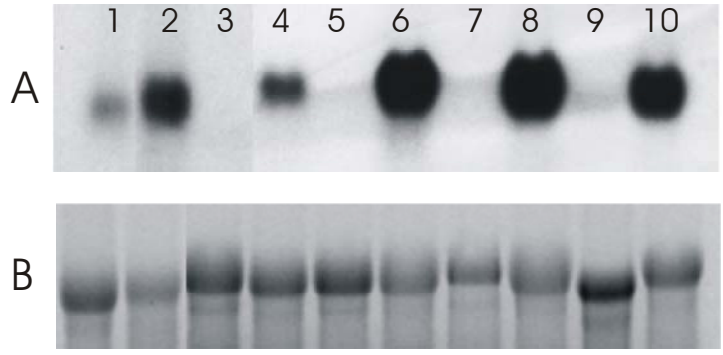

Fig. 19: A. Northern blot analysis of total RNA from and in young old leaves from several cultivars of *Malus domestica* hybridized with a Digoxigenin labeled metallothionein type 3 (*mt3*) probe. 1 and 2 – 'Remo' young and old leaves; 3 and 4 – 'Elstar' young and old leaves; 5 and 6 – 'Gloster' young and old leaves; 7 and 8 – 'Holsteiner Cox' young and old leaves; 9 and 10 – 'Pilot' young and old leaves. B. Corresponding ethidium bromide stained RNA gel.

4.1.6.3.2 Expression of mt3 in inoculated and uninoculated plants of *Malus domestica*

The involvement of *mt3* gene in the infection of *Malus* by *V. inaequalis* was investigated using Northern blot analysis. *In vitro* plants of *M. domestica* from 'Remo' and 'Elstar' were inoculated with *V. inaequalis*, and the RNA was isolated 24 and 48h after inoculation. The hybridization was carried out with the small fragment of *mt3* (151 bp) labeled with digoxigenin as a probe (Fig. 20).

A great difference between uninfected leaves from both cultivars could be observed. In 'Remo', the expression level decreased after inoculation, while in 'Elstar' it increased with the time.

4.1.6.4 Characterization of ESTs corresponding to metallothionein protein

The multiple sequence alignment of several metallothionein fragments was carried out.

Comparison of the predicted eight metallothionein fragments sequenced showed high homology among these fragments and metallothioneins isolated from other species (Fig. 21). All the metallothionein genes isolated, except R77 clone belong to the Type 3 (COBBETT and GOLDSBROUGH, 2002). All contained ten cystein residues, as expected for this type of protein, with exception of the predicted products of clones R77 and R142, which contain nine and eight residues, respectively. The R77 and R143 clones show another major difference, since their C-terminal extensions have a strongly helix breaking character, with one proline and two glycines followed by arginine or lysine residues.

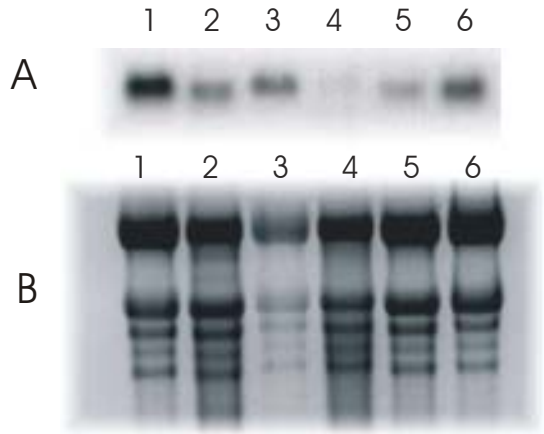

Fig. 20: A. Northern blot hybridization of total RNA from apple scab-resistant cv. 'Remo' and susceptible cv. 'Elstar' using *mt3* (metallothionein type 3) as a probe. B. Ethidium bromide stained RNA gel. 1 – Control - uninfected leaves of 'Remo'; 2 - leaves of 'Remo' 1 day after inoculation with *V. inaequalis*; 3 - leaves of 'Remo' 2 days after inoculation with *V. inaequalis*; 4 – Control - uninfected leaves of 'Elstar'; 5 -

leaves of ʿElstar' 1 day after inoculation with *V. inaequalis*; 6 - leaves of ʿElstar' 2 days after inoculation with *V. inaequalis*. Each lane was loaded with 10 µg of total RNA.

Fig. 21: Multiple sequence alignment of metallothionein proteins of type3. The sequences were obtained from *M. domestica* (Md_MT3 is NCBI Accession No. U61974; the R8, R77, R87, R119, R143 sequences were deduced from EST clones of *M. domestica* cv. ʿRemo'). The boxes indicate conserved cysteine residues, and the corresponding conserved cystein domains are indicated overlines. Asterisks indicate completely conserved residues; colons, highly conserved residues; dots, semiconserved residues.

4.2 DISCUSSION

Genome-sequencing projects lead to the identification of the complete catalogue of genes of an organism; however, they do not consider the gene expression patterns. Large-scale end-sequencing of cDNA library methods, like suppression subtractive hybridization (SSH), generates expressed sequence tags (ESTs) that can be used to obtain a precise gene expression pattern in a particular tissue and/or stage of development. EST sequencing has proved a popular and cost-effective method of isolating vast collections of coding sequences from a plethora of species. Until February 2004, the GenBank dbEST (database expressed sequence tags) contained 20,039,613 EST sequences from 611 organisms (MOYLE et al., 2005).

The identification and functional characterization of genes encoding proteins important in the host-pathogen interaction is a pre-requisite for comprehending the pathogen x plant interaction. While a large body of information is available about the induction of defense mechanisms in many plants, a molecular dissection in apple, involving several genes, has not up to date been carried out. Investigation of expressed genes of apple has mainly been carried out using pre-defined probes for specific genes (KOMJANC et al., 1999). cDNA libraries, based on the subtraction among different genotypes, has not been generated up to date.

Several monogenic dominant genes conferring resistance against apple scab, the most important disease of apple, have been characterized, such as *Vf*, *Vh2, Vh4, Vr2,* and *Vbj* (VINATZER et al., 2001 and 2004, XU and KORBAN, 2002, PATOCCHI et al., 2004, GYGAX et al., 2004, BUS et al., 2005). However, expression of resistance is often conditioned by minor or modifier genes, responsible for the difference in resistance observed among plants of the same progeny from the cross between a resistant (*Vf* carrying) and a susceptible cultivar (MACHARDY, 1996). Ontogenic non-race-specific resistance to scab has also already been described in *M. domestica,* where old leaves, but not aged/senescing leaves are resistant (GESSLER and STUMM, 1984).

By using the SSH method, two ESTs libraries were generated, that contained genes expressed either in the cv. ‘Remo’ or in the cv. ‘Elstar’. The *M. domestica* cv. ‘Remo’ is resistant against some important pathogens, among them *V. inaequalis*, the causal agent of apple scab (FISHER and FISHER, 1999). ‘Elstar’ is susceptible against this disease. Therefore, one can expect that the number of genes involved in the resistance response should be higher in

‘Remo’ in comparison to a susceptible cultivar, even in healthy plants. The proteins coded from these genes should be involved in a first response of the plant to the pathogen.

4.2.1 Characterization and comparison between both libraries

The libraries were generated for both cultivars, after subtraction between both cDNA pools using the Suppression Subtractive Hybridization method. From the sequenced fragments isolated in both libraries, transcripts belonging to the disease resistance genes class are constitutively present at a higher level in ‘Remo’ (clones corresponding to ten genes) than in ‘Elstar’ (three genes), including a number of proteins involved in detoxification of reactive oxygen species. A large amount of transcripts encoding metallothionein was also isolated in this library.

The high-level expression of mRNAs for several classes of disease resistance genes in ‘Remo’ is partly in accordance with recently published results showing the constitutive expression of PR proteins (such as chitinase, *β*-1,3-glucanases, osmotin, PR1 protein and thaumatin-like protein) in the apoplast of ‘Remo’ (GAU et al., 2004). Using SDS-PAGE and ESI-QToF mass spectrometry, chitinase and *β*-1,3-glucanase were detected in the apoplast fluid of ‘Remo’. However, total leaf extracts were not analysed. In the present work, by utilizing the SSH method, transcript levels in total leaf extracts were investigated. This allowed the identification of a number of additional transcripts that are constitutively expressed at higher levels in ‘Remo’ than in ‘Elstar’. The constitutive expression of PR proteins, especially in the apoplast, of ‘Remo’, and possibly also in other parts of the leaf, is most probably responsible for strengthening the cell wall, as well as for rapid degradation of *V. inaequalis* mycelium and for the failure of the fungus to complete its life cycle in the apoplast. It is well documented that plant defense against pathogens involves an oxidative burst and that the reactive oxygen species not only damage the pathogen but also the plant itself (APEL and HIRT, 2004). Therefore, it is not surprising that in ‘Remo’ population, a substantial number of clones containing a cDNA for a manganese superoxide dismutase could be identified.

The ELSTAR library was characterized by a large number of genes related to photosynthesis (34%). Fragments coding for three genes of this class were isolated in the REMO library, in comparison to ten genes in the ELSTAR library. These results suggest a higher photosynthetic activity in young leaves of ‘Elstar’, when compared to young leaves of ‘Remo’ and could indicate that ‘Remo’ leaves present an accelerated senescing process, which could positively influence the resistance against some diseases such as apple scab, once *Malus* present ontogenetic resistance against it.

Several sequences in both libraries did not have significant homology to coding sequences in the GenBank database and therefore could not be annotated by similarity. These sequences could contain some novel coding sequences that have not previously been isolated. However, it is unrealistic to suggest that all of the undiscovered EST subset encode for novel proteins with no significant homology to those in public sequence databases. Instead, many of such clones are likely to contain insufficient coding sequence to accurately assign an annotation based on homology. Furthermore, some of the undiscovered sequence ESTs may contain short stretches of protein coding sequence homologous to proteins in the non-redundant database, but due to a small open reading frame or short length of overlapping coding sequence, do not produce an acceptable expected value.

4.2.2 Analysis of expression profile of some genes isolated in REMO library

Semi quantitative analysis was carried out for some of the disease resistance genes isolated in the REMO library.

4.2.2.1 Cysteine Protease inhibitor

Three fragments of cysteine protease inhibitor were present in REMO library and showed to be highly constitutively expressed in this cultivar when compared to 'Elstar'. The protein sequence analysed by blastx showed 83% similarity to the *Pyrus communis* sequence in the database. Cysteine protease inhibitors (cystatins) have been characterized in a number of plant species such as rice (ABE et al., 1987), corn (ABE and WHITAKER 1988), cowpea (FERNANDES et al., 1993), sunflower (KOUZUMA et al., 1996), soybean (MISAKA et al., 1996), carrot (OJIMA et al., 1997) and bean (BRZIN et al., 1998). A cysteine protease inhibitor has already been purified from apple fruit and strongly inhibited papain, ficin and bromelian. However, the low values for cysteine protease inhibitor when compared with other seeds suggests that they may not have a defense function as postulated for many seed protease inhibitors but rather has an endogenous role in apple fruit development (RYAN et al., 2003).

Cysteine protease inhibitors are thought to protect cells from inappropriate endogenous or external proteolysis, and to be involved in the control mechanism responsible for intracellular or extracellular breakdown. Plant cysteine protease inhibitors are encoded by gene families (FERNANDES et al., 1993, WALDRON et al., 1993) but little is known about regulation of these genes. Expression of these genes is usually limited to specific organs or to particular periods during plant development like germination (BOTELLA et al., 1996), early leaf senescence

(HUANG et al., 2001), drought (WALDRON et al., 1993) or cold and salt stresses (PERNAS et al., 2000, VAN DER VYVER et al., 2003). Certain cysteine protease inhibitors genes are induced during different stresses such as wounding or in response to insect attack (WALDRON et al., 1993, BOTELLA et al., 1996, ZHAO et al., 1996). The enhanced expression of cysteine protease inhibitors mRNA observed during early leaf senescence in sweet potato suggests a role in preventing unwanted cell death in certain leaf tissues during the senescence process (HUANG et al., 2001). The expression analysis showed that a low level of expression in mature green leaves increased considerably at the early senescence stage and was maintained in the subsequent stages (HUANG et al., 2001).

Cystein protease inhibitor genes isolated from soybean were differentially expressed in different organs. The gene was induced by wounding or methyl jasmonate and was presumed to have a role in plant defense (BOTELLA et al., 1996). Genes coding for cysteine protease inhibitors have been used successfully against insect pests in transgenic plants (LEPLE et al., 1995; IRIE et al., 1996; COWGILL et al., 2002; RAHBE et al., 2003).

4.2.2.2 PR10b

Six fragments isolated from REMO libraries showed a high homology to the Ribonuclease-like PR-10b from *Malus domestica* by blastn comparison. Two copies in this library corresponded to the major allergen Mal d 1 (Mal d I), which shares a high homology to PR10b, were also isolated from *Malus domestica*. In the ELSTAR library, four ESTs showed high homology to the Bet v I allergen family protein, from *Arabidopsis thaliana*. This gene is also classified as PR10. These results suggest that genes belonging to this class are constitutively expressed and should probably present a wide range not specific resistance. The RT-PCR as well as real time PCR did not revealed any differences in expression pattern of ribonuclease-like PR-10b between both cultivars and thus this gene could be classified as a false positive isolation.

Most of the PR10 family genes have been shown to be induced upon microbial attack, fungal elicitors, wounding and stress stimuli. As is the case of the other PR-protein families, PR10-type are also expressed in a tissue-specific manner during development.

Plant interactions with fungal organisms have been investigated with regard to PR-10 gene expression. In compatible interactions studied, up-regulation was observed after plant contamination with fungi (MC GEE et al., 2001, PINTO and RICARDO, 1995). Other biotic interactions, such as mutual symbiosis between plant and microorganisms, also led to the induction of PR-10 gene members (FEUGEY et al., 1999, GAMAS et al., 1998).

In apple, several homologues of PR-10 genes have been identified, such as the major allergen Mal d 1 and its isoforms (SON et al., 1999, VANEK-KREBITZ et al., 1995) or as the *Ypr10 *Md.a* gene accumulating in the fruit during ripening (ATKINSON et al., 1996). Two subclasses of PR-10 genes (*APa* and *APb*) were characterized in leaves (ZIADI et al., 2001).

In leaves and stems of apple cvs 'Golden Delicious' and 'Gala', both susceptible to apple scab, a low basal level of the *APa* gene was constitutively expressed, while a high level was observed in roots (PÜHRINGER et al., 2000, POUPARD et al., 2003). The expression of two PR-10 genes of the *APa* subclass (*Ypr10*Md.b* and *Ypr10*Md.d*) in leaves of cv. 'Golden Delicious', was found to be activated in response to inoculation with *V. inaequalis* and the compatible and incompatible interaction between pathogen and host lead to a differential pattern of gene induction, indicating a strong gene induction occurring earlier in the compatible situation than in the incompatible one (POUPARD et al., 2003).

In other respects, the exact function of PR-10 proteins is still unknown and future investigations to understand the putative implication of PR-10 in mechanisms associated to apple defense are needed.

A ribonucleolytic activity was previously hypothesized for PR-10 members in plant species (BUFE et al., 1996, BANTIGNIES et al., 2000, PARK et al., 2004). WALTER et al. (1996) have proposed that cytosolic ribonucleases could be involved in selective and/or highly regulated degradation of existing mRNAs during stress or pathogen attack. It was supposed that such physiological function of PR-10 could be in part regulated by the binding of ligands of different nature such as phytosteroids, cytokinins, fatty acids or flavonoids (POUPARD et al., 2003).

PR-10 proteins have amino acid sequence similarity to the major food allergen of celery and pollen allergens of tree (WARNER et al., 1994, cited in PARK et al., 2004). The PR10 isolated from hot pepper is phosphorylated and the phosphorylated protein functions as a kind of RNAse being able to cleave viral RNA and as a result, may act as an antiviral protein and thus inhibit viral penetration and/or replication (PARK et al., 2004). These transcripts started to accumulate abundantly 48h after inoculation with the virus responsible in the incompatible interaction (PARK et al., 2004) and the accumulation occurred preferentially in resistant leaves, indicating specific induction during the HR upon bacterial inoculation (PARK et al., 2004). There have been many reports that PR-10 transcripts accumulate upon bacterial or fungal inoculation (MCGEE et al., 2001). Sequence analysis indicated that PR-10 proteins contain no signal peptide, suggesting that they are intracellular proteins located in the cytosol (PARK et al., 2004).

4.2.2.3 ADP-ribosylation factor

ADP-ribosylation factors (ARFs) make up a family of small GTP-binding proteins (BOMAN and KAHN, 1995) and can be divided in three classes: class-I (Arf1, Arf2 and Arf3), class-II (Arf4 and Arf5), and class-III (Arf6) (TSUCHIYA et al., 1991). They are involved in the regulation of intracellular membrane traffic (BALCH et al., 1992, RITZENTHALER et al., 2002), actin cytoskeleton (RADHAKRISHNA et al., 1996) and organelle structure (DONALDSON and JACKSON, 2000). Plant ARFs have been shown also to play a role in mitosis and cell cycle control during seed development (MCELVER et al., 2000).

One copy of ARF gene was identified in the REMO library. RT-PCR analysis showed a very slight difference in expression between 'Remo' and 'Elstar'. However, the differences were higher when the gene was analysed by real time PCR, indicating a threefold increase in the former cultivar. The deduced amino acid sequence of this clone shares 88% identity with ARFs from other plant species, including *Medicago truncatula* and *Triticum aestivum.* ARF genes have already been identified as house-keeping gene (KOBAYASHI-UEHARA et al., 2001), since they are constitutively expressed at a low level in control cells. However, the ARF isolated from rice also simultaneously demonstrated a role in defense. Rice ARF1 (RARF1) plays a role in plant disease response, as evidenced by a pathogen-induced rapid increase in gene expression (LEE et al., 2003). Moreover, the authors suggested that RARF1 induces a systematic resistance that relies on different signal transduction pathways (LEE et al., 2003). In addition, this ARF transcripts accumulated rapidly in rice cells inoculated with an avirulent pathogen, suggesting that RARF1 in susceptible cells responds with a slower onset of defense mechanisms following infection (LEE et al., 2003). The authors suggested that RARF1 activates plant disease resistance responses, and that constitutive expression of RARF1 confers enhanced resistance in transgenic plants (LEE et al., 2003).

4.2.3 Metallothioneins (MTs)

A large set of clones coding for metallothioneins type 3 (MT3) was detected in the REMO library. More than 90 clones were identified either by PCR or dot blot analysis. Although most of them could be discarded before sequencing of the clones, eight copies of the metallothionein type 3 (*mt3*) were sequenced, representing a gene family encoding slightly different proteins.

MT are low molecular weight proteins containing eight cysteine residues arranged in two domains that bind metal ions through clusters of thiolate bonds. They are found in a wide

variety of organisms including bacteria, fungi, animals and plants. MT from plants differ from the mammalian in the location and number of cysteins. According to the arrangement of Cys residues within domains, MT from plants are classified into three (ROBINSON et al., 1993) or four types (RAUSER, 1999). *Arabidopsis thaliana* contains four different gene families encoding metallothioneins (*mt1, mt2, mt3* and *mt4*) (COBBETT and GOLDSBROUGH, 2002).
Of the five families known, those with carboxy terminal glycine are the most widespread among plants, algae and certain yeasts (RAUSER, 1999). Type 3 MTs are characterized for containing four Cys residues in the N-terminal. The first three Cys are arranged in a Cys-Gly-Asn-Cys-Asp-Cys manner, while the fourth cysteine is not part of a pair cysteines, but is contained within a highly conserved motif, Gln-Cys-Xaa-Lys-Lys-Gly. Another six Cys residues are found in the C-terminal cysteine-rich domain and arranged in Cys-Xaa-Cys motifs. Approximately 40 amino acid residues separate the two domains from each other (COBBETT and GOLDSBROUGH, 2002).

The isolation of a large number of metallothionein fragments in the REMO library was not expected, once the SSH method should suppress the amplification of abundant transcripts (DIATCHENKO et al., 1996). However, this gene seems to represent a great part of the transcripts also in other species. A small-scale EST sequencing project from *Citrus unshiu* fruit library identified 20% of clone sequences as encoding MT (MORIGUCHI et al., 1998) and 40 clones of MT gene were isolated from pineapple fruit libraries (MOYLE et al., 2005).
A total of nine active MT genes and one pseudogene have been identified in the *Arabidopsis* genome (COBBETT and GOLDSBROUGH, 2002) and recently the analysis of various genomic databases revealed two additional MT genes in this species (GUO et al., 2003). There is only one *mt3* gene in the *Arabidopsis* genome (W. BUNDITHYA unpubl. data, cited in GUO et al., 2003).

4.2.3.1 Molecular characterization

The cDNA amplification of the EST isolated from cv. 'Remo' using the primers *mt3* fwd b and *mt3* rev, gave rise to a fragment containing 188 bp. The genomic DNA amplification using the same primers gave rise to a fragment of approximately 1,300 bp, revealing that the gene contains at least one intron. Once the amplification with the *mt3* fwd primer gave rise to cDNA and genomic DNA amplification fragments of the same size (151 bp), it is possible to infer that the intron is localized at the end of the gene. All type 3 MTs genes that have been characterized contain two introns, and the first lies in the same relative position after the end

of N-terminal cys-rich domain (COBBETT and GOLDSBROUGH, 2002). Analysis of the MT3 isolated from *Arabidopsis* revealed the presence of three exons and two introns (GUO et al., 2003), while MT from another types contained two exons interrupted by a single intron. The position of this intron is conserved and is located 1 bp before the last cysteine codon in the N-terminal cysteine-rich domain (ZHOU and GOLDSBROUGH 1995).

DNA from leaves of seven cultivars ('Remo', 'Rewena', 'Reglindis', 'Topaz', 'Elstar', 'Holsteiner Cox' and 'Golden Delicious') showing different resistance pattern against *Venturia inaequalis* was digested with *BamH*I. DNA hybridization revealed differences in copy number among cultivars. Although 'Remo' and 'Elstar' showed different expression profiles, the pattern of bands obtained was very similar in both cultivars. Different patterns were observed among 'Remo', 'Rewena' (carrying the *Vf* gene) and 'Reglindis' (carrying the *Va* gene), all coming from the same breeding program and all resistant against *V. inaequalis* (FISCHER and FISCHER, 1999). The cultivar 'Topaz', also resistant against this fungus, showed one additional band, when compared to 'Remo'. 'Golden Delicious', a susceptible cultivar also showed a pattern similar to that obtained for 'Remo' and 'Elstar' (susceptible against *V. inaequalis*). At least six bands were obtained for 'Remo' and 'Elstar', and although there was a difference in the number of bands among cultivars, it was not possible to conclude that the resistant cultivars contained more copies than the susceptible ones. However, the position of the genes in the genome can certainly influence the expression profile and other regulation factors such as gene silencing and methylation could be involved.

4.2.3.2 Protein characterization

The protein analysis of seven EST clones isolated in the library revealed differences in the sequence among them, suggesting that they belong to a family in the apple genome. The twelve Cys residues in *Arabidopsis* MT3, four in the amino terminus and eight in the carboxyl terminus, are highly conserved in other plant type 3 MT proteins (GUO et al., 2003). The resulting protein sequence in *Arabidopsis* contains six extra hydrophobic amino acids in the N-terminus and lacks two cysteines in the C-terminus (GUO et al., 2003).

REID and ROSS (1997) isolated two MT clones isolated from apple fruits that shared only 42 and 27% identity with each other in nucleic and amino acids (respectively), and showed high similarity with other MT-like sequences from plants. The banding pattern observed when MT1 insert was used as a probe was different from that observed with MT2 insert, suggesting that MT1 and MT2 are each representative of an MT-like gene family (REID and ROSS, 1997).

4.2.3.3 Functions

The expression profile of a *mt3* cDNA from apple, isolated in the EST library from cv. 'Remo' was investigated. Information and isolation of plant genes and cDNAs encoding MT proteins has not up to date been accompained by a corresponding increase in knowledge, and although several efforts have been done, the exact function of these proteins remains to be elucidated.

It is known that MTs sequester excess amounts of certain metal ions as part of a heavy metal detoxification mechanism. However, the regulation of this gene family under accumulation of certain metals differ among species and it has been suggested that the regulation and roles of the metallothionein-like genes in plants could be different from animals. The MT amount was strongly correlated with cadmium accumulation and transport in mammalian cells (BLAIS et al., 1999, CHUBATSU et al., 1992, KONDO et al., 1999, PEDERSEN et al., 1998), arbuscular mycorrhizal fungus (LANFRANCO et al., 2002), yeast (VAN HOOF et al., 2001) and *Caenorhabditis elegans* (SWAIN et al., 2004).

Up-regulation in response to copper has been reported in fungi (LANFRANCO et al., 2002, AVERBECK et al., 2001) and yeasts (SYRING et al., 2000, VAN HOOF et al., 2001, MIR et al., 2004). In contrast, the expression of MT1 is barely induced in *Caenorhabditis elegans* in response to copper (SWAIN et al., 2004) and overexpression of MT in tobacco plants did not significantly enhanced tolerance to cadmium (THOMAS et al., 2003). The addition of cadmium, manganese or zinc did not lead to a significant up-regulation of a MT1 transcription in the fungus *Podospora anserine* (AVERBECK et al., 2001). MT1 is involved in hyphal development and conidiation in *M. grisea* but is not associated with the response to metal toxicity (TUCKER et al., 2004). The induced level of MT-like mRNA by copper treatment was relatively mild when compared with wounding or virus infection in *Nicotiana glutinosa* (CHOI et al., 1996) and no significant differences in the GUS expression levels in seedlings of *Arabidopsis* grown on different copper concentrations were observed (BUTT et al., 1998). In apple fruits, no differences in steady state RNA level corresponding to a MT1 and a MT2 genes were detected in response to any of the metal or free radical treatments applied. Thus, the poor inducibility of plant MT genes by cadmium reported suggests a secondary role for plant MTs in cadmium detoxification (MIR et al., 2004).

Metallothioneins could also take part in regulation of gene expression and cell metabolism by donating/accepting Zn ion to/from Zn-dependent DNA binding proteins or metalloenzymes, being involved in normal processes of growth and differentiation (VALLE, 1995). MT1 isolated from the fungus *Magnaporthe grisea* displays a high affinity for zinc and is able to act as a powerful antioxidant because of its low redox potential and by virtue of its ability to release metal in the presence of reactive oxygen species. MT1 mutants, which did not express

MT1, did not show susceptibility upon exposure to increasing concentrations of copper, zinc or cadmium (TUCKER et al., 2004). However, plant MTs demonstrated a poorer capacity for binding Zn compared with animal MTs (MIR et al., 2004).
Furthermore, as expected for a thionein metal-binding protein, a MT1-deficient mutant failed to confer resistance against Ni, a metal with an exceedingly low affinity for thiolate groups (LANFRANCO et al., 2002).

4.2.3.3.1 Pathogen defense

MT3 expression after pathogen infection was analyzed by inoculation with *V. inaequalis* of *in vitro* plants of the cultivars 'Remo' and 'Elstar'. Northern blot analysis revealed that while in 'Remo' the expression level decreased in the first 24h after inoculation, in 'Elstar' it was up-regulated in the time analyzed (48h), revealing a difference in expression pattern between a resistant and a susceptible cultivar. These results confirmed the pattern obtained in a study with rice, where expression of a *mt* gene was suppressed after pathogen attack, thereby augmenting ROS accumulation and defense signaling (WONG et al., 2004).
Reactive oxygen species (ROS) are very important components of plant defense. ROS accumulation at the sites of fungal infection during early stages of plant defense signaling (LAMB and DIXON, 1997, MELLERSH et al., 2002 cited by TUCKER et al., 2004) is known as oxidative burst and may directly repel invading pathogens or serve as signaling molecules that activate defense response (HAMMOND-KOSACK and JONES, 1996, cited by WONG et al., 2004).
However, under oxidative stress, plants typically respond by activating a complex system of enzymatic and nonenzymatic ROS scavengers such as catalases, superoxide dismutase and ascorbate peroxidases, for maintaining redox homeostasis and protection against oxidative damage (MITTLER, 2002 cited by WONG et al., 2004). When these defenses fail to protect the plant from the ROS, cell death will result. Changes in gene expression measured during stress responses were found to be similar to some of the changes, which occur during leaf senescence (MILLER et al., 1999, JOHN et al., 2001, HANFREY et al., 1996, cited by NAVABPOUR et al., 2003, BUTT et al., 1998).
Therefore, activation of ROS scavengers during defense signaling would diminish ROS accumulation during the oxidative burst phase, and would thus be detrimental to disease resistance (WONG et al., 2004). MT is known as a ROS scavenger and may participate in redox regulation, and its levels may be transiently suppressed, thereby augmenting ROS accumulation and defense signaling (WONG et al., 2004). On the other hand, hydrogen

peroxide may synergize with nitric oxide (NO) to promote cell death (DELLEDONNE et al., 2001). MTs are also known to scavenge NO (SCHWARTZ et al., 1995).

Divergent results have already been obtained for other plant x pathogen interaction systems. The expression of LSC54 MT gene in *A. thaliana* in an incompatible interaction with *Peronospora* induced expression from the LSC54-2 promoter 12 h after infection at a time coincident with the appearance of the hypersensitive response and the expression appeared to be highly localized rather than systemic (BUTT et al., 1998). The expression was not evident until 24 h after inoculation with the compatible isolate (BUTT et al., 1998) and increased ROS was suggested to be the signal leading to the expression of the LSC54 gene (NAVABPOUR et al., 2003). In *Nicotiana glutinosa* MT mRNA was also induced after infection with tobacco mosaic virus (CHOI et al., 1996).

4.2.3.3.2 Maturation

In order to evaluate the differences in expression between young and old leaves of apple, Northern blot hybridization was carried out, using total RNA from five cultivars (‘Remo’ and ‘Pilot’ – resistant against *V. inaequalis;* and ‘Elstar’, ‘Gloster’ and ‘Holsteiner Cox’ – susceptible against this fungus). The *mt3* expression was strongly increased in old leaves in all cultivars evaluated. The results were confirmed by real time PCR amplification. A different pattern of expression was observed for MT2 analyzed by real time PCR. No differences were detected for this gene between ‘Remo’ and ‘Elstar’ and between young and old leaves, confirming that both MT show different pattern of expression, suggesting that they have different functions. The analysis of glutathione-S-transferase was also carried out by real time PCR, once this enzyme is known to be up-regulated during leaf senescence.

Leaf senescence is the final stage of leaf development, leading to cell death. During this process, loss of photosynthetic activity and hydrolysis of macromolecules, built up during the growth phase occur. This hydrolytic activity is concomitant with massive mobilization of the hydrolyzed compounds to the growing parts of plants (SMART, 1994).

The functions of metallothioneins during leaf senescence are not clear. It has been suggested that they are involved in the chelation of excess toxic metal ions released from metalloproteins during the cellular death and degradation processes. These metal ions may form a valuable resource for the future development of the plant. Before being shed from the plant, senescent leaves and tissues actively redistribute nutrients, including Zn and Cu ions, to other parts of the plant (ROBERT et al., 1996). So the presence of metal-binding proteins may have functions in sequestering, mobilization and transport from the senescing tissue to

developing areas of the plant (ROBINSON et al., 1993). MTs may also be involved in chaperoning released metal ions to protect cells from peroxide and metal-induced oxidative stress (BUCHANAN-WOLLASTON, 1994, BUTT et al., 1998) and/or scavenging the reactive oxygen species generated during the complex senescence program, thus acting as an antioxidant since it is a potent scavenger of hydroxyl radicals (CHUBATSU and MENEGHINI, 1993, HUSSIAN et al., 1996, MUIRA et al., 1997).

Increased expression of plant metallothioneins during leaf senescence has been reported in apple (REID and ROSS, 1997), tobacco (THOMAS et al., 2003), *Arabidopsis thaliana* (GARCIA-HERNANDEZ et al., 1998, HINDERHOFER and ZENTGRAF, 2001, GUO et al., 2003), rice (HSIEH et al., 1995), sweet potato (HUANG et al., 2001), pineapple (MOYLE et al., 2005) and *Brassica* (BUCHANAN-WOLLASTON and AINSWORTH, 1997). Transgenic plants of *Arabidopsis* expressing the *gus* gene under the control of the LSC54-2 promoter of a MT gene revealed that while young green leaves showed very little GUS expression, 750-fold increase was observed in leaves starting to senesce, with extensive blue staining detected in the mesophyll cells (BUTT et al., 1998). Only a few layers of cell, closest to the damaged tissue, showed enhanced expression with no systemic induction of the LSC54-2 gene detectable in other parts of the leaf (BUTT et al., 1998), as observed for pathogen-induced response.

MT2a and MT3 function in metal homeostasis in the mesophyll cells of leaves, especially young leaves, and in protecting the root apex, the first tissue to absorb excess Cu from the soil, from Cu toxicity (GUO et al., 2003). However, the sudden release of metal ions during leaf maturation could be highly toxic. The high affinity of plant MTs for metal ions allows MTs to function as chelators that protect cells from metal ion toxicity during senescence (GUO et al., 2003).

In a library of up-regulated genes from autumn leaves of *Populus tremula*, five MT encoding both type 2 and 3 MT, apparently originated from six different genes, were found, while in the young leaf library most genes with a high abundance of ETSs encoded proteins of the photosynthetic apparatus (BHALERAO et al., 2003).

High *mt3* mRNA level was noticed for both green and senescent leaves of buckwheat (*Fagopyrum esculentum* Moench). After H_2O_2/NaCl treatment, *mt3* mRNA level decreased in green leaves, contrary to senescent leaves where expression levels remained unchanged (BRKLJACIC et al., 2004).

Plant MT like transcripts have been detected in roots, stems, leaves, flowers, fruits and seeds of different plant species. The MT1 were mainly found in roots (EVANS et al., 1990, DE

FRAMOND, 1991) but have also been detected in etiolated seedling and leaf (ZHOU and GOLDSBROUGH, 1995). MT2 were found in leaves (HSIEH et al., 1996, FOLEY and SINGH, 1994), while MT3 and MT4 were mostly detected in ripening fruits (LEDGER and GARDNER, 1994, CLENDENNEN and MAY, 1997, GIRITCH et al., 1998). MT genes were up-regulated during fruit development in bananas (CLENDENNEN and MAY, 1997), apple (REID and ROSS, 1997), kiwifruit (LEDGER and GARDNER, 1994), grape (DAVIES and ROBINSON, 2000) and *Citrus* (MORIGUCHI et al., 1998). In *M. domestica*, the *amt1* RNA levels were high in flowers and fruit during the early stages of development, and tended to decrease as the fruit approached maturity. A contrasting pattern was observed for *amt2* RNA, which was barely detectable in flowers and young fruit, but accumulated with fruit development. Both *amt1* and *amt2* RNA were detectable in root tips (REID and ROSS, 1997).

In a study with the promoters of the genes MT1a, MT1c, MT2a, MT2b and MT3 fused with the *gus* open reading frame, the *mt3*:GUS transgene exhibited a similar pattern of expression to that of *mt2a*:GUS in leaves and root tips. A higher expression of *mt2*:GUS and *mt3*:GUS was detected in older leaves and was associated with senescence of these organs. A very low level of GUS activity in the pollen of *mt3*:GUS plants was observed (GUO et al., 2003). *Mt3*:GUS expression was observed throughout the mesophyll and was very low in phloem (GUO et al., 2003). The Cu treatment increased RNA expression of *mt3* in young expanding leaves but had no obvious effect on the expression of *mt2*:GUS and *mt3*:GUS in mature leaves (GUO et al., 2003). A high basal expression of *mt2b* and responsiveness of MT1a expression to Cu was observed and further suggest that MT2b is a housekeeping gene whereas MT1a may be responsible for dealing with rapid changes in Cu concentration in the phloem (GUO et al., 2003).

In ‘Remo’ leaves some genes that are normally over expressed in senescing leaves, like metallothioneins, are constitutively expressed. On the other hand, ‘Elstar’ showed a large number of genes related to photosynthesis, like ribulose-1-5-bisphosphate carboxylase, characteristic for new leaves. A similar distribution of these two transcripts was found in senescent leaves of *P. tremula*, where the subclass photosynthesis contained 5.2% of the clones in the autumn leaf library, compared with 33% in the young leaf library (BHALERAO et al., 2003). Thus, once apple presents ontogenetic resistance against *V. inaequalis*, one might speculate that the lower level of rubisco and other proteins related to photosynthesis and the high level of metallothioneins in young leaves turns ‘Remo’ unattractive for certain biotrophic pathogens, since in this respect the leaves resemble old leaves.

Beside metallothioneins, 'Remo' also presented a copy of the manganese superoxide dismutase that functions as a scavenger of O_2^- (ALSCHER et al., 2002). This gene typically increased its expression level after infection of *Gossypium barbadense* with *Verticillium dahliae* (ZUO et al., 2005).

The expression of glutathione-S-transferase gene was analysed by real time PCR and showed a higher expression in 'Remo' young leaves in comparison to 'Elstar' young leaves. The pattern of expression was also higher in old leaves when compared to young leaves in both cultivars. This protein have already been related to senescing up-regulated genes in other plant species, like *Arabidopsis* (HINDERHOFER and ZENTGRAF, 2001).

The results obtained demonstrated different patterns of expression of metallothionein gene between resistant and susceptible cultivars of apple against apple scab, the most important disease of this culture. The expression level was enhanced in both young and old leaves of several cultivars analyzed, while the inoculation with *V. inaequalis* demonstrated a different pattern of expression between the resistant and the susceptible cultivar. In both processes, it is possible that a ROS scavenger function can be attributed to MT3 once the pattern of expression was different in both cases. Moreover, the results suggest that probably the senescence and biotic stress induced this gene by different pathways, although in both cases several genes of the same classes are up- or down-regulated. Further work, based on RNAi plants could elucidate some of the lacks related to protein function.

5 CHAPTER 2

5.1 RESULTS

Transformation of plants is an important tool for studying gene function as well as to obtain plants carrying specific genes, which are not normally present in certain genotypes. *M. domestica* transformation has mainly been carried out via *Agrobacterium tumefaciens* using herbicide or antibiotic resistance genes as marker genes. However, the public concern is claiming for alternative genes, which should not present any risks to animals and to the ambient.

5.1.2 Sensitivity to mannose

Several plant species have been reported as not able to metabolize mannose. Up to date, the response of apple to mannose as a carbon source in *in vitro* organogenesis has not been documented. The sensitivity of the cultivar 'Holsteiner Cox' to this sugar was evaluated, in order to investigate the ability of apple explants to use mannose as a carbon source and to determine the mannose concentration that should be used in selection after transformation events. Several concentrations of sorbitol (the sugar normally used for adventitous shoot induction in apple *in vitro* culture) in combination with mannose were tested (Tab. 13).

The combination 30 g/L sorbitol in the absence of mannose (the concentration usually used in regeneration media), lead to regeneration rates near to 100% with 12.38 ± 1.4 shoots per explant. The combination of sorbitol and mannose lead to inhibition in shoots formation, and this inhibition grew with increasing concentrations (Fig. 22 and 23; Tab. 13). The explants regeneration showed to be negatively affected by adding mannose into the medium in a dosage dependent manner. The presence of mannose at higher concentrations in the medium was able to inhibit shoot formation, even at high concentrations of sorbitol.

Decreasing the sorbitol concentration lead to decrease in number of shoot formation, even in the absence of mannose, and the addition of mannose to the medium was not able to alleviate this effect, suggesting that this sugar can not be metabolized by apple explants (Tab. 13).

When mannose was tested as the sole carbon source no shoots were regenerated, and a severe browning was observed in the explants.

Tab. 13: Effect of various combinations of sorbitol and mannose on regeneration of apple leaf explants.

Sorbitol:mannose (g/L)	Regeneration rate (%)	Shoots per explant	Number of explants with partial necrosis (%)
30:0	97.5 ± 5	12.38 ± 1.4	0
30:0.25	94 ± 2	12.18 ± 5.6	0
30:0.50	96 ± 1	11.32 ± 1.5	n.e.
30:0.75	86 ± 11	8.31 ± 3.5	n.e.
30:1	58 ± 5	3.6 ± 0.3	n.e.
30:2	7.5 ±15	0.3 ± 0.6	5 ± 10
30:3	1 ± 2	0	n.e.
30:5	0	0	71.9 ± 9
30:10	0	0	100
15:0	90 ± 20	5.8 ± 1.2	0
15:0.25	58 ± 15	3.91 ± 1.7	n.e.
15:0.50	38 ± 18	2.05 ± 0.9	n.e.
15:0.75	20 ± 15	0.96 ± 0.8	n.e.
15:1	10 ± 1	0.4 ± 0.1	n.e.
15:2	0	0	60 ± 29.4
15:3	0	0	n.e.
15:5	0	0	80 ± 40
15:10	0	0	100
10:0	67.5 ± 34	2.9 ± 1.9	0
10:2	0	0	85 ± 12.9
10:5	0	0	100
10:10	0	0	100
5:0	0	0	0
5:2	0	0	100
5:5	0	0	100
5:10	0	0	100

Values represent the mean ± S.D. of two replications with a total of 40 explants per treatment.
n.e. – not evaluated

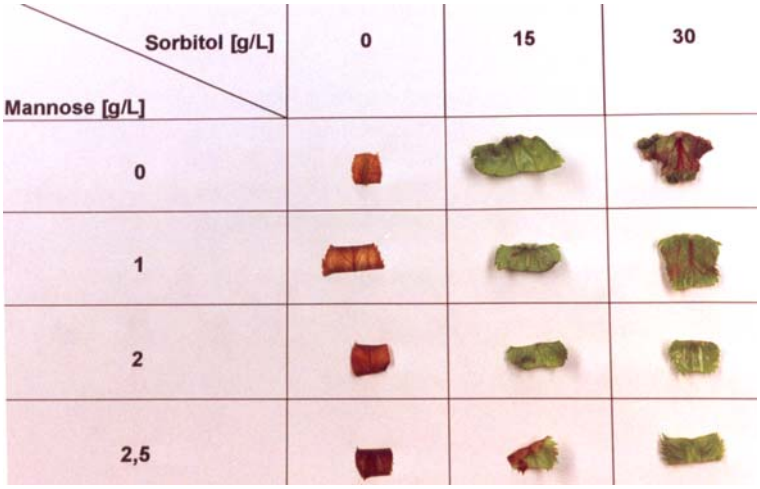

Fig. 22:Sensitivity of apple explants three weeks after cultivation on mannose containing medium.

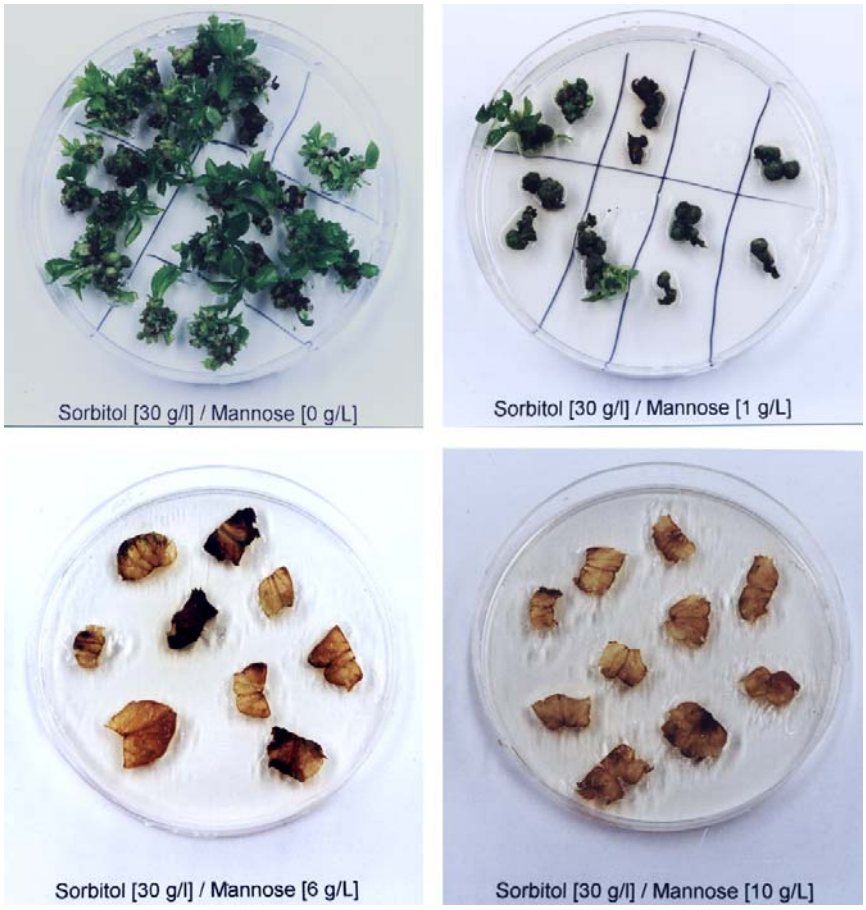

Fig. 23: Sensitivity of apple explants ten weeks after cultivation on mannose containing medium.

5.1.3 Mannose selection after Agrobacterium-mediated transformation

The use of mannose as the sole carbon source was not appropriate once it causes a severe browning of the explants within a few weeks. This process is probably too fast, and would not allow the regeneration of any transgenic shoots (as showed by the results of the 0 g/L sorbitol/5 g/L mannose). For this reason, sorbitol was included in medium in combination with mannose after transformation, to alleviate the effect of mannose-6-phosphate. Different concentrations of sorbitol (ranging from 5 to 30 g/L) and mannose (ranging from 0 to 10 g/L) were evaluated to obtain the best combination for apple.

5.1.4 Transformation rates

The selection conditions were first evaluated three weeks after infection with *Agrobacterium* by determining the proliferation of transgenic cells. For that, a GUS assay was carried out with 50 explants from each treatment. The expression was quantified by determining GUS expressing areas per explant. Assuming that T-DNA transfer rates are similar after coculture, an increase in GUS expression should be based on proliferation of transgenic cells (Fig. 24).

In average highest proliferation rates were obtained with the sorbitol/mannose (g/L) combinations 30/0, 30/2 and 15/0 (Fig. 24). This difference was confirmed by the statistical test "comparison with the best" according to what, all other sorbitol/mannose treatments led to significantly lower transformation rates.

Explants not used for GUS staining were further cultivated either on the same medium or with decreased/increased sorbitol/mannose concentrations. Shoots that regenerated on sorbitol (30 g/L) in the absence of mannose (without any selective pressure) were later on cultured on the respective sorbitol concentration supplemented with 2, 5, or 10 g/L mannose for selection. None of them survived on mannose-containing medium. A minimum of 15 g/L sorbitol was necessary to induce adventitious shoot development adequately after transformation. On medium containing only 5 and 10 g/L sorbitol explants were not able to develop shoots, although some explants showed transgenic callus three weeks after inoculation according to the GUS assay.

In a second set of experiments selection started with 30 g/L sorbitol/2 g/L mannose the selection pressure was increased by decreasing sorbitol concentration to 15 g/L and increasing mannose concentration to 2.5, 5 and 10 g/L after first bud development (Tab. 14). Transgenic plants were developed from explants initially selected on 30 g/L sorbitol and 1 g/L mannose or 30 g/L sorbitol and 2 g/L mannose. GUS staining of these shoots demonstrated these results (Fig. 25). Explants cultivated on 30 g/L sorbitol/2.5 g/L mannose all died on the subsequent media. Selection regimes (applied after 8 weeks on initial media) of individual experiments employed to recover transgenic plants are shown in Tab. 14.

5.1.5 Phosphinotricin as selection agent

The construct containing the *pat* gene, conferring resistance against phosphinotricin and the *gus* gene was used as a control, although the vector utilized was not the same. Transformations with pIBGUS using ppt selection were repeated twice with 150 explants respectively. Selection was carried out for four weeks on regeneration medium supplemented with 1 g/L PPT, two weeks on elongation medium supplemented with 3 g/L PPT, and C1 medium containing 5 and finally 10 g/L PPT. Only in one of these experiments it was possible to regenerate transgenic plants. The five lines regenerated were analyzed by GUS assay and PCR to analyse the presence of the *gus* and the *pat* genes. The transformation efficiency for that individual experiment was 3.3%.

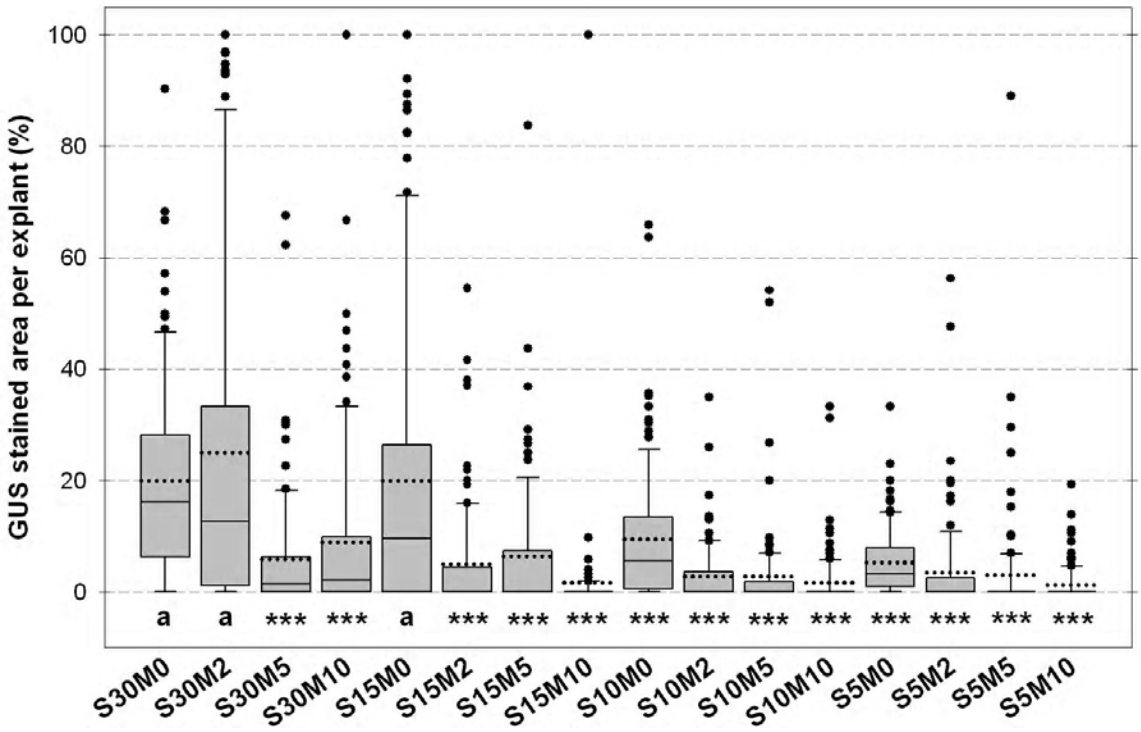

Fig. 24: Influence of different selection conditions on GUS expression assayed three weeks after inoculation of leaf explants with *A. tumefaciens* (pNOV2819 with *gus* gene). The expression is represented as the percentage of GUS stained area per explant. Each individual explant was studied and GUS stained area was related to the total size of the leaf. A minimum of 30 leaves per experiment was used. Experiments were repeated three times with the exception of the treatment 30S/2M, which was repeated five times. Box plots show the results of selection with different sorbitol/mannose concentrations and combinations. The line within the box shows the median and the upper and lower hinges represent quartiles. The dotted line is the mean. Whiskers mark the 90% percentile and dots represent outlying data. Combinations labelled with an a were the best groups according to the method "comparison with the best" (HSU et al. 2000), whereas all other combinations marked with a star were significantly worse.

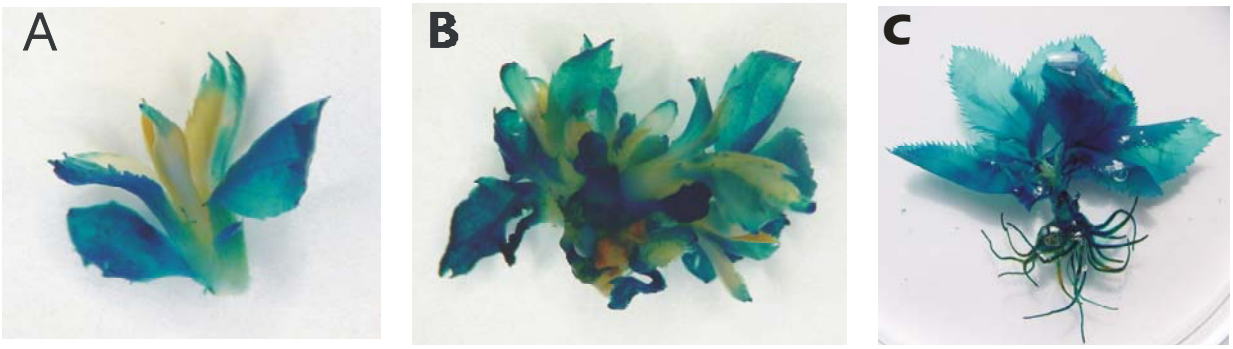

Fig. 25: GUS Assay. A and B: GUS stained shoots regenerated on medium containing mannose. C. GUS stained *pmi*-transgenic plant regenerated and rooted on mannose containing medium.

Tab. 14: Selection schemes and transformation frequencies of successful transformation experiments (M = mannose; S = sorbitol).

Exp. No	Number of explants	Mannose selection scheme	Number of regenerated explants	Number of PCR positive shoots	Number of southern positive shoots	Transformation frequencies*
T/HC04.5	100	30S/2M ⇨ 15 S/2.5 M	1	1	1	1%
T/HC04.6	25	30S/1M ⇨ 15 S/5 M	1	1	1	4%
T/HC04.6	25	30S/2M⇨ 15S/10M	5	5	5	20%
T/HC04.8	25	30S/2M⇨ 15 S/ 2,5 M	3	3	3	12%
T/HC04.8	25	30S/2M⇨ 15 S/5 M	6	6	6	24%
T/HC04.10	25	30S/1M⇨ 15 S/2.5 M	1	1	1	4%

*Transformation efficiencies were calculated for individual experiments as follows: transgenic lines x 100/number of transformed explants

5.1.6 Chlorophenol red assay

Plants of four putative transgenic lines growing on mannose selection media (15 g/L sorbitol/2.5g/L mannose) were randomly tested for PMI activity with the chlorophenol red (CPR) assay. Leaves of the plants were placed into a liquid medium containing the pH indicator CPR which conferred a deep red colour. The explants were scored after 4 days incubation in the dark. The yellow colour indicated an acidification of the medium, caused by the *pmi* activity, once tissues able to metabolize mannose acidified the medium and turned it from red to yellow, indicating that the explants were transgenic. As control, non-transgenic leaves from the cultivar ‘Holsteiner Cox’ were used. No colour change was observed in these explants (Fig. 26).

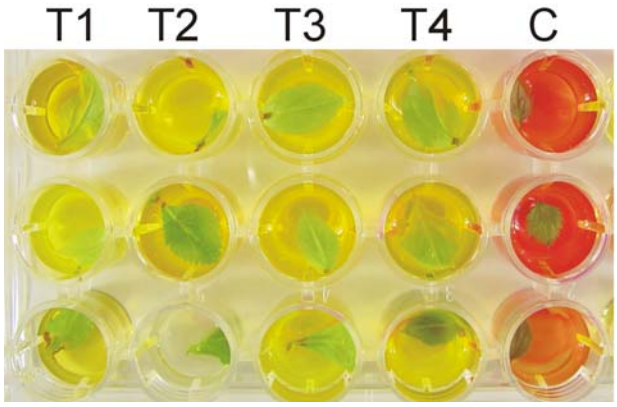

Fig. 26: Chlorophenol red assay of leaves from transformed (T1 - T4) and untransformed control plants (C). Tissue that is able to utilize mannose due to PMI activity acidifies the medium, which is indicated by a colour change from red to yellow.

5.1.7 Rooting

Rooting of *pmi*-transgenic plants from 16 lines was performed on media supplemented with different combinations of sucrose and mannose, according to the media where the plants had been cultivated (Tab. 15). In addition, plants of five lines were placed on rooting medium containing 5 g/L mannose in the absence of sucrose.

Tab. 15: Results of rooting of *pmi*-transgenic plants on media supplemented with different sorbitol and mannose concentrations.

Selection conditions for rooting (Suc:Man[g/L])	Total no. of plants	Rooted plants /Plants with GUS positive roots	Rooting frequency (%)
0:5	18	0/0	0
15:2.5	24	14/11	58.3
15:5	18	7/7	38.9
15:10	18	8/8	44.4

In average best rooting efficiencies were obtained on medium with 15 g/L sucrose and 2.5 g/L mannose. Root pieces were histochemically GUS stained to test transgenity of roots together with leaves of the same plant. On rooting medium supplemented with 5 g/L mannose shoots did not develop any roots.

Roots from all shoots, except three (all belonging to the line T/HC 04/8-41), showed GUS expression. However, this line proved to be positive in PCR and southern analyses.

5.1.8 Molecular analysis

Preliminary screening of putative *pmi*-transgenic plants was conducted with PCR or histochemical GUS assays. Shoots growing on a minimum mannose concentration of 2.5 g/L were screened for *pmi* integration via PCR and southern blot analysis. All tested lines showed the predicted band with the expected size of 514 bp, corresponding to the *pmi*-gene, while no fragment was amplified in untransformed control DNA sample (Fig. 27).

In order to verify transgene integration and to determine the respective copy number, genomic DNA of *pmi*-transformants and non-transgenic control plants were digested with *BamH*I, which presents a unique cleavage site in the *pmi*-gene, and subjected to southern blot analyses (Fig. 28). Multiple copies were found in two lines (lane 1 and 11), one line had at least three copies (lane 16), three had two copies (lane 2, 6 and 13) and ten lines had single integrations of the *pmi*-gene. No signals were detected in the negative control (lane C).

PCR analysis of phosphinotricin resistant shoots confirmed the presence of the *pat* gene. All tested lines showed the predicted band with the size of 412 bp, while no fragment was amplified in untransformed control DNA sample (Fig. 29).

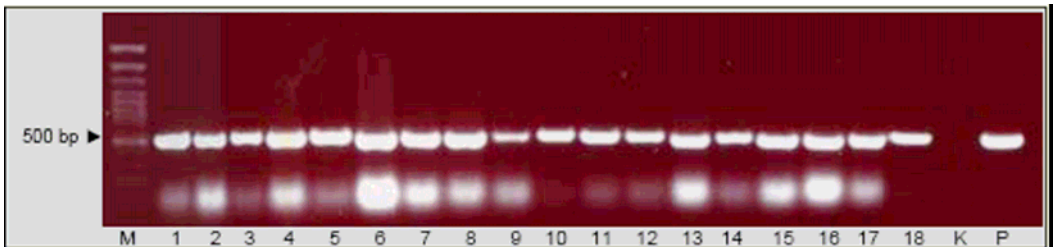

Fig. 27: PCR analysis of putative transgenic *pmi*-plants. Electrophoresis gel of the PCR products showing the expected 514 bp. M – Marker (100 bp+; MBI Fermentas, 1-18: putative *pmi*-transgenic lines of ˋHolsteiner Cox', K – wild type plants of ˋHolsteiner Cox', P – plasmid pNOV2819.

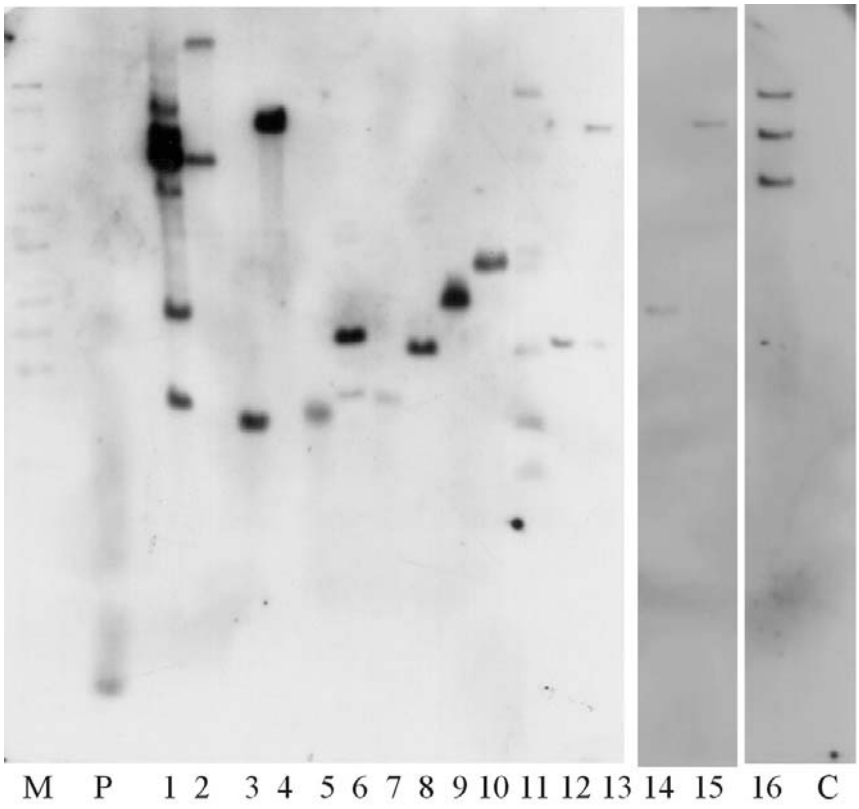

Fig. 28: Southern hybridisation of *BamH*I digested DNA isolated from PMI-transgenic apple plants and respective control plants. Genomic DNA was hybridised to a 514 bp *pmi*-probe. Lanes: M - molecular weight markers (1kb), P PCR fragment amplified from pNOV2819, 1-16 DNA isolated from transgenic lines: 1 – THC 04.5/3; 2 - THC 04.6/12; 3 - THC 04.6/13; 4 - THC 04.6/14; 5 - THC 04.6/15; 6 - THC 04.8/8A; 7 - THC 04.8/8C; 8 - THC 04.8/8; 9 - THC 04.8/10; 10 - THC 04.8/15; 11 - THC 04.8/41; 12 - THC 04.8/59; 13 - THC 04.8/58; 14 - THC 04.8/1; 15 - THC 04.6/6A; 16 - THC 04.6/16, C negative control (DNA from wild plants of ʿHolsteiner Cox'),

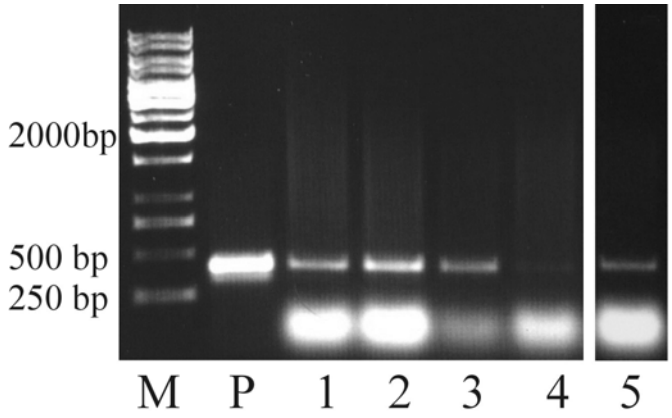

Fig. 29: PCR analysis of transgenic ʿHolsteiner Cox' plants transformed with pIBGUS. Gel electrophoresis of the *pat*-PCR product with the expected length of 412 bp. M: molecular weight markers (1 kb ladder, Fermentas), lane 1-5: transgenic HC clones, P plasmid pIBGUS.

5.1.9 Acclimatization of the plants

The transgenic lines obtained gave rise to phenotipically normal plants, which were transplanted to soil and maintained under greenhouse conditions (Fig. 30).

Fig. 30: A- Transgenic plant of ʿHolsteiner Cox' carrying the *pmi* gene. B- Wild type ʿHolsteiner Cox'.

5.2 DISCUSSION

There has been a growing concern worldwide regarding the use of genes conferring herbicide or antibiotic resistance as marker in transgenic organisms because they might pose an unpredictable hazard to the ecosystem as well as to the human health (PENNA et al., 2002). For this reason, the avoidance of such genes has been encouraged and several positive selection systems have been set up in recent years (HALDRUP et al., 1998a; 1998b, KUNZE et al., 2001, HE et al., 2004).

Generating marker-free transgenic plants would certainly contribute to the public acceptance of transgenic crops (DE VETTEN et al., 2003). Increasing research is focusing on marker-free transgenic plants via segregation or the use of heterologous site-specific recombination systems or transposition (SUGITA et al., 1999 and 2000, EBINUMA et al., 2001, JACOB and VELUTHAMBI, 2002). However, these are still in experimental phase and pose risks such as the creation of additional recombination spots (PENNA et al., 2002).

A new class of marker genes, the positive selectable marker genes, is also available, conferring metabolic advantage to the transgenic cells over the non-transformed ones. The major difference from selection based on antibiotics or herbicides, which kill non-transformed cells, is that the non-transformed cells in the positive selection system have their growth and development arrested by carbohydrate starvation (WANG et al., 2000) but still survive (HALDRUP et al., 1998b). The system based on *pmi* as the selectable marker gene and mannose as the selective agent was successfully applied here for apple transformation. Several independent lines expressing the *pmi*-gene and therefore able to convert mannose-6-phosphate into fructose-6-phosphate were produced.

5.2.1 Sensitivity to mannose

Apple explants were not able to metabolize mannose and develop any shoots on medium containing only mannose as a carbon source. In the presence of sorbitol, the inhibition of shoot formation was evident even in low concentrations as 1 g/L of mannose, indicating a deficiency in endogenous PMI activity in this species and suggesting that this inhibition, is probably due to a phosphate and/or ATP depletion rather than carbon depletion. However, it was observed that addition of sucrose alleviates the effect of mannose on growth (JOERSBO et al., 1998; LOUGHMAN 1966).

Phosphorylation of mannose triggered a signalling cascade that results in gene repression and energy depletion during seed germination of *Arabidopsis thaliana* (PEGO et al., 1999). The energy depletion via a hexokinase-mediated pathway was also suggested to cause a failure in rice embryo germination on mannose (HE et al., 2004). Incubation of spinach beet cells in the presence of mannose also resulted in starvation with respect to phosphate and ATP due to accumulation of unmetabolizable mannose-6-phosphate (SHEU-HWA et al., 1975, cited by JOERSBO et al., 1998). The phosphate depletion in turn resulted in significantly enhanced synthesis of starch and maltose (HEROLD et al. cited by JOERSBO et al., 1998). These reactions may channel available energy away from energy-dependent processes such as cell division and expansion, and could also be the cause of growth inhibition. On the other hand, cells transformed with a *pmi* gene are able to convert mannose-6-phosphate to fructose-6-phosphate which is readily metabolized.

Each species responds to mannose in a dosage dependent manner. For citrus several concentrations of this sugar were tested, and supplemented with 73 mM sucrose was chosen for selection after transformation (BOSCARIOL et al., 2003). For maize regeneration, 10 g/L mannose was lethal to tissue within the first two weeks of exposure; though maize protoplasts could grow on sucrose medium supplemented with up to 10 g/L mannose (WANG et al., 2000). This concentration is the osmotic equivalent of 20 g/L sucrose. The mixture of 10 g/L sucrose with 5 g/L mannose decreased growth of the tissue by 35% compared to 20 g/L sucrose (WRIGHT et al., 2001).

Shoot formation from cotyledons of pepper was not inhibited completely until mannose concentration reached 50 g/L in combination with 20 or 30 g/L of sucrose. This value is the highest quoted for selection of plants with mannose. Higher concentrations of sucrose had an additive effect lowering the shoot formation rate of pepper, contrary to other species. In this case, mannose itself does not seem to be sole inhibitor of shoot development, but sucrose should also be present in the medium in order to generate a selection pressure (KIM et al., 2002).

5.2.2 Transformation selection and frequencies

Medium containing only sorbitol (mannose-free) was used after transformation as a control, to evaluate the possibility of obtaining transgenic plants without any selection pressure. Although GUS assays performed three weeks after inoculation indicated high T-DNA transfer rates among the plants cultured on this medium (represented as the percentage of GUS expressing explants and size of GUS stained area), only escapes were obtained. None of the

regenerants from mannose-free medium was later on able to survive on selective medium, indicating that regeneration occurred preferably from non-transgenic cells. The generation of transgenic plants without the use of any selection pressure was described in only one study up to date. Using a virulent strain of *Agrobacterium tumefaciens* (AGLO), transgenic shoots of potato were obtained at an efficiency of 1-5%, depending on the genotype (DE VETTEN et al., 2003).

In order to determine the critical concentration threshold of mannose for transgenic adventitious shoot regeneration, dose response experiments were done with different sorbitol/mannose combinations. The addition of sorbitol prevented explants to become necrotic but could not suppress the inhibitory effect of mannose, reinforcing the theory that probably the phosphate and/or ATP depletion is the main reason for shoot regeneration, rather than carbon source. However, sorbitol positively influenced transformation efficiencies, and enabled shoot regeneration of transformed tissue, avoiding fast necrosis of explants. No transgenic plants were obtained when sorbitol concentration at the first weeks of selection was below 15 g/L.

For all species, where genetic transformation was carried out using the PMI/mannose selection system, a combination of mannose and sucrose was used. On medium with mannose alone, the scutellar tissue of maize not only failed to proliferate but also deteriorated in quality even at low levels (WRIGHT et al., 2001). Even for species were selection on medium containing only mannose was possible, like rice callus, transformation rates were lower than when sucrose was added to the medium (2.7% against 6% in the presence of sucrose) (HE et al., 2004).

The carbohydrate concentration varied among species. Compared to other species, apple can be considered very sensitive to mannose. For citrus transformation, mannose concentrations varying between 73 and 112 mM according to the cultivar transformed were used (BOSCARIOL et al., 2003). The concentration of this carbohydrate varied among 10 g/L and 20 g/L for maize (NEGROTTO et al., 2000, WANG et al., 2000), 15 g/L mannose for pepper (KIM et al., 2002) and 25 g/L for rice callus (HE et al., 2004). In rice transformation, fewer escapes were obtained at higher concentrations of mannose. This where suggested to be due to the fact that untransformed cells cultured on lower mannose concentration media had enough carbon source to survive through selection of 30 days and thus could recover on regeneration medium (HE et al., 2004). For apple transformation, concentrations as low as 1 g/L at the beginning of the selection process, proved to be efficient to obtain transgenic plants .

The transformation of *A. thaliana* on 2 mM mannose lead to a transformation rate of 2.5% (TODD and TAGUE 2001). In sugar beet, transformation frequencies of 0.34% were obtained after selection on medium containing 1.25 g/L mannose. At concentrations higher than 2.25 g/L no transformants were obtained (JOERSBO et al., 1998). The average transformation efficiency for recovering transgenic plants transformed via bombardment was 45%, while some experiments gave frequencies higher than 70% for maize (WRIGHT et al., 2001) and 20% (highest 45%) for wheat (WRIGHT et al., 2001). The efficiency rate of citrus transformation varied between 3 and 23%, depending on the variety and the concentration of mannose used (BOSCARIOL et al., 2003). Increasing sucrose concentration resulted in a higher number of citrus explants with shoots but a decrease in the number of PCR-positive plants (BOSCARIOL et al., 2003).

Response of untransformed explants of apple to mannose were not always consistent with results obtained after transformation and selection on the respective media. Although shoot regeneration of untransformed tissue was completely inhibited and high transformation efficiencies were detected after three weeks of culture on the lowest mannose concentration (2 g/L mannose), even in combination with 30 g/L sorbitol, subsequent selection on the same medium resulted in regeneration of some escapes, as indicated by histochemical GUS assays. The number of escapes was strongly reduced when selection pressure was enhanced during the process. The highest transformation efficiency (24%) was obtained after an initial selection of 30 g/L sorbitol and 2 g/L mannose followed by a selection with 15 g/L sorbitol and 5 g/L mannose. Also 2.5 g/L mannose was sufficient to regenerate transgenic shoots without escapes.

Escapes have also been controlled in other species by a stepwise increase in the mannose concentration during selection (JOERSBO et al., 1998; LUCCA et al., 2001) as well as by continued selection at the rooting stage (WRIGHT et al., 2001). When mannose concentration was increased to 5 g/L after the first six weeks of selection of sugar beet and subsequently enhanced to 10 g/L, the transformation frequency obtained was 0.94% and the number of escapes was dramatically reduced (JOERSBO et al., 1998). Increasing the mannose concentration after the first six weeks to 10 or 20 g/L reduced transformation frequencies; however, increasing the concentration from 10 to 20 g/L after 12 weeks only had a slight effect on transformation frequency suggesting that the tolerance of the PMI-transformed plants to mannose increases during selection (JOERSBO et al., 1998).

Increased resistance to selective agents such as kanamycin or ppt after *Agrobacterium tumefaciens* infection was also observed in carnation (ZHANG et al. 2005). Changes in gene expression of host as a response to the transfer of both T-DNA and *Vir* proteins during the transformation process (VEENA et al. 2003) might be responsible for that phenomenon.

Delayed necrosis was probably the main reason why transformation efficiencies were much higher compared to herbicide selection. However, since shoot development was even inhibited in the presence of sufficient amounts of a metabolizable sugar, the failure of regeneration was presumably due to depletion of orthophosphate required for ATP production and not to carbohydrate starvation. The same phenomenon was observed in papaya (ZHU et al. 2005), whilst mannose induced inhibition of seed germination in *Arabidopsis* (PEGO et al. 1999) and callus growth in maize (WANG et al. 2000) was reversed by adding metabolizable sugars, suggesting the occurrence of different inhibiting mechanisms depending on the plant species. For this reason, the system could not be defined as a conventional positive selection system, although it has been further on so described. Mannose does not lead to necrotic cells. Instead, untransformed explants and shoots grew very slowly and eventually lost vigor and acquired a light brown color (LINDSEY and GALLOIS, 1990). The herbicide induces strong necrosis in untransformed tissue, accompanied by the release of toxic compounds by the untransformed cells into the surrounding tissues that may adversely affect regeneration of transgenic cells and compromise the uptake of essential minerals and vitamins from the culture medium.
In rice callus transformed with the PMI/mannose selection system, necrotic tissues were rarely observed. The untransformed cells grew very slowly in this system and eventually lost vigour and acquired a light brown colour (HE et al., 2004). Thus, the deleterious effect of dying cells were to a large extent avoided using mannose system (HE et al., 2004). Dark brown or even black necrotic tissue often encountered in sugar beet transformation experiments, using kanamycin selection, were rarely observed when mannose selection was used (JOERSBO et al., 1998).

The transformation efficiencies obtained using the *manA* gene is clearly higher than that obtained using negative selection systems. The frequency rate of transformation obtained for the *pat* gene (3.3%) are in accordance to that previously reported (SZANKOWSKI et al., 2003). In this study the transformation efficiencies were 2.68% and 0.53% for 'Holsteiner Cox' using two different constructs. The transformation efficiency when the cultivar 'Elstar' was used was 0.17%. Although the plasmids used are not the same, similar results have been obtained

in another species (JOERSBO et al., 1998, WANG et al., 2000, WRIGHT et al., 2001). The transformation rates obtained for sugar beet were 10-fold higher when mannose selection was used, in comparison to kanamycin selection (JOERSBO et al., 1998) and a fourfold higher in comparison to herbicide selection for maize (WRIGHT et al., 2001). The transformation of maize using herbicide or antibiotic selectable markers gave rise to transformation efficiencies not higher than 12%, compared to more than 45% obtained with mannose (WRIGHT et al., 2001). Kanamycin resistance conferred by the *npt*II gene has been widely used as a selectable marker for apple transformation and efficiencies between 1.3 and 7.9 % were reported (KO et al., 2002, FAIZE et al., 2003, RADCHUK and KORKHOVOY, 2005).

The promoter strength could also present an influence on the efficiency rates. Different promoters were already tested in transformation using the *pmi* gene. However, no simple correlation between promoter strength and transformation frequency was apparent in sugar beet transformation (JOERSBO et al., 2000). The evidence for the beneficial effect of a strong promoter in front of the selectable marker gene is not unequivocal as it has been reported that a relatively weak promoter may be superior (MENGISTE et al., 1997) or the promoter strength may appear to be of less importance (LI et al., 1997).

5.2.3 Molecular analysis of gene integration

In order to verify the stability of transformation, 16 independent PCR-positive lines were analysed by Southern hybridization. The *BamH*I site in the pNOVGUS is upstream of the *pmi* coding region and cuts only once within the T-DNA. Digestion with this enzyme and subsequent hybridization with the *pmi* probe allowed the identification of border fragments between the T-DNA and plant DNA and provided an estimate of the copy number. The pattern of integration appeared to be simple with most of the events containing only one copy of the *pmi* gene and a few containing two copies.

Roots from one line did not show GUS expression although that line was confirmed to be transgenic by southern blot analysis. It is possible that GUS activity failed due to posttranscriptional gene silencing of the *gus* gene in that line. Multiple T-DNA integrations (6 copies, Fig. 28) support this hypothesis. Another possible explanation could be that this line is a chimera.

In maize transformation, the Southern hybridization of ten different transgenic lines indicated that at least in some cases, a single simple insertion event occurred. There was no correlation between signal intensity and the PMI activity of these lines, indicating that copy number has little effect on the overall level of *pmi* expression (TOOD and TAGUE, 2001).

The analysis of wheat transformation by Southern blot lead to the identification of multiple copies of the *pmi* gene in six of seven lines analysed after bombardment (WRIGHT et al., 2001). Two lanes gave very strong bands, suggesting that multiple copies of the transgene had integrated into the same loci as concatemers (WRIGHT et al., 2001). In citrus transformation, in most of the cases, transgenic plants presented only one copy of the gene (BOSCARIOL et al., 2003). Differences observed in the PMI activity between transgenic lines of maize suggested to have been caused by differences in chromosomal insertion sites of each transgenic event or by other factors such as DNA methylation and copy number (WANG et al., 2000).

5.2.4 Gene expression assays

Plants tested for PMI activity in the CPR assay showed a colour change to yellow, indicating the acidification of the medium caused by metabolic activity of the cells. The control plant tissues were not able to metabolize mannose, thereby maintaining the initial red colour of the medium.

In transgenic plants of some species, the PMI enzyme activity has also been measured by spectrophotometer at an OD of 340 nm. The OD value ranged from 0.17 to 1.32 in transformed transgenic sweet oranges, while no activity was observed in control plants (BOSCARIOL et al., 2003). The difference in the *pmi* expression level may have been caused by differences either in the position of the insertion or in gene copy number (WANG et al., 2000, BOSCARIOL et al., 2003). Enzymatic tests with transgenic plants indicated PMI activity ranging from about 165 to more than 1000 mU/mg protein in maize (TODD and TAGUE, 2001). In comparison between the lines showing the lower and the higher PMI activity on media containing different concentrations of mannose, both lines germinate to the same extent (73-80%) on all concentrations tested, and germination rates were not different from the germination rate of control plants on sucrose. The fivefold difference in activity between both transgenic lines did not translate into significant differences in growth or germination on high levels of mannose, indicating that low levels of *pmi* expression can support growth of maize on mannose (TODD and TAGUE 2001).

Also for sugar beet, at various mannose concentrations tested, the average PMI activities of the transformants were found to be quite similar. The average PMI activities of the transformants did not increase significantly when selection pressure was increased (JOERSBO et al., 1998). Transformed shoots with PMI activity only 20% above background level could tolerate mannose concentrations (5 g/L) which completely killed untransformed cells. This

suggests that a weak promoter in front of the *pmi* gene would be sufficient to obtain high transformation frequencies (JOERSBO et al., 1998).

5.2.5 Acclimatization of transgenic plants

Although no specific tests were carried out with the transgenic apple plants transplanted to the soil, the greenhouse-grown plants showed an apparently normal phenotype, when compared to control plants of 'Holsteiner Cox' and did not present any problems by the acclimatizytion. In another study, transgenic maize plants examined for different characteristics of plants and compositional analysis of grains also did not showed any statistical differences compared to control plants in any of the parameters evaluated nor presented any aberration in fertility (NEGROTTO et al., 2000, PRIVALLE et al., 2002).

The phenotype of PMI transgenic shoots of sugar beet was indistinguishable from clonal propagated non-transgenic shoot culture (JOERSBO et al., 1998). While the control plants were already killed on concentrations of 5 g/L mannose, transgenic shoots were completely resistant to mannose and apparently grew better on mannose containing media than on sucrose containing media. One transgenic line expressing less PMI activity showed some sensitivity to mannose at concentrations higher than 10 g/L. The results showed that only low levels of PMI activity are required to give resistance to the mannose concentrations employed during selection. The percentage of rooting was also was dramatically improved when the mannose system was used (JOERSBO et al., 1998).

6 GENERAL CONCLUSIONS

The Suppression Subtractive Hybridization method proved once more to be an efficient tool to generate cDNA libraries under specific circumstances. In the present study, it was used to subtract the entire cDNA pool of two *M. domestica* cultivars ('Remo' and 'Elstar') that differ in the level of resistance against some diseases, among them the apple scab.

The generation of REMO library (containing genes constitutively expressed in young leaves of the resistant cultivar) presented a higher amount of genes related to pathogenesis response, when compared to the ELSTAR library. By using different methods, a different pattern of expression for some of these genes in both cultivars was confirmed and characterized, while in other cases the difference was not evident, as for PR10b. All the disease resistance genes evaluated were constitutively expressed in both cultivars, although in different levels. This result suggests that these genes are probably important as part of a common defense mechanism, although the higher accumulation of their products in 'Remo' could also influence in rendering this cultivar more resistant against diseases.

The higher level of photossyntheis related genes in the cv 'Elstar', suggested that young leaves of this cultivar show a higher photosynthetic activity, and/or that 'Remo' leaves present an accelerated leaf maturing process when compared to 'Elstar'. The possible higher concentration of carbohydrates in 'Elstar' young leaves could turn it more attractive to pathogens. If 'Remo' leaves mature earlier than 'Elstar' leaves, it also could suggest that the ontogenetic resistance is earlier induced in 'Remo' leaves. Another support for this idea is the higher concentration of Metallothionein type3 in 'Remo' leaves. This pattern of balance among rubisco and *mt3* is reported as being characteristic of senescing leaves (BHALERAO et al., 2003).

The study of *mt3* expression under pathogen inoculation and senescing suggested different pathways for both processes. When *in vitro* plants of 'Remo' and 'Elstar' were inoculated with *Venturia inaequalis*, the expression in 'Remo' decreased in the first 24h and turned to increase in the next 48h, while in 'Elstar' the expression was enhanced during the process. In the senescing process, the expression level in old leaves was similar in all cultivars, while in young leaves it was lower in 'Elstar'. This results again support the idea that 'Remo' leaves could start the senescing process earlier than 'Elstar'. Old leaves of all cultivars present ontogenetic resistance, and *mt3* could be some how involved in this process, once the

expression is expressively higher in such leaves (30 fold higher than in young leaves). This gene can be involved in the oxidative burst process by chaperoning released metal ions to protect cells from oxidative stress and by scavenging the reactive oxygen species generated during the complex senescing program (CHUBATSU and MENEGHINI, 1993, BUCHANAN-WOLLASTON, 1994, HUSSIAN et al., 1996; MUIRA et al., 1997; BUTT et al., 1998). The transformation of plants with this gene would be very important in determining its functions and could also be tested as a source of resistance against diseases.

In both libraries some of the isolated genes could be identified as known genes and could be divided in different classes in both libraries. Most of the clones, however, did not showed homology to any sequences in databases, or were characterized as 'hypothetical proteins'. These results emphasize the importance of studies aiming the determination of gene functions, once several of these unidentified genes are probably important keys in different pathways of plant defense and development. From the characterized genes, some present protection function against pathogens, and could be introduced in known cultivars, in order to enhance their resistance against important pathogens, among them *V. inaequalis.* These genes, even if they do not present a major source of resistance against apple scab, could be used in pyramidizing processes, along with other genes, such as *Vf,* which has been already isolated. The use of such genes, isolated from the same specie, in transgenic plants is desirable once it could enhance the acceptance of such plants.

Another aspect that should be taken into account in the generation of transgenic plants is the use of harmless marker genes in the transformation process, such as those systems that use metabolizable sugars. By using these systems, the use of negative markers, such as herbicides or antibiotics resistance genes could be avoided, once this is an important public claim.

The results obtained using the PMI/mannose selection system demonstrates that this is a superior system for apple transformation, when compared to either antibiotic or herbicide selectable marker genes. In this and in previous studies (SZANKOWSKI et al., 2003) transformation efficiencies obtained were between 0.17 and 3.3% using herbicide selection. However, direct comparison between the efficiencies obtained was not feasible, once the backbones of used plasmids were different. pNOV2819 contains an additional *vir*G copy that might increase virulence of bacteria and thus T-DNA transfer (GHORBEL et al., 2001). DE BONDT et al. (1996) and RADCHUK and KORKHOVOY, (2005) also used herbicide resistance genes for apple transformation, but did not obtain any transgenic shoots after ppt selection. Kanamycin resistance conferred by the *npt*II gene has been widely used as a selectable marker

for apple transformation. Efficiencies ranging from 1.3 to 7.9 % were reported (KO et al., 2002, FAIZE et al., 2003, RADCHUK and KORKHOVOY, 2005).

By using the PMI/mannose system, it was possible to recover transgenic apple plants with an efficiency up to 24%. Transformation rates ranged between the results obtained for other crops, such as sugar beet (0.94%) (JOERSBO et al., 1998), maize (45%) (WRIGHT et al., 2001), rice (44%) (LUCCA et al., 2001) and sweet orange (3% to 23%) (BOSCARIOL et al., 2003).

Mannose selection works equally well in soil and in plates. It is a very inexpensive selection system, if compared to kanamycin or phosphinotricin. It has also likely the lowest toxicity to humans. Toxicity tests were carried out with mice. Doses of 5050 mg/kg body weight were administered and the results did not showed any clinical signals of toxicity. No abnormalities were observed (PRIVALLE et al., 2002).

Kanamycin has excellent selective properties in plates and is relatively inexpensive. But selection in soil by spraying is problematic, once often complete killing is not possible except at high concentrations (TODD and TAGUE, 2001). Phosphinotricin is an excellent marker for soil-grown plants, particularly given the low cost of commercially prepared herbicide formulations. However, selection on plates is difficult for most of the species, usually requiring purified phosphinotricin. Additionally, a large number of escapes was noticed when selecting on plates containing phosphinotricin (TOOD and TAGUE 2001).

The PMI/mannose selection system represents a highly efficient tool to recover transgenic apple plants, which is devoid of the disadvantages of herbicide and antibiotic selection.

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Erklärung

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

Hannover, im März 2006.

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