UNIVERSIDADE TÉCNICA DE LISBOA
INSTITUTO SUPERIOR DE AGRONOMIA

Alternative strategies to fight apple scab

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Doutoramento em Engenharia Agronómica

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"Dissertação apresentada neste Instituto para obtenção do grau de Doutor"

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Estratégias alternativas de combate ao pedrado da macieira

Resumo

O pedrado da macieira, causado pelo fungo *Venturia inaequalis*, é controlado essencialmente por luta química, eficaz mas problemática em termos económicos e ecológicos. Assim, uma estratégia de melhoramento genético para resistência ao pedrado, com promotores indutíveis pelo fungo a exprimir genes que inibem o seu desenvolvimento, pode apresentar significativo interesse.

Dois promotores que regulam a expressão de diferentes genes que codificam PGIPs (proteínas inibidoras de poligalacturonases) foram isolados de uma biblioteca genómica de macieira e fundidos com o gene codificador da ß-glucuronidase (GUS). A sua indutibilidade após elicitação com produtos de origem fúngica, foi verificada em tabacos transgénicos.

Para identificar genes participando na resposta de cultivares resistentes, a interacção entre a cultivar 'Florina' e o pedrado foi analisada. A análise da expressão diferencial do RNA isolado de folhas infectadas e não infectadas revelou a acumulação de mRNAs mostrando homologias com genes envolvidos em mecanismos de defesa, na síntese da parede celular e cutícula ou com genes sem função conhecida.

Dois genes codificadores de quitinases da classe III em macieira foram isolados da mesma biblioteca genómica. Para avaliar as suas propriedades antifúngicas através da sobreexpressão em macieiras transgénicas, condições que podem influenciar a transformação de discos foliares em macieira foram analisadas.

Palavras-chave: *Venturia inaequalis*, *Malus* x *domestica*, quitinase, *pgip*, expressão diferencial

Abstract

Apple scab caused by the fungus *Venturia inaequalis* is the most important disease occurring in apple orchards. Control measures comprise mainly repeated pesticide applications, being problematic in terms of high costs, environmental protection and human health. Thus, scab resistance breeding using a biotechnological approach, with pathogen-inducible promoters expressing genes that could impair fungal development, might confer a considerable benefit for a more sustainable agriculture.

Two apple promoters of different genes encoding PGIPs (polygalacturonase-inhibiting proteins) were isolated from an apple genomic library and fused to the ß-glucuronidase (GUS) gene. Their inducibility with fungal elicitors was investigated in transgenic tobaccos and both promoters proved to be inducible.

In order to identify genes involved in the defense mechanism of scab-resistant cultivars, the interaction between ´Florina´ and *Venturia* was analysed. Differential display analysis of RNA from non-infected and infected leaves revealed the accumulation of transcripts showing homologies with genes involved in plant defense, synthesis of cell walls and cuticular layer and of transcripts with unknown function.

Two apple genes encoding a class III chitinase were isolated from the same genomic library. In order to evaluate their antifungal properties by overexpression in transgenic apples, conditions affecting the success of apple leaf discs transformation were analysed.

Key-words: *Venturia inaequalis*, *Malus* x *domestica*, chitinase, *pgip*, differential expression

List of common abbreviations

β-ME	β-mercaptoethanol
4-MU	7-hydroxy-4-methylcoumarin
4-MUG	4-methylumbelliferyl-ß-D-glucuronid
A	adenine
A. dest.	distilled water
A_{260}	absorbance at 260nm
aa	amino acid
Acc. no.	accession number
ACLSV	apple chlorotic leaf spot virus
AFLP	amplified fragment length polymorphic DNA
Amp^r	ampicillin resistance
ApMV	apple mosaic virus
APS	ammonium persulfate
ASGV	apple stem grooving virus
ASPV	apple stem pitting virus
BAC	bacterial artificial chromosome
BAP	6-benzylaminopurine
BIBAC	binary vector for BAC
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CAM	calmodulin
CaMV	cauliflower mosaic virus
CAPS	cleavable amplified polymorphic sequence
cDNA	complementary DNA
CDP-Star™	disodium 4-chloro-3-(4-methoxyspiro(1,2-dioxetane-3,2´-(5´-chloro) tryciclo[3.3.1.1^{3,7}]decan)-4-yl)phenyl phosphate
chi	chitinase
CI	chloroform/isoamyl alcohol
cM	centiMorgan
CTAB	hexadecyltrimethylammonium bromide
DDRT-PCR	differential display reverse-transcription PCR
DEPC	diethylpyrocarbonate
DH	Denhardt's Reagent
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleosides triphosphates
dpi	days post-infection
DTT	dithiothreitol
e.g.	for example
EDTA	ethylenediamine-tetracetic acid
ENFVN	Estação Nacional de Fruticultura Vieira Natividade
EtBr	ethidium bromide
EtOH	ethanol
$EtOH_{abs}$	absolute ethanol
EU	European Union

FAO	Food and Agriculture Organisation
Fig.	Figure
G	guanine
GA	galacturonic acid
GA_3	gibberellic acid
GUS	β-glucuronidase
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid
i.e.	that is
IAA	indole-3-acetic acid
IAM	Institute of Applied Microbiology
IBA	3-indolebutyric acid
IPTG	isopropyl β-D-thiogalactopyranoside
Kan^r	kanamycin resistance
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertoni
LRR	Leucine-Rich Repeats
MEA	malt extract agar
MEAm	malt extract agar modified
min	minute
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog
NaAc	sodium acetate
NCBI	National Center for Biotechnology Information
nt	nucleotide
OD	optical density
P_{35S}	35S promoter
PAA	polyacrylamide
PCR	polymerase chain reaction
PDA	potato dextrose agar
pfu	plaque forming units
PGA	polygalacturonic acid
PGIP	polygalacturonase-inhibiting protein
pI	isoelectric point
P_{pgip}	*pgip* promoter
PR	pathogenesis-related
pv.	patovar
RACE	rapid amplification of cDNA ends
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse-transcription PCR
SAR	systemic acquired resistance

SCAR	sequence characterised amplified regions
SDS	sodium dodecyl sulfate
sec	second
SN	supernatant
sp.	species
SSR	simple sequence repeats
STS	sequence-tagged site
T	thymine
TAE	tris-acetate-EDTA
TAIR	The Arabidopsis Information Resources
T_{ann}	annealing temperature
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TBE	tris-borate-EDTA
T_{CaMV}	CaMV termination sequence
T-DNA	transfer-DNA
TDZ	thidiazuron
TE	tris- EDTA
TEMED	N,N,N´,N´-tetramethylethylenediamine
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
U	unit
uv	ultraviolet
vol	volume
w/v	weight/volume
WA	water agar
X-Gal	5-bromo-4-chloro-3-indolyl ß-D-galactoside acid
X-Gluc	5-bromo-4-chloro-3-indolyl ß-D-glucuronic acid

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I INTRODUCTION

1. Fruit production in European Union and Portugal

Fruit production acquired in european agriculture a very important role, not only by supplying primary goods but also by providing raw materials for food industry. Apples, pears and plums are grown in nearly all countries of the European Union while peaches, apricots, table grapes and nuts exist essentially in southern countries as France, Spain, Portugal, Italy and Greece.

In 1986, the surface occupied by orchards in the 15 countries that constitute nowadays the European Union covered about 6 369 887 ha. The annual production reached about 61 716 531 ton, considering mainly pome, stone and citrus fruits, but also nuts, table grapes, berries, kiwi, figs and table olives. For these numbers contributed primary the apples, with 471 827 ha and 10 530 753 ton, corresponding to 7.4% of orchard area and 17.1% of total production, followed by oranges (275 281 ha, 5 298 046 ton) and peaches and nectarines (239 266 ha, 3 160 713 ton) (FAO Database, 2001).

In the last 15 years the orchard area and the annual production decreased slightly in the European Union, achieving in the year 2000, 5 723 660 ha and 61 450 756 ton, respectively. Important decreases of orchard surface occurred particularly in Italy, Greece, France, Spain and United Kingdom. Increasing tendency shows fruit culture in Denmark, Belgium and Luxembourg, as well as in Finland. As before, in 2000, most important fruit crops remain the apples, with 10 163 389 ton and 353 515 ha (6.2% of the surface, 16.5% of the total production), together with oranges (303 611 ha, 6 259 789 ton) and peaches and nectarines (255 499 ha, 4 350 609 ton) (FAO Database, 2001).

Considering the production amounts (Table 1), Italy and France were the largest producers in 1986, followed by Spain and Germany and, after Greece, Portugal. In the year 2000, Italy and Spain were the most important producers in the European Union, followed by France and Germany. Portugal accompanied the trend, decreasing slightly the orchard surface and the total of primary fruit production (FAO Database, 2001).

Table 1: Evolution of primary fruit production and cultivated orchard surface of Portugal and major producers in European Union (FAO Database, 2001).

Country	1986		2000	
	Surface (ha)	Production (ton)	Surface (ha)	Production (ton)
France	1 242 455	13 707 150	1 053 290	11 155 103
Germany	386 734	6 176 654	361 011	5 688 396
Greece	358 729	4 035 860	315 800	4 180 229
Italy	1 561 565	20 349 866	1 373 463	19 412 167
Portugal	452 242	1 830 463	446 704	1 803 316
Spain	2 143 311	12 824 981	1 990 560	16 119 425

1.1 Apple production

Most of apple cultivars derive from *Malus pumila* Mill., small-fruited species that exists spontaneous in Europe. Recent cultivars were obtained from hybridisation between different species of the genus *Malus* (Gautier, 1988) and are referred as *Malus* x *domestica* Borkh.

In Northern hemisphere, apple orchards exist between latitudes of 30º and 60º. In the Southern hemisphere, they exist mainly in New Zealand, Australia, South Africa, Argentina and Chile (Gautier, 1988). According to FAO, apple world production in 1985 was 38 905 235 tons, four times more than in 1955 (8 700 000 tons), mainly from the Northern hemisphere. In 1985, Europe contributed in total with slightly less than one third of world total production, Portugal contributed with 95 100 tons (FAO Database, 2001).

In the year 2000, total production of apples in the 15 EU countries achieved 10 163 389 ton, more than 50% of it coming from France, Germany and Italy (each country over 2 000 000 ton/year). Portugal contributed with 253 816 ton, about 2.5% of the global value (FAO Database, 2001). The increase of yields due to improved agricultural practices, as well as the introduction of more productive cultivars gave origin to a surplus of apple production. For the year 2001, predicted values point out a total amount slightly lower than in 2000, with about 7 497 016 ton expected (Pinon, 2001). Nevertheless, increases of production are estimated in Great Britain, Spain and particularly in Portugal, where an increase of 30% is expected, with total production reaching about 316 000 ton (INE Database, 2001).

Apple remains the basis of european orchards, mainly due to the broad spectrum of cultivars available, adapted to different soil and climatic conditions. The most important cultivar is ´Golden Delicious`, with over 33% of the total production (Pinon, 2001). Although in the last

years cultivar renovation in the orchards was intensified and its presence in the orchards is decreasing, this cultivar is expected to keep its dominating position in the near future. ´Red Delicious` is also suffering a significant decrease, as well as ´Cox`s Orange´ and ´Elstar`, while ´Granny Smith` and ´Rome Beauty` found apparently their market and remain constant (Nicetto, 1998). ´Jonagold` has been the primary cultivar in Belgium, Germany and Netherlands and it is fighting for the second place on Europe`s list of cultivars (de Coster, 1998). In great development, not only but also in Europe are 'Pink Lady$^{R}$', ´Fuji` (surface increased 13%) and, above all, ´Royal Gala` (surface increased 11%) (Pinon, 2001).

1.2 Important limitations to apple production

The dispersion of apple orchards through different climatic regions indicates a huge "plasticity" of this culture. Minimal needs consist of average temperatures not below 15ºC between May and October, sufficient to allow flowering and fruit production, and precipitation of 600mm. Apple trees prefer deep soils, with good aeration and drainage, but survive also in chalk soils (Gautier, 1988). A high diversity of cultivars, with different needs of chilling to break the dormancy and different flowering periods, allows the adaptation of this species towards different climatic conditions and altitudes.

The diversity of soil and climatic conditions in Portugal allows the successful growth of several world wide appreciated and also national cultivars, with fruits developing the desired color and size (Silva, 1993). Thus, the most important limitations to apple culture in Portugal, as well as in Southern Europe, remain the phytopathogens, agents of plagues and diseases that are responsible for decreases in production but also in quality, since they cause deformed, cracked fruits, bad taste, or give origin to toxic substances for mankind (Gautier, 1993).

Causal agents of plagues are insects, acari and nematodes; the most important plagues in apple orchards are the woolly apple aphid (*Eriosoma lanigerum*) and the codling moth (*Cydia pomonella*), followed by the glasshouse spider mite (*Tetranychus urticae*), red spider mite (*Panonychus ulmi*), rosy apple aphid (*Dysaphis plantaginea)*, green apple aphid (*Aphis pomi*) or San José scale (*Quadraspidiotus perniciosus*) (Amaro, 1993).

Infectious diseases can be caused by virus, phytoplasmas and also by fungi or by bacteria. Diseases caused by phytoplasmas (e.g. apple proliferation) and viruses (e.g. ACLSV, ASGV, ASPV, ApMV) are specially problematic, since they cannot be removed once installed in the orchard, as it does not exist any curative treatment. In this case is extremely important the

establishment of orchards with certified non-infected material (IAM Plant Pathology Webpages).

Different bacterial diseases affect apple orchards, being the most important the fire blight, disease caused by *Erwinia amylovora*. The fire blight disease was first identified around 1780 in Hudson Valley (USA), and then recognised in Europe, in Kent (United Kingdom) in 1957 in pear orchards. Although it appears more frequently in pear orchards, fire blight attacks also apple trees (Gautier, 1993). Cultivars have different sensitivity to this disease. Cultivars like ´Jonathan` and ´Fuji` are quite sensitive (Ogawa and English, 1991).

In spite of the damage caused by bacterial and viral diseases, special relevance in the orchard acquire fungal diseases like powdery mildew (caused by *Podosphaera leucotricha*), canker (wound disease caused by *Nectria galligena*) and, mainly, apple scab (caused by the fungus *Venturia inaequalis*), the most important cause of production decrease in the apple orchards (Gautier, 1988; Biggs, 1990; Agrios, 1997).

2. The apple scab disease

Apple scab disease has been extensively studied and described in numerous references. A very complete review is presented in the book ´Apple scab´ (MacHardy, 1996). Here only a brief overview on apple scab disease is presented, with focus on the interaction apple-fungus, the aspect that was more relevant for the work developed.

2.1 Importance of apple scab

Apple scab disease is the most economically important fungal disease that attacks apple orchards in Europe, in Asia, in South and North America. It was first described in Sweden by Fries in 1819 (Ogawa and English, 1991; Biggs, 1990), but scab has plagued apple growers for many centuries, symptoms of the disease are evident on fruit in still-life paintings dating back to the 14th century (Merwin *et al*., 1994). It appears in all temperate regions where apples are grown, being less frequent in semiarid regions in western North America, Australia, New Zealand and South Africa (Biggs, 1990).

Apple scab occurs through all the vegetative period, starting when the buds develop the first green leaves (C3-D) (Gautier, 1988) up to the post-harvest period. Losses result directly from fruit or pedicel infections, and indirectly from decrease of photosynthetic activity and repeated defoliation, reducing tree growth and yield (Biggs, 1990). Nevertheless, it is in the fruits that the losses are more important: young fruits appear with brown spots, do not grow, get deformed and are therefore not sellable (Gautier, 1988). When it is not controlled, scab can cause almost total destruction of an apple crop (Ogawa and English, 1991), and also poor fruit bud development for the next year (Agrios, 1997). Scab also reduces the period that infected fruits can be kept in storage (Agrios, 1997). Factors like sanitation, topography, cultivar susceptibility or frequency of infection periods influence the disease progression and final severity, but the loss can achieve up to 70% when humid and mild weather occurs during spring months (Biggs, 1990). In Portugal, favourable temperatures during almost all the vegetative period conjugated with occurrence of rain determine an epidemic behaviour of this disease (Rosa, 1993).

2.2 Hosts

The most important host of apple scab is the cultivated apple, *Malus* x *domestica* Borkh., but this disease affects also several flowering crab apples, different species of the genus *Malus* (MacHardy, 1996; Palmiter, 1934). With different degrees of severity, apple scab disease

affects most of the apple cultivars with commercial interest. Cultivars like 'McIntosh' (Amaro and Baggiolini, 1982; Milaire, 1993) or 'Gloster' (Milaire, 1993) are considered extremely susceptible, followed by ´Golden Delicious´, ´Red Delicious` and related, described as quite susceptible, ´Cox´s Orange`, ´Jonathan´, ´White Canada´s Reinette´ as susceptible, ´Grey Canada´s ´Reinette´ and ´Belle de Boskoop`, 'Jonathan' and 'Granny Smith' as less sensitive (Gautier, 1988). Noteworthy is that the classification of the cultivars concerning their scab susceptibility is highly dependent on the local, with different classifications being attributed to the same cultivar according to the particular regions (Gessler, 1994). There exist also cultivars issued by breeding that are resistant (see I 3.4.2).

2.3 Symptoms

Apple scab can be observed on leaves, petioles, blossoms, sepals, fruits, pedicels and, less frequently, young shoots and buds. It is characterised by the formation of brown-olive green spots in the green parts of the vegetative tissues (Gautier, 1988), whereas most obvious symptoms occur on actively growing leaves and fruits (Biggs, 1990). Fully expanded leaves at the time of the inoculation show usually no symptoms (ontogenic resistance) (Biggs, 1990; Hering *et al.*, 1993; Valsangiacomo and Gessler, 1988)

As leaves emerge in spring, their lower surfaces are exposed and thus the first lesions are often found on them. Later, as the leaves unfold, both surfaces are exposed and become infected (Biggs, 1990). In the upper surface, first lesions appear as a lighter shade of green compared with the healthy surface of the leaf. They are circular and become covered with numerous darkened lines. The lesions increase in size, become olive-colored and acquire a velvety appearance, with feathery indistinct margins (Biggs, 1990; MacHardy, 1996) (Fig. 1). With disease progression, the lesions become darker, and margins become distinct (Biggs, 1990; Rosa, 1993). The inner portion of a lesion may become brown or grey. Some spots are nearly black (MacHardy, 1996). In the lower surface, lesions are more frequent in the vicinity of the midrib and are diffuse, with irregular poorly-defined borders (MacHardy, 1996).

Young leaves with several infections can become curled, dwarfed and distorted. When an infected leaf ages, the tissues close to a lesion thicken and the leaf surface becomes deformed. The lesions mainly remain on the upper or lower leaf surface for the entire growing season; ocasionally, the underlying cell becomes brown and dies, and lesions become visible on both surfaces. The number of lesions per leaf may range from one to several hundred (Biggs, 1990).

Fig. 1: Apple scab lesions on ´McIntosh´ leaves. Leaf yellowing is followed by defoliation (Jones and Aldwinckle, 1990).

In the flowers, the symptoms appear in the sepals and in the pedicels. Infections on petioles and pedicels result in premature abscission of leaves and fruits, respectively (Biggs, 1990).
In the young fruits, lesions appear initially to be similar to those in the leaves; as the infected fruit enlarges, the symptoms are slightly different, consisting of brown to dark scabby lesions, sometimes cracked (Fig. 2).

Fig. 2: Scab infected fruit of apple cultivar ´Golden Delicious´ .

When the young meristematic tissue near the fruit surface is affected, which occurs with infections early in the season, the fruit can develop unevenly, since uninfected parts continue to grow, giving origin to deformation, or even cracking, in the skin and flesh. Although the entire surface of the fruit is susceptible to attack, infections early in the season usually cluster around the calyx end. Infections occurring in late summer or early fall may only become visible during storage. Symptoms are then described as pin-point scab, with rough, black circular lesions with a diameter ranging from 0.1 to 4 mm (Biggs, 1990).
Scab disease develops in the diverse hosts in different manners, therefore defined reaction classes for each group of symptoms developed under greenhouse conditions were established (Shay and Hough, 1952). These classes identified distinct differences in host response and considered the extent of fungal sporulation. These classes (0-5) are as follows: 0, no macroscopic evidence of infection; 1, pin-point pits and no sporulation; 2, irregular chlorotic or necrotic lesions and no sporulation; 3, few restricted sporulating lesions; 4, extensive

abundantly sporulating lesions. Since the original reaction classes were described, another class (M) has been added. This is described as a mixture of necrotic, nonsporulating and sparsely sporulating lesions (Williams and Kuc, 1969).

These classes of symptoms were also used to characterise susceptibility of the host. As these observations were made in greenhouse assays, in extreme favourable conditions to the pathogen, plants with symptoms comprised in classes 0, 1, 2, M and 3 are considered as showing resistance. Only class 4 is considered as field susceptible (Messiaen, 1981; Williams and Kuc, 1969).

2.4 Causal organism

This disease is caused by the fungus *Venturia inaequalis* (Cooke) Wint. (anamorph *Spilocaea pomi* Fr.). This fungus belongs to the class *Ascomycetes*, subclass *Loculoascomycetidae*, order *Pleosporales* and family *Venturiaceae*. The fungus produces pseudothecia in a stroma in overwintered leaves or fruits in the orchard ground. These structures are negatively geotropic, separate, dark brown to black and spherical (90-150μm in diameter). In the pseudothecium, there are about 50-100 fasciculate, cylindrical, short-stipitate and eight-spored asci (55-75 x 6-12μm) with a thin and bitunicate wall. Ascospores (11-15 x 5-7μm) are yellowish green and unequally two-celled, with the upper cell shorter and wider than the lower cell (the name of the species derives from the unequal size of the two cells in the ascospore). Conidia (12-22 x 6-9μm) are yellowish olive, one- or two-celled, single, terminal and ovated to lanceolate. Sometimes they have an irregular shape. They are produced sequentially, visible by a series of abscission ridges on the conidiophore. The conidiophores arise from short, erect, closely septate, brown mycelium and are nonseptate or closely one-septate, brown (Biggs, 1990) and sometimes swollen at the base (Ogawa and English, 1991). Mycelia are septate and branched and the cells are uninucleate, except at the growing tips of the hyphae (Boone, 1971).

The fungus is bipolar heterothallic. Fungal strains of opposite mating types produce ascogonia and antheridia, and, following fertilisation, pseudothecia form (Agrios, 1997).

Several distinct physiological races have been primarily identified, based on their phytopathogenicity in different apple cultivars (Boone, 1971; Crosby *et al.*, 1992; Parisi *et al*., 1993).

2.5 Disease cycle

This fungus shows an evolution in two phases: a saprophytic phase, in the dead leaves or fruits detached from the tree, and a parasitic one, on the living organs in the plants.

It overwinters as immature pseudothecia in infected leaves and fruits on the orchard floor. Following a distinct rest or dormant period, with temperatures about 0ºC, the pseudothecium continues to mature with the development of asci and ascospores. Moisture is necessary for the development of pseudothecia. The optimal temperature range for ascogonial development is 8-12ºC, and the optimum for ascospore maturation is 16-18ºC (Biggs, 1990). The pseudothecia development does not occur simultaneously: in the same leaf, the maturation occurs at first in the structures on the side exposed to the light (Rosa, 1993).

When old infected leaves overwintered in the orchard become wet, mature asci expand through the ostiole and discharge ascospores, which are disseminated by the wind (air dispersion during daylight), and will germinate in the water droplets existing on the surface of the newly developed green organs from the trees, starting the primary infections (Gautier, 1988). Necessary conditions for germination are a range of temperatures about 1ºC to 26ºC, the presence of green organs in the orchard (state C3-D) and enough rain to make wet the plant tissues (Gautier, 1988). In most years and locations, the first ascospores are mature and capable of causing infections at about the time of budbreak. They continue to mature and are discharged over a period of 5-9 weeks. The peak period of ascospores discharge usually occurs between the pink and the full-bloom stages of bud development (Biggs, 1990). In case there are not favourable conditions to germinate, ascospores can maintain their vitality for some period, even some months in case of lower temperatures and dry air (Rosa, 1993). Germination begins when the ascospore contacts with a wet surface of a leaf or a fruit. Free moisture is required for the initiation of the germination; after the initiation, germination proceeds as long as the relative humidity is higher than 95%. The time required for infection to take place is a function of the number of hours of wetness and the temperature, ranging from 21 hours at 6ºC to 9 hours at 16-24ºC. Germinated ascospores develop a mycelium under the cuticle from which new conidia will be formed (Biggs, 1990). After an incubation period varying from 9 to 20 days, the first olive green spots appear and get covered by a velvety surface which contains the newly formed conidia (Gautier, 1988; Biggs, 1990).

Conidia (up to 100 000/leaf lesion) are disseminated by splashing rain and wind to new leaf or fruit surfaces, and are the principal inoculum involved in the build up of the disease during summer. They germinate, penetrate the host and give rise to new lesions, as the ascospores did, originating secondary and further infections, keeping the disease in the orchard during all growing season (Biggs, 1990). The rate of germination and appressorium formation of both

ascospores and conidia is directly proportional to temperatures from 5ºC to 20ºC, but conidia germinate and form appressoria faster than ascospores (Turner *et al.*, 1986).

Visible lesions appear usually within 9-17 days, depending on the temperature and the relative humidity. The minimum relative humidity for sporulation to occur is 60-70%, but periods of lower humidity are not letal for the fungus (Biggs, 1990). The rain has also a very important role in the dissemination of the conidia, and therefore, in the progression of scab disease in the orchard (Gautier, 1988). Several cycles can occur during the growing season, depending on the frequency of infection periods and the susceptibility of the host tissue. Following leaf fall in autumn, the fungus enters the saprophytic phase (Biggs, 1990). It penetrates the epidermal cells and grows into the interior of the leaf. If the leaf remains moist, the initiation of pseudothecia soon follows in the interior of the leaf (Wilson, 1928).

2.6 Interaction plant-fungus

The apple scab pathogen has a type of parasitism with features in common with those of the obligate parasites (Boone, 1971; Messiaen, 1981; Agrios, 1997), coexisting the fungal structures with the living host tissue in the course of disease development. During the parasitic phase, the fungus establishes a biotrophic relationship with the host (Koller *et al.*, 1992), remaining limited to a position between the cuticle and outer epidermal cell walls and deriving its nourishment from the underlying host cells (Williams and Kuc, 1969).

On both susceptible and resistant hosts, spore germination, appressorial formation and cuticle penetration are similar (Williams and Kuc, 1969; Hering *et al.*, 1993; Yepes and Aldwinckle, 1993a). In the susceptible cultivars, the germination tube is short and seldom ramified; it ends in one single appressorium that will join preferentially to an intercellular junction. In the resistant cultivars, the germination tube is longer and ramified, developing up to 6 appressoria (Chevalier and Lespinasse, 1994). After the formation of the apressoria, these structures are apparently held fast to the leaf surface by means of a mucilaginous sheath. Direct cuticular penetration is accomplished by means of a penetration peg, an infection hypha which develops through a thin walled pore-like area about 2µm in diameter in the lower surface of the apressorium (Williams and Kuc, 1969; Yepes and Aldwinckle, 1993a).

The mechanism by which the infection peg penetrates the cuticle is not clearly known. Nevertheless, it was verified that the cuticle does not represent an effective preformed physical barrier for the pathogen, infection structures originating from ascospores or conidia gain entrance through the intact cuticle (Valsangiacomo and Gessler, 1988).

The fungitoxic action of fatty acids derived from hydrolysed cutin was taken as evidence against an enzymatic penetration of the cuticle. It was speculated that these compounds, once released by the action of the enzyme, would suppress the growth of the invading scab fungus (Martin, 1964). However, based on the ultrastructural appearance of the cuticle at the site of penetration, enzymatic hydrolysis rather than mechanical force has been suggested as the means of cuticle penetration (Valsangiacomo and Gessler, 1988). Furthermore, transitory esterase activity was detectable only during the early stage of the scab infection. The enzymatic activity observed in the appressorium might represent the localisation of hydrolytic enzymes necessary for host penetration (Nicholson *et al.*, 1972). It was suggested that the fungus produces a cutinase, that can dissolve the cuticle and aid in penetration (Valsangiacomo and Gessler, 1988; Köller and Parker, 1989). Experiments with mycelium from *Venturia inaequalis* grown on polymeric cutin as the sole carbon source, revealed the production of cutinase as the exclusive serine esterase released into the extracellular fluid (Köller *et al.*, 1991). In addition, in the same study, the application of a specific cutinase inhibitor resulted in the delay of penetration and formation of appressoria, but mainly in the complete and lasting block of stroma development beneath the cuticle, indicating a crucial role of cutinase in cuticle penetration and subcuticular growth (Köller *et al.*, 1991). In early studies (1915) Wiltshire had already concluded that cutin hydrolysis was not only required by the fungus to "eat its way through the cuticle until it arrives between the cuticle and epidermall cell wall" but also during its parasitic phase of subcuticular growth, suggesting a more permanent role of cutinase in disease development, besides its normal function as a penetration enzyme. A possible principle for the cutinase model would be that small quantities of cutinase, most likely with a cuticle-sensing function are released from conidia germinating in water, giving origin to cutin monomers, which are inducers of *de novo* cutinase production in both germinating conidia and mycelium of the fungus (Köller *et al.*, 1991). Indirect evidence of enzymatic degradation of the cuticle during penetration and subcuticular growth of the fungus was obtained by electron microscopy (Yepes and Aldwinckle, 1993a).

After penetration of the surface, the invading fungus is limited to a position between the cuticle and outer epidermal cell walls (Williams and Kuc, 1969). As soon as the penetration peg reaches the epidermal cell walls, it flattens into an irregularly shaped primary hypha, that ramifies into secondary hyphae, branching radially to form a subcuticular stroma, consisting of short thick-walled cells (Williams and Kuc, 1969; Yepes and Aldwinckle, 1993a). It is at

this stage that a differential interaction occurs, with colonisation and establishment depending on the susceptibility of the host. Resistance will be expressed when the infectious hypha is in contact with the epidermal cell wall (Chevalier and Lespinasse, 1994).

In a susceptible host, at the onset, the hyphae bind to the epidermal cell wall, without incrustations; the epidermal cell does not undergo any histological changes and also the palisade tissue is not modified (Chevalier *et al.*, 1991; Chevalier and Lespinasse, 1994). The fungus derives its nutrition from the underlying host cells without the development of intracellular haustoria. The mycelial cells usually adhere firmly to the upper epidermal wall with gaps evident only beneath the anticlinal walls of the fungal cells (Corlett *et al.*, 1976). They fill in the gaps and irregularities of the epidermal surface, appearing sometimes the cells of the host and parasite closely interlocked (Corlett *et al.*, 1976). The existence of cytoplasmic links (called ectodesmata), which extend through outer epidermal walls, could provide contact between host cytoplasm and fungus, serving as bridge (Franke, 1961). During the first days after inoculation, host cells show little abnormality (Boone, 1971). After this period, that can vary upon the cultivar, but 10-12 days are common (Chevalier and Lespinasse, 1994), the fungal pseudoparenchymatous stroma develops and produces conidiophores in the uppermost cell layer that push through the overlying cuticle, and conidia are formed, giving origin to a visible (velvety) lesion (Williams and Kuc, 1969; Corlett *et al.*, 1976). In the host, the cytoplasm of the epidermal cells beneath a sporulating lesion becomes depleted and virtually disappears, resulting in cell death. Accompanying this effect, depletion of plastids (the organelles loose their integrity and organisation as structure and coalesce into larger disorganised masses), and vacuolation occur in the palisade mesophyll cells. With the progression of the disease, the leaf tissue underlying the subcuticular fungus stroma becomes chlorotic to necrotic, resulting eventually in the collapse of the entired infected area (Williams and Kuc, 1969; Boone, 1971; Corlett *et al.*, 1976).

There are indications that the fungus enzymatically degrades the cuticle and epidermal cell wall. Electron-microscopy studies on mature sporulating lesions in susceptible apple leaves infected with *Venturia inaequalis*, demonstrated extensive degradation of the upper epidermal layer adjacent to the mycelial cells (Corlett *et al.*, 1976). Later, similar studies revealed modifications in the wall of epidermal cells of the host, as electron transparent zones, believed to be caused by cell wall degrading enzymes, were evident in portions of cell wall in direct contact with the fungal stroma (Valsangiacomo *et al.*, 1989; Müller *et al.*, 1994).

In the resistant cultivars, modifications on host and parasite tissue depend upon the type of resistance involved. The resistance mechanism leading to class 1 symptoms is considered a hypersensitive response (Shay and Hough, 1952).

In case of the hypersensitive response, the infection process is interrupted due to early necrosis of infected tissue. Within a very short period (20 to 72 hours) after penetration, many wall appositions are setting in the epidermal cells underlying the point of penetration and the cells collapse bellow the infection peg (Chevalier and Lespinasse, 1994), leaving their anticlinal walls protruding (Chevalier *et al*., 1991). Shortly thereafter the fungus is killed (Shay and Hough, 1952). In the host, the intercellular spaces are reduced, the cells become filled with a granular deposition of material (Chevalier *et al*., 1991; Chevalier and Lespinasse, 1994) and immediately beneath these cells, the membranes of plastids in the palisade cells are apparently altered, with a loss of definition in the plastids (Williams and Kuc, 1969). There is also an accumulation of darkly staining spheroid bodies in the cytoplasm of the palisade cells (Williams and Kuc, 1969). It was suggested that the collapse of cells in host tissues triggers a series of metabolic events which produce substances that inhibit extracellular enzymes produced by the fungus, and thereby inhibit its growth (Raa, 1968). This collapse could derive from the toxic action of an extracellular metabolite produced by the fungus, that might cause a loss of compartmentation of enzymes and substrates in the epidermal cells. This loss would initiate the mixing of phloridzin, phenoloxidase and ß-glycosidase, leading to the formation of fungitoxic (highly toxic to germinated spores of the fungus) and phytotoxic oxidation products and to host cell collapse (Williams and Kuc, 1969; Pellizzari *et al.*, 1970). *In vitro* experiments showed that a high concentration of phloretin, one of the products derived from metabolism of phloridzin, inhibit the fungal growth (Holowczak *et al.*, 1962). At the same time, the cytoplasm of the fungal hyphae strongly degenerates and parasite hyphae become necrotic. Primary stroma remains reduced to some hyphae, and the fungus is limited in its progression by the presence of the cell wall appositions (Chevalier *et al*., 1991; Chevalier and Lespinasse, 1994). The fungal wall incrusts strongly in the host wall, which becomes thinner (Chevalier and Lespinasse, 1994). On the areas that remained healthy, numerous conidia germinate, forming one or several appressoria but not producing any subcuticular stroma; the epidermal cells remain identical. The mesophyll cells and low epidermis do not undergo important modifications (Chevalier *et al*., 1991).

With type 2 resistance, macroscopic symptoms are not expressed until 6-12 days after inoculation, when the infected area becomes necrotic. At this stage, although tissue aspect

varies among plant hosts, very often the upper epidermis is partially collapsed, cells are plasmolysed, cytoplasm is necrotic and the vacuoles enclose dense granulations. The palisade tissue, with high cellular density, sometimes has multiple layers of small cells with a large cytoplasm where the small-sized vacuoles frequently enclose polymorphic granules (Enochs, 1964; Chevalier *et al*., 1991; Merwin *et al.*, 1994). The fungus remains viable in these lesions, sometimes up to 21 days (Merwin *et al.*, 1994); the subcuticular hyphae whose sections are collapsed are either isolated from each other or grouped in uniseriated, limited stromata, but conidiophore formation and sporulation does not occur (Enochs, 1964; Chevalier *et al*., 1991).

In leaves resistant to scab, unstructured and poorly developed stroma was observed and little or no cell wall degradation was detectable close to it (MacHardy, 1996).

It was suggested that resistant and susceptible apple leaves have the same potential defense system, but due to insensitivity of the latter to the toxic proteins formed by *Venturia inaequalis*, their defense apparatus is not mobilised in the presence of the pathogen, which can grow freely in susceptible leaves (Raa, 1968). In contrast, the resistant host would be capable to react by necrosis to an unknown heat stable metabolite deriving from germinating spores (Noveroske *et al*., 1964).

A crucial role for cell wall-degrading enzymes during subcuticular growth has been suggested (Wagner *et al.*, 1988; Kollar, 1998). The ability of *Venturia inaequalis* to metabolise cell wall components of plants had been reported already in 1962, after *in vitro* experiments indicated that *Venturia inaequalis* could metabolise cellulose and pectin, two components commonly found in the walls of plant cells (Holowczak *et al.*, 1962). Lately, plant cell wall degradation was associated with fungal pectinolytic activity (Valsangiacomo *et al.*, 1989). Pectinolytic activity was essentially detected in the mycelial extracts, suggesting that plant cell wall-degrading enzymes from *Venturia inaequalis* were not generally released into the medium, which was considered to be related to the particular type of parasitism of this fungus (Kollar, 1994), characterised as biotrophic while parasitising the plant tissue. This activity was first associated with an exopolygalacturonase, as this enzymatic activity was detected in mycelial extracts of one isolate of *Venturia inaequalis*. However, although enzyme extracts were able to degrade apple cell wall preparations, cell wall material was not hydrolysed efficiently even after extensive incubation with excess of enzyme (Valsangiacomo *et al.*, 1992). Later, an endopolygalacturonase (pectinase that degrades the homogalacturonans from the pectins that form the cell wall) with extracellular activity was identified. This constitutively expressed

enzyme showed to disintegrate apple cell wall and was suggested to be one of first enzymes involved in the infection process (Kollar, 1998). *In vitro* studies showed that a high production of this pectinase was detectable at the early phase of mycelial growth. The fast release of high pectinase activity shortly after penetration of the cuticle might be a prerequisite for the establishment of the pathogen, enhancing fast release of nutrients or removal of physical barriers, and subsequently, an improved degradability of the cellulosic fibrils, which may be set free from the pectin matrix by the pectinolytic activity (Kollar, 1998).

2.7 Genetics of the interaction plant-fungus

The interaction scab-apple is considered to be an example for the gene-for-gene interaction, that suggests that to each resistance gene in the host corresponds a virulence gene in the pathogen (Flor, 1956; Gessler, 1994). A certain number of virulence genes has been identified in the parasite in close relation with the resistance genes in the host and several physiological races of *Venturia inaequalis* have been defined with basis on their specific pathogenicity towards the different host genotypes, that showed differential resistance (Shay and Williams, 1956; Williams and Kuc, 1969; Crosby *et al.*, 1992).

It was verified that the different races can cause different degrees of disease incidence and severity to the different genotypes. Seven races were identified, basing on the differential virulence that they show in each host, some of them infect and overcome certain genes for resistance (resistance genes are presented in I 3.4.1). Already in 1956, in a differential range of resistant parents, Shay and Williams (1956) defined races 2, 3 and 4 of *Venturia inaequalis*.

Race 1 is the race commonly encountered throughout the world that attacks *Malus* x *domestica*. Race 2, collected in South Dakota, USA, successfully attacks *Malus baccata* ´Dolgo´, ´Alexis´ and ´Bittercrab´, and certain segregates of the russian seedling R12740-7A, but not Vr (see I 3.4.1), among others. Race 3, collected in Nova Scotia, Canada, overcomes the resistance found in ´Geneva´. Race 4, found in Indiana, USA, attacks certain segregates of the russian seedling R12740-7A (Crosby *et al.*, 1992), suggesting a third *locus* for qualitative resistance different from that overcome by race 2 or Vr (Williams and Kuc, 1969). Race 5 was found at the John Innes Institute, Norwich, United Kingdom (Williams and Brown, 1968) and overcame Vm, the pit type resistance found in *Malus micromalus* and *Malus atrosanguinea* 804 (Williams and Kuc, 1969; Parisi *et al.*, 1993). Race 6 overcomes the Vf-

introgressed resistance (opposite to all the previous) and it was named after studies where all Vf gene cultivars or selections, previously segregating in the 5 classes of symptoms (resistant genotypes showing symptoms distributed through 4 classes, the susceptible genotypes in the fifth class), were completely susceptible to a certain scab inoculum – Ahrensburg inoculum (Parisi *et al.*, 1993). Another scab isolate (race 7) showed to be capable of rendering the resistance of *Malus floribunda* 821 ineffective, but not that of some of later selections (Roberts and Crute, 1994).

Noteworthy is that the race 6 does not attack susceptible cultivars like ´Granny Smith´, ´Reinette Clochard´ or *Malus baccata jackii*, *Malus pumila* R 12740-7A or ´Antonovka´ PI 172623 (Parisi and Lespinasse, 1999). More recently, Koch *et al*. (2000) have shown that every apple cultivar has resistance genes against all genotypes of *Venturia inaequalis*, except those missing the corresponding avirulence alleles. In nature, however, the scab inoculum is always a mixture of virulent and avirulent genotypes. Consequently these resistances are not visible in the field and, for this reason, are called ephemeral.

3. Control of apple scab disease

3.1 Cultural methods of fight against apple scab

Orchard sanitation practices can contribute to prevent the formation of pseudothecia during winter, lowering the inoculum level and, thus, the threat of early attack in the next growing season. Overwintering inoculum can be reduced by applying nitrogen (e.g. as urea) to leaves in autumn, in order to increase the rate of leaf decomposition under mild winter conditions (Carisse et al., 2000; Thakur and Sharma, 1999), as well as by removal of all the organs (fruits, leaves) from the floor (Merwin *et al.*, 1994; Biggs, 1990).

Some cultural practices like spacing the trees at plantation, as well as regular pruning, allow a better air circulation and a better spraying coverage, contributing to reduce the risk of infection (Biggs, 1990). The mixture in the orchard of different cultivars contributes also for a higher pressure towards the pathogen and to a general lower susceptibility (Gessler, 1994).

3.2 Chemical control

Until the end of the 19th century, there were no substances available for effective chemical control against apple scab. By the late 1970s there were at least 17 different fungicides available for controlling this disease (Merwin *et al.*, 1994). Nowadays, in orchards, apple scab disease is effectively controlled with fungicides. Substances that are commonly used include phtalimides (e.g. captan), ethylenebisdithiocarbamates (e.g. mancozeb, maneb, thiram, zineb), benzimidazoles (e.g. benomyl, thiophanate-methyl, carbendazim), ergosterol biosynthesis inhibitors (e.g. bitertanol, fenarimol, nuarimol) and dodine (Gautier, 1988; MacHardy, 1996). Fungicides from the group of benzimidazoles, ergosterol biosynthesis inhibitors and dodine are nowadays less used, since they gave origin to fungicide resistance (Biggs, 1990; Milaire, 1993).

Fungicide control programs are usually integrated with control measures for powdery mildew, rusts and various diseases and plagues. In conventional agriculture, treatments are often made preventive, basing on the cultivar, phenological state of the trees, inoculum that exists in the orchard and on the climatic conditions, factors with direct influence on the severity of the attack (Gautier, 1988; Milaire, 1993). Narrow-spectrum fungicides (e.g. ergosterol biosynthesis inhibitors) are usually combined with broad-spectrum products (e.g. ethylenebisdithiocarbamates) to increase efficacy and minimise the selection of resistant strains (Merwin *et al.*, 1994).

Since it is very difficult and expensive to control the secondary cycles of the disease, the strategies for controlling this pathogen are mostly concerned with the destruction of the primary inoculum. For control of primary infections, sprays are often applied according to host phenology: first application occurs normally at silver tip, followed by further applications at green tip, 1-cm green, tight cluster, full pink, bloom and petal fall. The number of fungicide applications prior to petal fall varies with factors like weather conditions, cultivar, rate of tissue development, fungicide used and also the density of ascospore inoculum present in the orchard, evaluated by the infection severity of the previous year. Cover sprays are applied one week after petal fall and are repeated every 10-14 days until 2-3 weeks before harvest (Biggs, 1990).

Although it is still highly spread over the world, the importance of repeated fungicide treatment as control method was reduced, due to fungicide resistance (e.g benzimidazoles), as well as its high costs and the increasing environmental concerns (Biggs, 1990). This reduction occurs in agreement with new devised strategies for orchard management. Indeed, pesticide usage has been substantially reduced where integrated pest management strategies have been implemented. They devised a reduction of disease inoculum and eradication of incipient infections by making use of the advantages of a precise timing of application of fungicides. Various models have been proposed for predicting key developments in the epidemiological cycle of scab (Merwin *et al.*, 1994) and the warning systems, estimating the probability of scab attack in function of the clima (temperature and humidity) achieved great importance in the control of scab disease, avoiding an excessive application of fungicides (Biggs, 1990).

In many countries, warning services have been established to monitor infection periods and advise growers within a few hours after conditions for infection have occurred in the region. In these services, temperature and duration of wet periods are monitored in the orchards. Weather data are then analised and, together with the different established models, used to determine whether infection can occur (Biggs, 1990; Milaire, 1993). The first model available was the Mills curve (Mills, 1944). This curve (Fig. 3) establishes the duration of humid period necessary to cause infection in function of the temperature, considering that the germination of the ascospores or conidia is only assured if, after contamination, the leaf remains wet during a certain period which is function of the temperature (Gautier, 1988). According to the values determined for duration and average temperature of the wet period, Mills classified the severity of the potential infection risk in three categories (light, moderate or heavy), within a range of temperatures between 5 and 26°C (Xu *et al.*, 1995).

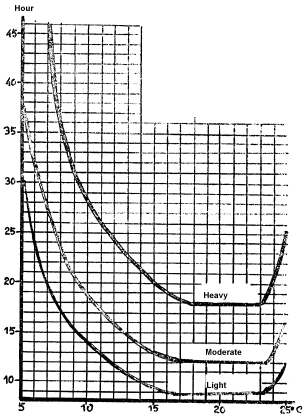


Fig. 3: Mills´ curve. Period during which the leaves should remain wet at different temperatures in order that light, moderate or heavy contaminations can occur (adapted from Matias and Gonçalves, 1993).

Mills´criteria referred to a static model and did not represent infection and spore mortality as dynamic processes, suggesting identical risk levels for periods of equal wetness duration and average temperature, regardless of the temperature profile during the wet period(s) (Xu *et al.*, 1995). In order to improve scab forecasting, these criteria were revised, incorporating the time of ascospore discharge, ascospores longevity and dose, and also cultivar susceptibility and vegetation condition (MacHardy, 1996). Different models were established, regarding specific local conditions (Xu *et al.*, 1995; Rosenberger, 2000). Electronic environment-monitoring equipment for use in orchards has been developed to automatically alert growers to infection periods.

Nowadays, in practice, growers frequently use weather forecasts to modify the timing of treatments based in calendar schedules. Protective fungicides are applied only when extended periods of wetting are forecast. Thus, the frequency of application is decreased during dry weather and increased during humid conditions. To achieve the greatest efficiency from applications based on weather forecasts, growers must be aware of the rate of host development and the rate of loss of fungicides residues due to weathering. The availability of reliable and specific forecasts is critical to the success of modified calendar-based schedules. (Biggs, 1990). Postinfection fungicides are also used in modified calendar-based schedules when critical applications are missed because of incorrect weather forecasts (Biggs, 1990).

Control programs based on a postinfection schedule are another alternative to calendar-based schedules. Postinfection schedules are common in arid regions, where infection periods are less frequent. To use a postinfection schedule, growers must know when infection has occurred and which fungicide is suitable to control scab when applied 24-96 hours after the initiation of an infection period (Biggs, 1990).

Some fungicides, applied in autumn before leaf fall or to the leaves in the orchard ground, can also inhibit the development of pseudothecia in the overwintering infected leaves (Gadoury *et al.*, 1989).

3.3 Biological control

Biological control has focused mainly in the search of antagonists that inhibit fungal development when applied in susceptible organs, and also that impair the sexuated stage of the pathogen, reducing potential inoculum for the following season. This approach has shown to be less effective against summer scab (Andrews *et al.*, 1982; Cullen *et al.*, 1984), but has provided interesting results concerning the reduction of the overwintering inoculum (Young and Andrews, 1990). The establishment of *Athelia bombacina* Pers., a fungal saprophyte, on apple leaves has shown to contribute to the reduction of pseudothecia in overwintering scabby leaves and to inhibit over 98% of the ascospore formation, in both controlled laboratory experiments and in the field (Gadoury *et al.*, 1989; Philion *et al.*, 1997). Six fungal isolates from genera *Trichoderma*, *Phoma*, *Coniothyrium*, *Ophiostoma* and *Diplodia* were tested *in vitro* for their ability to inhibit ascospore production, and proved to be efficient potential antagonists of the saprophytic phase of *Venturia inaequalis* (Philion *et al.*, 1997). Some *Microsphaeropsis* sp. provided as well a significant and consistent reduction in ascospore production (Carisse *et al.*, 2000).

Burr *et al*. (1996) reported also the finding of several promising antagonistic microrganisms, including one strain of *Pseudomonas syringae*, that appeared to control apple scab as effectively as captan under greenhouse conditions. Some strains of *Pseudomonas fluorescens* and one strain of *Bacillus pumilus* showed also strong inhibition of mycelial growth and conidial germination of *Venturia inaequalis*, and were proposed as potential biocontrol agents of apple scab (Kucheryava *et al.*, 1999).

Ascospores of *Chaetomium globosum* applied to apple leaves showed a strong inhibiting effect on scab progression (Andrews *et al.*, 1982; Cullen and Andrews, 1984) but this effect was not reproduced in field assays, where the efficacy was much lower (Cullen *et al.*, 1984).

Although the results observed in experiments *in vitro* are positive, they are not yet enough consistent to advise the use of biological strategies to impair scab disease in the orchards (Rosa, 1993; Agrios, 1997).

3.4 Genetic approaches to fight apple scab

The first genetic approach to fight apple scab considered the existence of natural sources of resistance within the genus *Malus*, and the possibility to use them in breeding programs.

3.4.1 Natural sources of resistance

In 1934 and 1935, Rudloff and Schmidt (Rudloff and Schmidt, 1934; Rudloff and Schmidt, 1935) established that four small-fruited *Malus* species, *Malus atropurpurea*, *Malus micromalus*, *Malus spectabilis* and *Malus spectabilis Kaido*, possessed resistance towards scab, as they were entirely free of scab or showed very light infection during field inoculation tests. A very high level of resistance was also presented by the commercial cultivar ´Antonovka´. These findings suggested the possibility of developing scab resistant varieties.

Among the collection established by Crandall, Hough found also six small-fruited selections of asiatic *Malus* species that exhibited field immunity to scab: *Malus atrosanguinea* 804, *Malus floribunda* 821, *Malus prunifolia* 19651, *Malus ringo*, *Malus toringo* and *Malus zumi* (Hough, 1944).

Two types of scab resistance in *Malus* were identified: quantitative or multiple factors (polygenic resistance) and qualitative or single major factors (monogenic resistance) (Williams and Kuc, 1969). The former type was considered to be sensitive to the effect of environment, host conditioning or both, while the latter was defined as expressed under all conditions, usually as single dominant genes. Both types may condition field immunity in that no macroscopic evidence of infection is present, or resistance may be expressed as a reduced number and size of sporulating lesions (Williams and Kuc, 1969).

The identified sources of resistance are summarised in Table 2, comprising six *loci* for qualitative resistance (defined gene pools).

Table 2: Genetic sources for resistance breeding against apple scab (adapted from Sansavini, 1993).

Qualitative resistance (*locus*)	**Polygenic resistance**
Malus baccata Jackii (Vbj)	*Malus baccata*
Malus floribunda 821 (Vf)	*Malus sargentii* 843
Malus micromalus (Vm)	*Malus sieboldii* 2982-22
Malus pumila R12740-7A (Vr)	*Malus toringo* 852
´Antonovka` PI172623 (Va)	*Malus zumi calocarpa*
Hansen´s *baccata* n° 2 (Vb)	

Another important source of scab resistance is derived from the accession *Malus atrosanguinea* 804 (Dayton and Williams, 1970). This genotype appears to have two dominant genes affecting resistance (Shay *et al.*, 1953). The major gene produces a pit-type (type 1) resistance reaction and is designated Vm (Dayton and Williams, 1970). The second gene, generating a type 3 reaction, is often referred to as the "masked" gene, because its phenotype is not expressed in plants that contain the pit gene (Williams and Brown, 1968).
Minor resistance traits were identified in different commercial cultivars from *Malus* x *domestica* (Rousselle *et al.*, 1974; Gessler, 1994), but these traits were only effective towards a part of the heterogenous inoculum derived from pseudothecial development in the orchard and are not useful in orchards containing only two or three cultivars (Messiaen, 1981). They were considered not interesting to be included in breeding programs. One example was found in ´Golden Delicious´, scab susceptible cultivar carrying a resistant gene that has been recently called Vg (Bénaouf and Parisi, 1997).

3.4.2 Breeding based on introgression of resistance trait through backcrossing

Some of the resistant wild genotypes (I 3.4.1) were used in several breeding efforts. These programs consisted on the cross between wild species of genus *Malus*, with natural scab resistance, and the susceptible cultivars. The aim was the introgression of the resistance character in susceptible cultivars through repeated backcrossing, assuming that no major resistance genes are present in *Malus* x *domestica* (Williams and Kuc, 1969; Crosby *et al*., 1992).
In 1934, Rudloff and Schmidt, believing that cross-breeding presented the most likely solution for developing scab resistant varieties, proceeded to initiate a suitable program. They verified that it was possible to transmit the original level of resistance but they were discouraged in that factors controlling small fruit size seemed to be dominant and the program was interrupted by World War II (Williams and Kuc, 1969).
In 1945, a cooperative program of scab resistance breeding was initiated by Purdue University and the University of Illinois, in USA, by Shay and Hough, after Hough evaluated the collection of *Malus* species and hybrids previously assembled by Crandall in the beginning of the 20th century, and verified that one progeny derived from the cross (*Malus floribunda* 821 x ´Rome Beauty´) x (*Malus floribunda* 821 x ´Rome Beauty´) aproximated a segregation ratio of 1 scab resistant: 1 scab susceptible (Williams and Kuc, 1969; Crosby *et al*., 1992). Two resistant selections from this cross (F_2 26829-2-2 and F_2 26830-2) were used

in further crosses, forming the basis for this breeding program and the source of resistance for most of today´s resistant cultivars (Valsangiacomo and Gessler, 1988). Since then, this program, known as PRI program (Rutgers University was included in 1950), has released several highly resistant cultivars, being followed by different programs also in USA, in France, Germany, Switzerland, Canada, Rumania and Brazil (Crosby *et al*.,1992).

Within these programs, dominant genes for scab resistance were transferred by a repeated series of hybridisations of genotypes heterozygous for resistance with adapted, high quality genotypes homozygous for scab susceptibility. In these modified backcrosses, half of the progeny contained dominant resistance in the heterozygous condition and could be clearly identified in the seedling stage (Hough *et al*., 1953). The use of different susceptible parents was important due to inbreeding depression in apple, that makes repeated backcrossing with the same parent not desirable (Merwin *et al.*, 1994). Between 1970 and 1992, 48 scab-resistant cultivars were released, among them one with Vf from *Malus atrosanguine*a 804, 5 with the Vm gene from *Malus atrosanguinea* 804, one with a Vr gene from a russian apple seedling and 37 of them deriving from the small-fruited ornamental species *Malus floribunda* (Sieb. ex Van Houtte) clone 821 (Crosby *et al.*, 1992). The most important resistant cultivars obtained until 1994 are shown in Table 3.

Although several sources of apple scab-resistance have been identified, *Malus floribunda* 821 was the most frequently used source of scab resistance (see Table 3), as it conferred resistance to most identified *Venturia inaequalis* races (Hough, 1944; Parisi *et al*., 1993). The original clone itself produces a pin-point resistant reaction to *Venturia inaequalis* (Chevalier *et al.*, 1991), whereas the resistant reaction in later generations of *Malus floribunda* backcrosses with susceptible commercial cultivars may be expressed as a class 1, 2, 3 or M. The usual resistant reaction is a chlorotic lesion with a variable level of sporulation (Manganaris *et al.*, 1994). The most important cultivar derived from this genotype was ´Florina´, obtained by INRA in 1977 (Gautier, 1993) (see I 3.4.5.2.1).

By the year 2000, a total of 18 scab-resistant cultivars containing the Vf gene were released by PRI alone or jointly with other institutes and about 50 cultivars derived from PRI germplams have been released by breeders world wide (Janick, 2000).

Table 3: Scab resistant apple cultivars released by different breeding programs (compiled from Crosby *et al.*, 1992 and Merwin *et al.*, 1994).

Cultivar	**Resistance gene**	**Year of release**	**Country of origin**
´Prima´	Vf	1970	USA
´Priscilla´	Vf	1972	
´Sir Prize´	Vf	1975	
´Liberty´	Vf	1978	
´Jonafree´	Vf	1979	
´Redfree´	Vf	1981	
´Mc Shay´	Vf	1981	
´Freedom´	Vf+polygenic	1983	
´Dayton´	Vf	1988	
´William´s Pride´	Vf	1988	
´Enterprise´	Vf	1992	
´GoldRush´	Vf	1992	
´Mac Free´	Vf	1974	Canada
´Nova Easygro´	Vf	1975	
´Novamac´	Vf	1978	
´Moira´	Vf	1978	
´Trent´	Vf	1979	
´Britegold´	Vf	1980	
´Murray´	Vm	1980	
´Rouville´	Vm	1983	
´Richelieu´	Vf	1983	
´Nova Spy´	Vf	1986	
´Generos´	polygenic	1972	Rumania
´Pionier´	Vf	1983	
´Romus 1´	Vf	1984	
´Romus 3´	Vf	1984	
´Voinea´	Vf	1985	
´Gavin´	Vf	1977	United Kingdom
´Primicia´	Vf	1988	Brazil
´Priam´	Vf	1974	France
´Florina´	Vf	1977	
´Judeline´ (juice)	Vf	1986	
´Judaine´ (juice)	Vf	1986	
´Baujade´	Vf	1989	

However, although resistant cultivars for fresh apples consumption are not yet grown on a large scale by commercial growers (only for cider apples in France) due to reduced fruit quality (see I 3.4.4), some cultivars appear to be useful commercially. ´Liberty´ has been recommended to growers who wish to reduce or eliminate their fungicide application (Garcia *et al.*, 2000). ´Golden Lasa´ was also considered a good alternative for ´Golden Delicious´, especially for organic growers (Bergamini and Giongo, 2000a), as it showed to be very productive and with a high processing ability. Recently, several scab resistant cultivars were evaluated as positive (´Florina´) and promising (´Summerfree´, ´Topaz´, ´Golden Lasa´, ´Primiera´, ´Golden Orange´, ´Delorina´, ´Enterprise´, ´GoldRush´) for a commercial growing in Italy (Fideghelli, 2000). ´Topaz´ is also very successful among the scab resistant cultivars; due to its resistance also to powdery mildew, this cultivar obtained in the Check Republic is being largely used in orchards under biological management (Seguin, 2000). ´Netta´ and ´Ciosa´ are also under evaluation in Italy, appearing very promising for their quality traits (Bergamini and Giongo, 2000b). ´Red Earlib´, a scab-resistant variety ´Red Delicious´ type is described as a very productive cultivar with very attractive red colour (Bergamini and Giongo, 2000c). Cultivars that are resistant to scab and powdery mildew and show good taste characteristics, like ´Golden Mira´ or ´Brina´, with good adaptation to storage conditions (Bergamini and Giongo, 2000d, e) seem also interesting.

3.4.3 Efforts towards the identification of integrated resistance mechanism

It was observed that the reaction of the progeny from a cross between a heterozygous Vf-resistant plant and a susceptible cultivar upon inoculation with *Venturia inaequalis* could not be clearly classified into two discrete classes, susceptible and resistant, but rather in several groups from very susceptible to symptomless (Chevalier *et al.*, 1991; Gessler, 1992). This observation supported the assumption that not only one single gene is involved in the resistance mechanism, but rather a group of more or less closely linked genes. This complex should integrate a major and some minor genes, in which the phenotype from Vf gene is enhanced or augmented by additional genes (Williams and Kuc, 1969; Lamb and Hamilton, 1969; Rousselle *et al.*, 1974; Hough *et al.*, 1988; Gessler, 1992). Rousselle *et al*. (1974) suggested that modifiers of major genes in *Malus* appeared to be inherited independently of major genes and to act in a quantitative manner with a mostly cumulative effect. These minor genes could be transmitted either by the resistant or the susceptible parent. The possible contribute of the susceptible parent to the resistance of apple progenies to scab had also been

suggested by Lamb and Hamilton in 1969, and confirmed by Kellerhals and Meyer (Kellerhals and Meyer, 1994), after the observation that highly-susceptible parents generally induced a lower percentage of resistant seedlings in the progeny.

It was hypothesised that Vf is a simply inherited gene that confers a class 3 reaction type and that additional genes enhance the effect of Vf to give class 1, 2 and M reactions (Hough *et al.*, 1988). Similar interpretation had been made by Williams and Kuc (1969), suggesting that the original level of resistance was due either to a group of rather closely linked quantitative genes or to a class 3 reaction qualitative gene closely linked to one or more quantitative genes.

These findings reinforce the importance of the identification of the resistance complex in order to use it in plant breeding involving engineered transfer to cultivars with commercial interest. The development of tools like molecular markers, linked to the genome segment of interest and, therefore, suitable for rapid and detailed genetic analysis (Gianfranceschi *et al.*, 1994) is an important contribution. They permit mapping genes coding for agronomically important characters, thus increasing the efficiency and reducing the time-scale of plant breeding (King *et al.*, 1991). In breeding against scab disease, the analysis of the progeny plants by marking the corresponding genome segment constituted a very attractive approach towards the identification and early selection of resistant genotypes, less laborious and less time-consuming (Yang *et al.*, 1997a), avoiding the selection based on greenhouse assays of the progenies with different pathogen races. A further advantage is its higher objectivity, since it is independent of the variability due to inoculum used or environmental conditions (Koller *et al.*, 1994; Tartarini, 1996). Additionally, the availability of scab markers tightly linked to physiologically different scab resistance genes would allow their combination in the same genotype in order to achieve a more durable resistance (Tartarini, 1996).

In this view, programs aiming the isolation of the Vf- and Vm-resistance complexes by map-based cloning were established, basing on the identification of molecular markers that co-segregate with the resistance genes in a bulk segregant analysis (Michelmore *et al.*, 1991). The goal was the construction of a linkage map of molecular markers around the resistance *loci*.

One STS (sequence tagged site) marker showing correlation with Vm system has been identified (Cheng *et al.*, 1998). For map-based cloning and isolation of the Vf gene, gene-flanking or closely linked molecular markers, mostly RAPD (random amplified polymorphic DNA) markers (Yang and Krüger, 1994; Koller *et al.*, 1994; Yang and Korban, 1996;

Tartarini, 1996; Yang *et al.*, 1997a,b) were identified as starting points for chromosome walking. One isozyme marker was also identified (Manganaris *et al.*, 1994). Some RAPD markers were converted in more reliable and reproducible markers, like SCAR (sequence characterised amplified region) (Gianfranceschi *et al.*, 1996; Yang *et al.*, 1997b), or CAPS (cleaved amplified polymorphic sequence) (Gianfranceschi *et al.*, 1996). These markers permit the identification of homozygous plants for the Vf gene (Tartarini *et al.*, 2000).

A first map around Vf was constructed with eight genetic markers on both sides of the resistance gene, spreading over approximately 28 cM (Gardiner *et al.*, 1996). Later, the isozyme marker - PGM-1, 17 RAPD markers, 6 RFLP (restriction length fragment polymorphisms) markers, all reported to be associated with the Vf gene, were used to construct a detailed map covering 54 cM of the ´Prima´ linkage group containing the Vf gene (King *et al.*, 1998).

Recently, a BAC (bacterial artificial chromosome) library was constructed from the scab resistant cultivar ´Florina´ (Vinatzer *et al.*, 1998) in order to isolate Vf scab resistance gene through map-based cloning. Screening of this large-insert apple genomic library with a molecular marker closely linked to the Vf gene (Tartarini, 1996) resulted in six positive clones (Vinatzer *et al.*, 1998). A physical mapping of the Vf gene region (Patocchi *et al.*, 1999a) and the construction of a BAC contig spanning the entire Vf *locus* (Patocchi *et al.*, 1999b) were reported. On BAC clones derived from the Vf scab resistance *locus*, a cluster of receptor-like genes with homology to the *Cladosporium fulvum* (Cf) resistance gene family of tomato was identified (Vinatzer *et al.*, 2001). Two BAC clones of the Vf region were subcloned in a BIBAC vector for use in transformation experiments (Tartarini *et al.*, 2000), in order to analyse if any of the single isolated genes is the determinant for Vf-resistance.

Another BAC library was constructed for the scab resistant apple cultivar ´GoldRush´. The library has been screened with 14 PCR-based SCAR markers (Xu *et al.*, 2000) derived from AFLPs (amplification fragment length polymorphisms) tightly linked to the Vf gene (Xu *et al.*, 2001).

Since 1998, the efforts from different research institutes in Europe were integrated in the frame of the european project D.A.R.E. (Durable Apple Resistance in Europe). Aims of this project regarding scab resistance are the characterisation of new apple cultivars carrying durable resistance, the assessment of risks of resistance breakdown related to the appearance of new virulences, the genetic dissection of polygenic resistance taking into account pathogen variability, the development of new breeding strategies and also market study and analysis of

consumer preferences for new resistant cultivars (Lespinasse *et al.*, 2000). An european collection of *Venturia inaequalis* was settled (Lespinasse, 2000). A core-orchard including partial and complete scab resistant cultivars was planted in each participating country, in order to evaluate plant resistance stability and the presence of new scab races. A saturated map for the population ´Prima´x ´Fiesta´ has been constructed and other 4 genetic maps are in progress mainly with SSRs (Simple Sequence Repeats), RFLPs and AFLPs markers (Lespinasse, 2000).

In the search for resistance gene analogues in apple, candidates with high degrees of similarity to major resistance genes or receptor-like kinases from other plants have been identified and they can be used as RFLP probes or CAPS markers for mapping resistance *loci* (Lespinasse *et al.*, 2000).

A major resistance gene to *Venturia inaequalis* race 7 has been identified in the cultivar ´Prima´and has been mapped close to a RFLP marker. This gene is supposed to be the Vg gene from ´Golden Delicious´(Bénaouf and Parisi, 2000), one of the ancestors of ´Prima´, as ´Prima´ and ´Golden Delicious´ showed to be resistant towards some strains that can overcome the Vf resistance, developing the same typical necrotic resistance reactions.

3.4.4 Limitations of this approach and interest of a broad-range mechanism

In spite of the large number of cultivars showing resistance against scab that was obtained by breeding based on introgression of resistance trait through backcrossing, this approach showed some disadvantages. The long duration of the breeding programs and the reduced commercial interest of the cultivars obtained (Parisi and Lespinasse, 1999), when compared with susceptible cultivars like ´Jonagold´, ´Golden Delicious´, ´Gala´ or ´Braeburn´ (Anonyme, 2001) were an important aspect, since fruit trees are species where the cultivar recognition is quite important, opposite to crops. Although some of the obtained resistant cultivars are considered promising (I 3.4.2), fruit quality, evaluated as highest parameters in firmness, juiciness and flavour, precocity and cropping performance (Kellerhals and Meyer, 1994) are goals that would need to be achieved.

Another very important limitation is the apparent erosion of the resistance during successive backcrossing, particularly when very susceptible parents are chosen (Kellerhals *et al.*, 1993). The original *Malus floribunda* 821 resistance was expressed as class 1 reaction. Selections F_2 26829-2-2 and F_2 26830-2 gave a class 2 reaction. In the modified backcross progeny, the resistant reaction classes can range from 2 to M to 3 (Williams and Kuc, 1969), indicating an

increased sensibility in comparison with the resistant parent. This erosion of the resistance trait was even more visible when scab symptoms have been observed in the field on apple cultivar ´Prima´ and some of its seedlings, that had been selected as resistant in the greenhouse, whereas the *Malus floribunda* 821 remained resistant (Parisi *et al.*, 1993). Symptoms of scab had already been observed in the orchards in Ahrensburg on cultivars selected as resistant in the greenhouse, particularly ´Prima´, but these observations were not interpreted as a breakdown on the resistance coded by Vf, but rather as an expression of modifier genes influencing the reaction of Vf-gene of ´Prima´(Krüger, 1991). Although Vf-resistance was considered durable, since Vf cultivars had been free of scab for over 50 years in the different countries where they had been grown, this study was the first demonstration that Vf- derived resistance can be overcome by a race of *Venturia inaequalis* (Parisi *et al.*, 1993). In addition to evidence the importance of using strain mixtures as inoculum during resistance assays, this study emphasised the difference between the genetic background of *Malus floribunda* 821 (resistance not overcome) and that of new selections deriving from it (resistance overcome) (Parisi *et al.*, 1993). It was suggested that *Malus floribunda* 821 contains an additional gene for hypersensitivity, which was lost early in breeding efforts (Parisi and Lespinasse, 1999).

The overcoming of Vm- and Vf-resistance derived mechanisms showed the limitations of the introgression of a race-specific resistance mechanism (Messiaen, 1981) and the importance of diversifying the sources of resistance in new breeding strategies, as the epidemiology and rate of spread of new virulent isolates remains to be determined (Parisi *et al.*, 1993). A possible strategy could be the combined use (pyramiding) in the same cultivar of different resistance genes, deriving from other wild *Malus* sources as well as from old cultivars from *Malus* x *domestica*, that carry functional resistance genes (Lamb and Hamilton, 1969; Gessler, 1994) complemented with appropriate planting strategies. Breeding strategies which associate major resistance genes and polygenes in the same genotype showed already promising results (Lespinasse *et al.*, 2000). Progenies were developed from crosses with parents that carry different resistances (Vf x Vb; Vf x Vm; polygenic resistance x Vf) and tested with scab inoculum as well as evaluated concerning the presence of specific markers (Lespinasse, 2000). However, polygenic resistance (under the control of several genes) is partial and is more difficult to select, particularly as it is influenced by climatic conditions (Evans *et al.*, 2000). Moreover, recents reports showed that scab infection through artificial inoculum in greenhouse is not totally reproducible in field (Sansavini *et al.*, 2000).

Another alternative, as a durable protection system, might consist in an engineered mechanism promoting the inhibition of fungal development, that would be non race-specific and independent of variation in the pathogen. Genes that might impair basic processes of fungal growth could be used in such a mechanism. This approach, outside a gene-for-gene system and equally effective against different races (Vanderplank, 1963), would overcome the limitations of a mechanism based on a dominant oligogenic source of resistance with characteristics of vertical resistance. The level of accumulation of products derived from defense-related genes or the rapidity of the induction of these genes in a host plant has been correlated to the degree of its disease resistance (Anuratha *et al.*, 1996; Lawrence *et al.*, 1996). In this view, pathogen-inducible promoters could play a major role in the control of disease resistance by modifying the regulatory factors for the expression of introduced defense genes.

In this mechanism (I 3.4.5), the protective function of fungus-inhibiting genes would be regulated by a fungus-inducible promoter (I 3.4.5.1). This promoter would recognise the invading pathogen and lead to the activation of the genes conferring impairment of fungal growth. Genes that are involved in metabolic pathways activated during the defense reactions shown by resistant cultivars (I 3.4.5.2.1) or genes that encode proteins acting on an essential component of the cell wall of the fungi (e.g. chitin) (I 3.4.5.2.2) could be regarded as candidate genes.

Although there still remains the possibility of breakdown of protection, this approach could contribute for a more durable defense mechanism than the race-specific resistance, where the resistance gene plays a major role in the initial event of recognition response. The direct introduction of the designed mechanism into apple cultivars could confer natural resistance to these cultivars, while retaining their blend of characteristics.

In a similar view, alternative approaches including the isolation of genes encoding LRR (Leucine-Rich Repeats) proteins that might be involved in resistance against *Venturia inaequalis* and, more in general, fungal pathogens (Komjanc *et al.*, 1999) or the use of genes encoding proteins with antifungal properties isolated from the fungus *Trichoderma harzianum*, frequently used in biocontrol (Bolar *et al.*, 2000), were recently reported. In the former case, one gene was isolated, that is likely to participate in defense-related signalling and is activated in ´Florina´ leaves upon infection with an avirulent strain of *Venturia inaequalis* in a higher level than in the susceptible cultivar ´Golden Delicious´ (Komjanc *et al.*, 1999). In the latter, a fungal endochitinase active against *Venturia inaequalis* and

Gymnosporangium juniperi-virginianae Schwein. was integrated into the apple genome and showed to confer increased resistance to apple scab under the regulation of the 35S promoter (Bolar *et al.*, 2000).

3.4.5 Engineered strategy to impair apple scab development

3.4.5.1 Participation of fungal endopolygalacturonases in pathogenesis and value of *pgip* promoters as fungus-inducible promoters

Endopolygalacturonases (poly(1,4-alfa-D-galacturonide) glycanohydrolase; E.C.2.1.15) are the first detectable enzymes secreted by phytopathogenic fungi when they are grown *in vitro* on plant cell walls (Jones *et al.*, 1972) and are also described to be secreted by phytopathogenic microrganisms on growth initiation (Karr and Albersheim, 1970). These enzymes hydrolyse pectic compounds of the middle lamella and of the primary cell walls, since polygalacturonic acid, their preferential substrate, is the polymer that forms the backbone of the pectin molecule in the intracellular matrix and primary walls (Jarvis, 1984) and, thereby, assist in the colonisation of plant tissues (Cervone *et al*., 1989; Favaron *et al.*, 1994). Endopolygalacturonases do not only supply the fungus with a nutrient source for growth, they also facilitate the ability of other fungus-secreted plant cell wall-degrading enzymes to attack their substrates (Karr and Albersheim, 1970; Cervone *et al.*, 1990). The importance of microbial polygalacturonases in pathogenesis has been established not only in plant diseases characterised by rapid and extensive degradation of host cell walls, but also in some diseases where only a minimal breakdown of cell wall polysaccharides occurs during penetration and colonisation of host tissue, as in the case of diseases caused by biotrophs (Cooper, 1984; Hahn *et al.*, 1989). An endopolygalacturonase from *Venturia inaequalis*, a fungus with a parasitic phase similar to biotrophs (Boone, 1971), is described to participate on the onset of the fungal development (Kollar, 1998) (see I 2.6).

In plant-fungus interactions, fungal endopolygalacturonases are also considered potential avirulence factors, since oligogalacturonides derived from endopolygalacturonase degradation of cell wall pectin have been found to be potent elicitors of the plant defense response (Cervone *et al*., 1989; Cervone *et al.*, 1992). It was suggested that fungal pathogens released some signals that activated one gene encoding a PGIP (polygalacturonase inhibiting protein), and that fungal polygalacturonases could be one of these signals (Yao *et al.*, 1999). PGIPs are extracellular LRR proteins that bind to and inhibit the activity of fungal endopolygalacturonases (Nuss *et al.*, 1996), being described as the primary binding site of

these enzymes to host cell walls (Cervone *et al.*, 1986; Johnston *et al.*, 1994). They have been defined by their inhibition *in vitro* of fungal polygalacturonase activity (Stotz *et al.*, 1993) and are constitutively expressed in low levels in the cell walls of healthy plants (Cervone *et al.*, 1989). Induction of *pgip* mRNA after addition of oligogalacturonides to suspension-culture bean cells was demonstrated, suggesting the higher accumulation of the PGIP in the plant tissue at the onset of the fungal infection, in consequence of the accumulation of oligogalacturonides after the interaction between low levels of fungal endopolygalacturonases with low levels of PGIP present in the plant cell wall (Cervone *et al.*, 1992). Accumulation of PGIP in *Phaseolus vulgaris* L. was found also to be induced by fungal infection, besides by wounding and elicitors (Bergmann *et al.*, 1994). The accumulation of PGIP upon treatment with elicitor (bean) and wounding (bean, soybean) was a consequence of an increased accumulation of *pgip* mRNA (Bergmann *et al.*, 1994; Favaron *et al.*, 1994).

In bean hypocotyls infected with *Colletotrichum lindemuthianum*, the level of PGIP increased upon fungal inoculation, preferentially accumulating in those cells immediately surrounding the infection site. Immunocytochemical experiments revealed that a basal level of PGIP occurs in the cell walls of uninoculated resistant and susceptible *P. vulgaris* plants, and that this constitutive level increases significantly upon fungal attack (Bergmann *et al.*, 1994). However, in contrast with these results, where no indication of a differential increase of PGIP levels in compatible and incompatible race-cultivar combinations was found, a more rapid and intense accumulation of *pgip* mRNA in the incompatible interaction was observed (Nuss *et al.*, 1996; Devoto *et al.*, 1997), while in the compatible interaction, no substantial accumulation of *pgip* mRNA was observed in hypocotyls and only a slight accumulation was observed in leaves during lesion formation (Devoto *et al.*, 1997). Transcriptional accumulation of *pgip* following fungal infection was also observed in hypocotyls of soybean seedlings (Favaron *et al.*, 2000). Opposite to what was described to bean, much higher accumulation of *pgip* transcripts occurred in compatible interaction (Favaron *et al.*, 2000), suggesting the presence of different regulation patterns. In immature and non-stored ripe apples, the *pgip* gene was rapidly upregulated by fungal infections. Increase of *pgip* transcript levels after fungal infection with *Botrytis cinerea* and *Penicillium expansum* was detected in decayed areas and in the tissue adjacent to inoculation sites (Yao *et al.*, 1999).

These results suggest the possibility to use an apple *pgip* promoter as a pathogen-inducible promoter in the regulation of an engineered protection mechanism against apple scab.

3.4.5.2 Putative ´defense´ genes

3.4.5.2.1 Genes involved in defense responses in resistant types

In an approach towards the unravelling of processes participating in the defense mechanism developed by resistant types, the analysis on a molecular level of the events occurring during the interaction between scab and resistant host could provide valuable knowledge concerning the pathways activated in the host defense response. A good candidate for this analysis would be the resistant cultivar ´Florina´.

´Florina´ is a resistant cultivar obtained in France in 1977, that has been widely used in scab resistance studies. It carries the Vf-resistance trait, derived fom *Malus floribunda* 821, resulting from 4th backcrossing with different susceptible cultivars (Fig. 4) and also the Vg-resistance trait (Bénaouf and Parisi, 1997). This cultivar shows no clear hypersensitive response (Komjanc *et al.*, 1999), being its resistance associated with class 0-2, ranging from no symptoms to chlorosis or necrosis without sporulation (Ivanicka *et al.*, 1996).

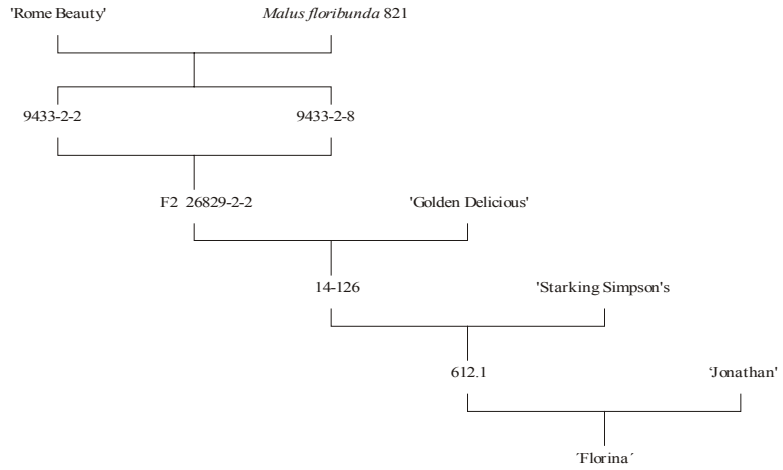


Fig. 4: Obtention of resistant cultivar ´Florina´ (Sansavini, 1993).

In a plant-fungus interaction, differential gene expression occurs in both organisms from the attempted fungal invasion onwards, since the establishment of the infection results from changes in the pathogen (activation of mechanisms for penetration and colonisation of the host tissues) and in the host (activation of plant defense responses). Pattern changes in the fungus may be identified by comparing the expression pattern from the fungus *in planta* with the expression pattern from the fungus cultivated *in vitro*. Plant genes regulated in response to the pathogen may be identified through the comparison with the expression pattern of healthy plants or plants infected with non-related pathogens (Benito *et al.*, 1996). The technique of

DDRT-PCR (differential display reverse transcription-PCR) would be a possible tool to analyse this interaction.

DDRT-PCR enables to display both qualitative and quantitative differences of gene expression in different cell stages or tissues by comparison of their cDNA pattern (Liang and Pardee, 1992). A common application is to identify genes that are regulated during differentiation or development or to isolate genes involved in signal cascades. It can also be used to study cell and life cycles, in order to identify genes which allow the cell or the organism to inhabit different environments or initiate pathogenesis (Sturtevant, 2000). In the context of plant-microbe interaction, this technique was used to analyse symbiotic (Vögeli-Lange *et al.*, 1996; Martin-Laurent *et al.*, 1997; Lapopin *et al.*, 1999) and parasitic interactions. In plant pathology it was first used to isolate genes participating in the interaction between tomato and the fungus *Botrytis cinerea* (Benito *et al.*, 1996). Several other interactions involving plant-fungus (Truesdell and Dickman, 1997; Munoz and Bailey, 1998; Mazeyrat *et al.*, 1998; Yi and Hwang, 1998; Collinge and Boller, 2001), plant-bacteria (Seehaus and Tenhaken, 1998), plant-insect (Hermsmeier *et al.*, 2001), plant-virus (Geri *et al.*, 1999; Chen and Chen, 2000), plant-phytoplasma (Jagoueix-Eveillard *et al.*, 2001) and plant-nematode (Hermesmeier *et al.*, 1998; Hermesmeier *et al.*, 2000; Vercauteren *et al.*, 2001) were investigated by differential display. These results indicate the potential interest of the application of DDRT-PCR to analyse the gene expression pattern in resistant cultivar ´Florina´ upon fungal inoculation, in order to identify host genes that are activated in response to fungal invasion.

3.4.5.2.2 Genes encoding enzymes degrading fungal cell walls

Enzymes that degrade fungal cell walls like chitinases and ß-1,3-glucanases are suggested to be part of the inducible defense response of higher plants elicited by plant-pathogen interactions and a hypersensitive response (Kim *et al.*, 1998), and might be considered interesting as resistance factors. Particularly, in a strategy for breeding against apple scab, plant chitinases appear as good candidates, as they degrade chitin, one of the most important components of the fungal cell wall of several fungi, including ascomycetes.

Chitinases are commonly found in a wide range of organisms including bacteria, fungi, higher plants, insects, crustaceans and some vertebrates (Melchers *et al.*, 1994). Chitinases (E.C 3.2.1.14) are glycanohydrolases that catalyse the hydrolysis of chitin, a ß-1,4-linked homopolymer of N-acetyl-*D*-glucosamine, which is a major component of the cell walls of

many fungi but does not exist in plants (Chang *et al.*, 1995). Although plants lack chitin, several chitinase isoforms have been characterised in a large variety of plant species including monocotyledonous and dicotyledonous species (Derckel *et al.*, 1996). Their expression is regulated in a developmental and also organ-specific pattern, but stress conditions such as fungal, viral or bacterial challenge, elicitor treatment or exposure to ethylene also induce their expression (Boller, 1988; Derckel *et al.*, 1996; Busam *et al.*, 1997).

Six classes of plant chitinases have been described based on structural and functional properties as percent identity, characteristics of N-terminal or C-terminal amino acid sequences, specific domains or cellular localisation (Chang *et al.*, 1995). Class I chitinases are usually basic proteins localised in the vacuole. They contain a cysteine-rich N-terminal domain with chitin-binding properties which is connected to the catalytic domain via a proline-rich hinge region, and a short C-terminal hydrophobic domain providing the information for targeting to the vacuole (Neuhaus *et al.*, 1991a; Büchter *et al.*, 1997). Tobacco class I chitinases inhibit the growth of many fungi *in vitro* by causing lysis of hyphal tips (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Class II chitinases share highly conserved catalytic domains with class I chitinases, but they are usually acidic, extracellular proteins which lack the cysteine-rich domain and the C-terminal targeting signal (Büchter *et al.*, 1997; Busam *et al.*, 1997). Although they were considered as not having antifungal activity by themselves (Sela-Buurlage *et al.*, 1993; Melchers *et al.*, 1994), antifungal activity of a barley or tomato class II endochitinases was already observed (Andersen *et al.*, 1997; Tabaeizadeh *et al.*, 1999; Oldach *et al.*, 2001). Class III chitinases are also extracellular proteins but lack sequence similarity to the class I and II enzymes and are therefore grouped into a separate family of PR (pathogenesis-related) proteins, the PR8 family (van den Bulcke *et al.*, 1989; van Loon *et al.*, 1994; Neuhaus *et al.*, 1996). They represent bifunctional, lysozymal enzymes (Metraux *et al.*, 1989), but it is not known whether they exhibit fungal growth-inhibiting activities *in vitro* (Melchers *et al.*, 1994). However, a chitinase from *Rhizopus oligosporus* conferring antifungal activity to transgenic tobacco, was found to be similar to class III chitinases (Terakawa *et al.*, 1997). Class IV chitinases resemble class I chitinases as they have a cysteine-rich domain, a hinge region and a related catalytic domain, but due to a few gaps they are smaller proteins. Class V is characterised by the duplication of the N-terminal chitin-binding domain (Robert *et al*., 2002). Class VI is represented by two proteins from tobacco with rather low chitinase activity that are unrelated to other plant

chitinases but share sequence similarity to bacterial exochitinases (Melchers *et al.*, 1994; Robert *et al*., 2002).

Considerable interest in chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing organisms, such as insects, nematodes and fungi (Cohen, 1987). Resistance to undesired organisms can derive from degradation of vital structures, as the cell wall in fungi, or by release of chitin oligomers from fungal pathogens, active elicitors that subsequently induce other type of defensive responses by the host (Kurosaki *et al.*, 1988; Ren and West, 1992; Uknes *et al.*, 1993; Nishizawa *et al.*, 1999a).

Since chitinase activity in plants drastically increases after infection, it is assumed that chitinases are involved in protecting plants against fungal pathogens (Mauch *et al.*, 1984), and that enhanced chitinase activity is functionally implicated in the defense response directed towards chitin, as a major cell wall component of most fungi (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). Furthermore, in many plant-fungus interactions, chitinase enzymatic activity, protein and mRNA levels have been reported to be higher in resistant cultivars and at early stages after inoculation than in susceptible cultivars (Rasmussen *et al.*, 1992; Collinge *et al.*, 1993).

Evidence supporting an antifungal role for plant chitinases comes from studies showing the ability of different chitinases to inhibit growth of fungal hyphae *in vitro* alone (Collinge *et al.*, 1993; Melchers *et al.*, 1994; Derckel *et al.*, 1998), or in combination with ß-1,3-glucanases (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988; Broekaert *et al.*, 1988; Leah *et al.*, 1991; Sela-Buurlage *et al.*, 1993; Gomes *et al.*, 1996). These data were further supported by results showing that enhanced resistance against certain fungal pathogens derived from the chitinase expression in transgenic plants (Broglie *et al.*, 1991; Logemann *et al.*, 1993; Lin *et al.*, 1995; Grison *et al.*, 1996; Asao *et al.*, 1997; Tabei *et al.*, 1998; Tabaeizadeh *et al.*, 1999; Nishizawa *et al.*, 1999b; Yamamoto *et al.*, 2000; Datta *et al.*, 2001; Waniska *et al.*, 2001; Rohini and Sankara, 2001), although contrary results were also reported (Neuhaus *et al.*, 1991b; Nielsen *et al.*, 1993). Enhanced resistance to fungal pathogens has also been demonstrated in transgenic plants overexpressing chitinases and ß-1,3-glucanases, with a synergistic benefit where both genes are present (Zhu *et al.*, 1994; Jach *et al.*, 1995; Jongedijk *et al.*, 1995), maybe derived from synergistic effect of hydrolases on the pathogen cell wall. In a similar approach, genes encoding chitinases isolated from fungi (Terakawa *et al.*, 1997; Bolar *et al.*, 2000) and bacteria (Jones, 1988; Suslow *et al.*, 1988) have been introduced in plants by

genetic engineering and the transformants showed higher resistance against some fungal pathogens.

The extracellular deposition of chitinases may have the advantage that they make an earlier contact with invading microrganisms, and can therefore be effective in defending plant cells from the early features of invasion by pathogens (Ishige *et al.*, 1993). Thus, acidic chitinases, putatively secreted to the cell wall region should be more effective than basic chitinases, accumulating in the vacuole (van den Bulcke *et al.*, 1989), in the defense reaction.

Due to the characteristic growth pattern of apple scab during its parasitic phase, remaining limited to the extracellular space between cuticle and epidermal cells of the host, the extracellular deposition of enzymes degrading the fungal cell wall could constitute a further benefit.

II OBJECTIVES

In apple scab disease, the growth of the mycelium of *Venturia inaequalis* is confined to the subcuticular space, where it retrieves its nutrition from the underlying epidermal cells without forming intracellular haustoria. Genes encoding proteins which are expressed extracellularly and participate in the degradation of fungal cell wall, as well as genes involved in defense responses occurring in resistant genotypes, might contribute to the impairment of fungal growth and, thus, represent promising candidates for resistance breeding using gene transfer technology. In addition the use of pathogen-inducible promoters, which are activated during the early stages of the infection process, could confer a further benefit in the regulation of introduced ‘defense‘ genes.
In this view, the aims of this work were:

1. To establish a scab-infection system under controlled conditions

In order to analyse molecular mechanisms that are involved in the interaction between apple and apple scab fungus, the establishment of a functional scab-infection system under controlled conditions was a prerequisite.
Conditions that allow maintenance and sporulation of scab isolates in *in vitro* culture were investigated, in order to obtain sporulating isolates available for artificial infections. To evaluate the efficiency of the artificial inoculation and the virulence of a conidial suspension used as inoculum, infection assays with different susceptible apple cultivars were performed with *in vitro* and *in vivo* plant material.
For infections *in planta*, it was necessary to assure that no other pathogens were present, not only to avoid pesticide treatment, but also to ensure that the responses observed in the host plants derived from the interaction with *Venturia inaequalis*. To accomplish this prerequisite, a system was developed that allows the preservation of apple plants under low phytopathogenic pressure throughout the growing season.

2. To isolate a fungus-inducible promoter

This task comprised the isolation of apple *pgip*-promoters from an apple genomic library and the investigation of the inducibility of *pgip* genes upon fungal elicitation. For this purpose, the characteristics of isolated *pgip*-promoters fused with the reporter gene GUS, as well as their inducibility upon fungal elicitation had to be investigated in transgenic tobacco plants.

3. To explore putative ´defense´ genes

Two strategies were developed towards the identification of genes that could be used in an engineered protection mechanism: the identification of different genes activated during the interaction between a resistant host and apple scab, and the isolation of genes encoding proteins which are expressed extracellularly and are involved in the degradation of fungal cell wall.

To gain knowledge about the defense responses developed by scab-resistant cultivars upon fungal attack, differential display reverse-transcription PCR (DDRT-PCR) in artificially inoculated ´Florina´ plants was carried out in order to isolate apple genes with differential expression during fungal interaction. Confirmation of differential expression of the isolated cDNAs upon scab infection was performed by RT-PCR and Northern analyses, prior to the identification of full-length clones.

In another approach an apple genomic library was screened for class III chitinase genes and further, the expression pattern of this gene family was investigated in apple. In order to evaluate the antifungal characteristics of the chitinases encoded by the isolated genes, a construct comprising the class III chitinase gene under the control of a constitutive promoter has been generated for overexpression in transgenic apples. Conditions contributing to a successful apple leaf discs transformation with *Agrobacterium tumefaciens* were tried to work out.

III MATERIAL AND METHODS

1. Plant material and tissue culture

1.1 Culture media

The different culture media were based on Murashige and Skoog (MS) medium as shown in Table 4. Media used were either liquid or solidified with agar. Composition of MS is referred in Appendix 1. Culture media were sterilised by autoclaving 20min at 120°C.

Antibiotic stock solutions from kanamycin (50mg/ml) and cefotaxime (62.5mg/ml) were prepared by dissolving the antibiotic in A. dest. and filter sterilising (0.22µm pore). Antibiotics were added to the culture media after autoclaving.

Table 4: Plant tissue culture media.

Medium designation	Reference	Macronutrients	Saccharose (g/l)	Hormones (mg/l)	Agar (g/l)	pH
MS0	Murashige and Skoog, 1962	MS	20	-	8	5.7
52G	Laimer *et al.*, 1988	MS	20	0.36 BAP 0.03 IBA	8	5.7
EC3	Laimer *et al.*, unpublished	¼ MS	15	0.3 IBA	-	5.7
46A	Laimer *et al.*, unpublished	½ MS	20	-	-	5.7
MSreg	da Câmara Machado *et al.*, 1992	MS	20	0.8 BAP 0.1 IBA	8	5.7
TE3	da Câmara Machado *et al.*, 1995	MS	20	2.0 TDZ 3.0 IAA	8	5.7
J3	da Câmara Machado *et al.*, 1995	MS	20	3.0 BAP 3.0 IAA	8	5.7

1.2 *In vitro* plant material

All *in vitro* cultures were grown under sterile conditions at 25°C with a 16h photoperiod and a light intensity of 50µM$m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

Non-transgenic *in vitro* shoot cultures of *Nicotiana tabacum* cultivar ´Samsun´ NN were kept on MS0 medium and subcultured every 6-8 weeks.

Non-transgenic *in vitro* shoot cultures of apple cultivars ´Maschanzker´, ´Elstar´, ´Golden Delicious´, ´Royal Gala´, ´McIntosh´, ´Jonagold´ were maintained on shoot propagation medium 52G and subcultured every 6-8 weeks.

For root induction, apple shoots were transferred to EC3 and incubated 7-10 days in the dark at 25°C, and subsequently transferred to a glass jar with sterile perlite and 46A for root formation. Shoots were kept at this stage for 4-6 weeks at 16h light/day.

1.3 *In vivo* plant material

1.3.1 *Nicotiana tabacum*

3-4 weeks after the last subculture, rooted plantlets of *Nicotiana tabacum* were acclimatised and grown in the greenhouse at 22°C with 16h light/day. To prevent wilting plants were covered with a plastic bag. When plants started to form new leaves, bags were removed.

1.3.2. *Malus* x *domestica*

Apple plants were grown under controlled conditions, i.e. under low pathogen pressure although omitting pesticide spraying. For this purpose, two boxes were designed and constructed (Fig. 5a). The boxes had entrances for water and filtered air (filter:1µm; flow: 15l/min) and an air exit. The system was kept under slight overpressure to minimise the risk of pathogen entrance during operation. The boxes were kept in a climatised compartment at 20°C with 14h light/day.

Rooted *in vitro* plantlets of apple cultivars ´Golden Delicious´ and ´Jonagold´ were transferred to sterile soil in autoclaved ceramic pots in a laminar flow hood and acclimatised to the greenhouse as described in III 1.3.1 (Fig. 5b). After first leaves formed in the greenhouse, the plastic covers were removed and plants were further grown in the boxes (Fig. 5c). Scions from ´Florina´ trees were grafted on rootstock ´Bittenfelder´ and grown under the described conditions.

Fig. 5: Box (a) where plants were acclimatised (b) and kept during the growing season (c).

1.4 Leaf discs transformation with *Agrobacterium tumefaciens*

1.4.1 *Nicotiana tabacum*

Leaves, excised from *in vitro* propagated shoots 4 weeks after the last subculture, were cut 3 or 4 times across the midrib and the leaf discs were placed with their abaxial side onto regeneration medium MSreg (see Table 4). Leaf discs were incubated for 24h at 23°C in the dark before transformation.

Liquid cultures of *Agrobacterium tumefaciens* carrying the desired plasmid were grown overnight in LB medium (see Appendix 1) supplemented with 250µg/ml streptomycin and 50µg/ml kanamycin at 28°C. The overnight bacterial cultures were diluted in fresh medium supplemented with antibiotics and further grown to an OD_{600nm} of 0.4-0.6. For the transformation, bacterial cultures were diluted 1:50 in a sterile solution of 3% saccharose. Leaf discs were immersed in the bacterial suspension for a few seconds, blotted on a sterile paper towel to remove excess liquid and placed on MSreg. After 48h of cocultivation at 23°C either in the dark or with 16h light/day, leaf discs were transferred to MSreg supplemented with 250µg/ml cefotaxime for Agrobacteria elimination, and 100µg/ml kanamycin for selection of transformed cells. Explants were kept under the same light regime for 3 more weeks. After this period, all explants were submitted to a regime of 16h photoperiod. Regenerated shoots were subcultured in MS0 (see Table 4) supplemented with 100µg/ml kanamycin.

Tobacco leaf discs transformation was carried out with *Agrobacterium tumefaciens* LBA4404 harbouring the different *pgip*::GUS constructs.

1.4.2 *Malus* x *domestica*

Leaves from 6 weeks old *in vitro* propagated shoots of cultivar ´Jonagold´ were used for one transformation experiment with *Agrobacterium tumefaciens* EHA105 harbouring the *CaMV 35S*::Chitinase construct. A control regeneration experiment was performed in parallel with the transformation assay and incubated in similar conditions.

The 4-5 youngest leaves were excised, cut 3-4 times across the midrib and placed with the adaxial side onto regeneration medium (J3 or TE3, see Table 4) for 24h at 23°C in the dark before transformation. Bacterial culture grown overnight as described in III 1.4.1 was diluted in fresh LB supplemented with antibiotics and 20µM acetosyringone and further grown to an OD_{600nm} of ~0.6. Bacterial culture was diluted 1:50 in sterile 3% saccharose. Leaf discs were immersed in this suspension, blotted dry on a sterile paper towel and placed on the

regeneration medium. Explants were cocultivated with Agrobacteria during 48h in the dark at 23°C. After cocultivation, leaf discs were transferred to regeneration medium supplemented with 250µg/ml cefotaxime, incubated 10 days in the dark, and subsequently subjected to a regime of 16h light/day at 23°C. After 4 weeks infected leaf discs were passaged to fresh regeneration medium supplemented with 250µg/ml cefotaxime and 100µg/ml kanamycin. Passage to fresh regeneration medium supplemented with both antibiotics was repeated every 4 weeks. Formed shoots in the regeneration experiment were transferred to shoot propagation medium 52G (see Table 4).

1.5 Germination of seeds from transgenic *Nicotiana tabacum* (Moser, 1997)

Tobacco seeds were surface-sterilised by washing them 1 x 1min in 70%EtOH and 2 x 5min in 20%Danclor (1% active Cl^-). The last washing step was performed under a laminar flow hood. Seeds were washed 6 x 1min with sterile A. dest. and treated with 0.1mg/ml GA_3 overnight at RT. Seeds were plated under sterile conditions on cotton+filter paper soaked with a 350µg/ml kanamycin solution and incubated at 25°C with 16h photoperiod. Germinated green plantlets were transferred to MS0 (see Table 4) supplemented with 100µg/ml kanamycin and clonally propagated *in vitro* on this medium.

2. Work with fungi

2.1 Fungal isolates and culture of fungi

Isolates of *Venturia inaequalis* were kindly provided by Eng. Teixeira de Sousa from the collection of ENFVN (Portugal). Isolates 6, 8 and 19 were obtained from diseased leaves of distinct apple cultivars collected in orchards in the north and west region of Portugal. The isolate MA652 of *Botrytis cinerea* was kindly provided by Prof. Dr. Prillinger from the collection of the IAM (Austria).

Isolates of *Venturia inaequalis* were grown and maintained in Potato Dextrose Agar (PDA), malt extract agar (MEA) or in a modified malt extract agar (MEAm), without tryptone and supplemented with 0.24% $MgSO_4$ and 0.68% KH_2PO_4 to buffer the medium (Palmiter, 1934). Composition of the different media is shown in Table 5. All the media were sterilised by autoclaving at 120°C for 20min. Scab isolates were cultivated at 20°C, according to Kollar (1994) with 14h photoperiod. The isolate of *Botrytis cinerea* was grown and maintained in PDA at 23°C with 16h light/day.

Table 5: Culture media for growth and maintenance of fungi.

PDA	39g Potato dextrose agar/ 1000ml A. dest.; pH6.0
MEA	30g Malt extract, 1.5g tryptone, 15g agar/ 1000ml A. dest.; pH6.3
MEAm	25g Malt extract, 4.9g $MgSO_4.7H_2O$, 6.8g KH_2PO_4, 15g agar / 1000ml A. dest.; pH5.0

2.2 Infection experiments with apple scab

2.2.1 Preparation of a conidial suspension for infection assays

Fungal mycelia from sporulating colonies (approximately 6 cm in diameter) were homogenised in a common kitchen blender (Severin) with sterile A. dest.. The mycelial extract was slowly shaked at 23°C for 24h, to enable the release of spores into the liquid phase and subsequently filtered under sterile conditions. Filtration was done through cotton, a nylon mesh, 3 layers of Miracloth tissue (Calbiochem) or a filter paper (Schleicher & Schuell 597). Spores in the filtrate were collected by centrifugation at 4200rpm 12min at RT and resuspended in sterile A. dest.. The titer of the suspension was evaluated by microscopical observation and adjusted to 200-300 conidia/µl for artificial infections.

2.2.2 Inoculation of plant material with apple scab

2.2.2.1 Infection of detached leaves

Leaves from *in vitro* propagated shoots of cultivars ´Maschanzker´, ´Elstar´, ´Golden Delicious´, ´Royal Gala´, ´McIntosh´ and ´Jonagold´ were incubated on ½MS, ½MSm, 1%WA, 0.6%WA (see Table 6) or on a moist filter paper, with the abaxial side in contact with the medium. Conidial suspension was pipetted (1-2µl) or sprayed with a small glass sprayer (Desaga NS14.5/23) under sterile conditions. Leaf explants were incubated at 20°C with 14h photoperiod during 8 weeks.

Table 6: Incubation media for assays with detached leaves.

Medium designation	Macronutrients	Micronutrients	Vitamins	Saccharose (g/l)	Agar (g/l)	pH
½ MS	½ MS	MS	MS	20	6	5.7
½ MSm	½ MS	MS	MS except myo-inositol	-	6	5.7
1% WA	-	-	-	-	10	-
0.6% WA	-	-	-	-	6	-

Leaves from apple plants of cultivars ´Jonagold´ and ´Golden Delicious´ grown in the greenhouse were surface-desinfected by the following procedure: a) incubation 2 x 5min in desinfection solution (0.25% active Cl^-, 0.1%Tween20) with gentle agitation, b) rinse in sterile A. dest., c) incubation 3 x 10min in sterile A. dest. to remove any traces of desinfecting compounds that could inhibit scab growth. Scab inoculation of surface-desinfected leaves, placed with the abaxial side in contact with the medium ½MSm (see Table 6), was performed under sterile conditions by spraying the conidial suspension. Infected leaf explants were incubated for 6 weeks as described for *in vitro* material.

For the analysis of *pgip* expression, similar infection assays were carried out with detached leaves from plants grown in the boxes. Mock infections were performed with sterile A. dest.. Leaves were collected at different time points, shock-frozen with liquid nitrogen and kept at –80°C, until extraction of total RNA was performed.

2.2.2.2 Infections *in planta*

Plants of cultivars ´Jonagold´ and ´Florina´ grown under controlled low-pathogen conditions (see III 1.3.2) were used. Scab inoculations were carried out by spraying the conidial

suspension on the four apical, fully unfolded leaves of each plant. Plants were kept in a plastic tunnel (Fig. 6) for 8 weeks at 22-24°C with high humidity and 16h light/day.

Fig. 6: Plastic tunnel where infected plants were maintained.

Scab development at the early stages of infection was assessed by microscopical observation of samples taken from susceptible cultivar ´Jonagold´. Progressive disease development was observed macroscopically.

For gene expression analysis in the cultivar ´Florina´, parallel with scab inoculation, mock infections with sterile A. dest. were performed. Leaf material from infected and mock-infected ´Florina´ plants was collected 11dpi, immediately shock-frozen in liquid nitrogen and kept at –80°C until extraction of total RNA was performed.

2.2.3 Staining of lesions for microscopical examination (Preece, 1959)

Green pigments of infected leaves were extracted with cold methanol and leaf material was rinsed in A. dest. before staining of the lesions. Leaf tissue was immersed 5min in 1% periodic acid, washed 5min in A. dest., immersed 5min in decolorised basic fuchsin, washed 5min in sulphurous acid solution and finally immersed 5min in A. dest.. The composition of the different solutions is shown in Appendix 2. The tissue was mounted in A. dest. or 10%glycerol for examination with a Leitz Diaplan light microscope. The fungus is visible in a bright red, while the host tissue remains colourless.

2.3 Production of supernatants from *Botrytis cinerea*

The composition of the different liquid media used for cultivation of *Botrytis cinerea* is shown in Table 7. Media differed mainly in salt and vitamin composition and carbon sources.

Composition of basal MSf, Czapek-Dox and Visser solutions is presented in Appendix 1. All the media prepared were sterilised by autoclaving 20min at 120°C.

Table 7: Culture media used in liquid cultures of *Botrytis cinerea*.

Medium designation	Salts and vitamins	Carbon source	pH
MSfpH5.0	MSf	2% saccharose	5.0
MSfpH5.7	MSf	2% saccharose	5.7
A	Czapek-Dox	1% glucose + 1% pectin non-esterified	5.0
B	Czapek-Dox	1% glucose + 1% PGA	5.0
D	Visser	1% glucose + 1% PGA	5.0
G	Czapek-Dox	0.5% PGA	5.0
H	Czapek-Dox	0.5% pectin esterified	4.5
I	Czapek-Dox	2% GA	5.3
J	Czapek-Dox	2% glucose	4.8
L	Czapek-Dox	2% tryptone	5.0

25ml cultures with the different media were inoculated with mycelial discs cut from the margins of 8 days old colonies of *Botrytis cinerea* and incubated at 23°C with gentle agitation and 16h photoperiod. Supernatants were harvested after 3 days for culture H, J, L, after 4 days for culture B, D, after 5 days for culture G, I and after 7 days for culture A, MSfpH5.0, MSfpH5.7, filtered through filter paper and assayed regarding their polygalacturonase activity (III 4.1).

25ml cultures with MSfpH5.7 or MAMm (Appendix 1) were inoculated with *Botrytis cinerea* and incubated for 7 days as described above. After this period, the culture was supplemented with 25ml of fresh medium and further incubated for 7 days. Supernatants were filtered through filter paper and used in elicitation assays with tobacco explants.

3. Molecular biology work

3.1 Solutions and reagents used in molecular biology

All chemicals used for the preparation of buffers and gel solutions were purchased from Merck or Sigma if not specified elsewhere. PCR chemicals and dNTPs were purchased from Promega, primers from GibcoBRL and restriction endonucleases and other enzymes used in DNA modifications were purchased from MBI. Composition of buffers and solutions is listed in Appendix 2.

3.2 Bacterial strains

E. coli	KW251 LE392	used as host for bacteriophage λ
	DH5α INVαF´	used for plasmid transformation, permit α-complementation with the N-terminus of ß-galactosidase encoded in pBluescript and pCRII vectors by the *lac*Zgene
A. tumefaciens	LBA4404 EHA105	used for binary plasmid transformation and plant transformation

3.3 Vectors

pBluescript II KS Phagmid	2961bp	Stratagene	Amp^r	contain inducible *lac* promoter and the *lacZ*gene interrupted by the multiple cloning site thus providing α-complementation for blue/white color selection of recombinant phagmids
pCRII.1/pCR2.1	3948bp	Invitrogen	Amp^r Kan^r	
pBSGUS	5293bp	Pühringer *et al.*, 2000	Amp^r	modified Bluescript vector containing the *uid*A gene encoding the ß-glucuronidase (GUS) of *E. coli* and a poly(A) termination sequence from CaMV(Cauliflower Mosaic Virus). The GUS gene has a 172bp intron from the potato ST-LS1 gene, starting at position 338, so that the bacteria cannot translate the gene into a functional enzyme (Vancanneyt *et al*.,1990)
pRT104	3340bp	Töpfer *et al.*, 1987	Amp^r	carries the 35S promoter and the polyadenylation signal of CaMV
pBIN19	11777bp	Bevan, 1984	Kan^r	binary vector used for transfer of constructs into plants by *Agrobacterium*-mediated transformation

3.4 Primers used

Lyophilised primers were dissolved in TE pH8.0 to a 1mM stock concentration and 10µM or 25µM (in case of degenerate primers) working dilutions were prepared with sterile A. dest.. The indicated melting temperature (Tm) was calculated considering 2ºC for each A or T and 4ºC for G or C. For degenerate primers, minimal melting temperature was estimated considering the lower temperature of the wobbling nucleotide.

3.4.1 Vector primers

Primer designation	Vector	Length	Sequence	**Tm (°C)**
T3SEQ	pBS/pCRII.1	21 nt	5′ ACTAAAGGGAACAAAAGCTGG 3′	60
T7SEQ	pBS/pCRII.1	21 nt	5′ CGTAATACGACTCACTATAGG 3′	60
M13pUC-48rev	pBS/pCRII.1	24 nt	5′ CGCCAGGGTTTTCCCAGTCACGAC 3′	78
M13pUC-47	pBS/pCRII.1	24 nt	5′ AGCGGATAACAATTTCACACAGGA 3′	68
pCRII(-39-48)	pCRII.1	20 nt	5′ CTCTAGATGCATGCTCGAGC 3′	62
JH029	pRT104	24 nt	5′ CACTATCCTTCGCAAGACCCTTCC 3′	74
JH030	pRT104	24 nt	5′ CATGAGCGAAACCCTATAAGAACCC 3′	74

3.4.2 Anchor primers for reverse-transcription

oligo(dT) anchor primer	Length	Sequence
dT_{GG}	13 nt	5′ TTTTTTTTTTGG 3′
dT_{AG}	13 nt	5′ TTTTTTTTTTAG 3′
dT_{CC}	13 nt	5′ TTTTTTTTTTCC 3′
dT_{VN}	13 nt	5′ TTTTTTTTTT(G/C/A)(G/C/T/A) 3′

3.4.3 10-mer random primers used in differential display

Primer designation	Length	Sequence	Primer designation	Length	Sequence
DD1	10 nt	5′ TACAACGAGG 3′	DD6	10 nt	5′ AAACTCCGTC 3′
DD2	10 nt	5′ TGGATTGGTC 3′	DD7	10 nt	5′ TCGATACAGG 3′
DD3	10 nt	5′ CTTTCTACCC 3′	DD8	10 nt	5′ TGGTAAAGGG 3′
DD4	10 nt	5′ TTTTGGCTCC 3′	DD9	10 nt	5′ TCGGTCATAG 3′
DD5	10 nt	5′ GGAACCAATC 3′	DD10	10 nt	5′ GGTACTAAGG 3′

3.4.4 *pgip* primers

Primer designation	Length	Sequence	Tm (°C)
5´PGIP deg	22 nt	5´ G(T/C)AA(T/C)CC(T/C/A/G)GA(T/C)GA(T/C)AA(A/G)AA (A/G)GT 3´	56
Int PGIP deg	20 nt	5´(G/T)T(C/T/G/A)A(G/A)(C/T)TT(C/T/G/A)GC(T/G/A)AT(C/T/G/A)GC (C/T/G/A)GG 3´	52
PGIP-F	21 nt	5´ ATGGACGTCAAGTTCCCCACC 3´	66
PGIP-R	21 nt	5´ TTTGCAGCTTGGGAGTGGAGC 3´	66
PGIP0397-R	18 nt	5´ ATGAGGTCAAGACGTAGG 3´	54
PGIP0397-F	19 nt	5´ TACAGGTCATATTCCGAAG 3´	54
PGIP0998-R	19 nt	5´ GCCAGTGAGATTGGGTTGC 3´	60
MDPGIP-F	19 nt	5´ CAACCCGCCATTGCCAAGC 3´	62
MDPGIP-PRO1	23 nt	5´ ATTCTTTGTATAACATAATCTGG 3´	58
MDPGIP-PRO2	20 nt	5´ TCAGCCCAAGGATCTACTGG 3´	62
MDPGIP-PRO3	22 nt	5´ TATACGTGCAATTCTCTCTACG 3´	62
MDPGIP-PRO4	23 nt	5´ TTGCAAGTACTACCAGATTTAGG 3´	64

3.4.5 *chitinase* primers

Primer designation	Length	Sequence	Tm (°C)
5´CHI	24 nt	5´ A(T/C)TGGGG(C/A/T)CAAAA(C/T)GG(C/A/G/T)(A/G)(A/G)(T/C/A) GA(A/G)G 3´	66
3´CHI	26 nt	5´ TT(G/A)TA(G/A)AA(C/T)TG(C/A)A(T/C)CCA(C/G/A)ACATA(A/G)TC 3´	62
CHI0200-R	23 nt	5´ AGAGTA(T/G)CTCCCAGAAGCTCCTC 3´	70
CHI0300-F	21 nt	5´ CAGA(A/G)ATCA(A/G)GTCATGCCAAG 3´	66
CHI2300-F	20 nt	5´ AGCAGTGGACTTCAGCCATC 3´	62
C1-F	30 nt	5´ NNNGAATTCATGGCTCCAAAGTCCACAATG 3´	62*
C1-R	30 nt	5´ NNNGGATCCCTATAGAAGAGCTCGATCATC 3´	60*
C5-F	26 nt	5´ GAATTCATGGCTTCAAAGTCCACAGC 3´	60*
C5-R	22 nt	5´ CTAGACATCATTCTTGATGGAG 3´	62
C20-F	21 nt	5´ ATGGCT(T/C)CAAAGTCCACAATG 3´	60

* For primers C1-F, C1-R and C5-F, comprising a restriction site at the 5´end, Tm was calculated considering only the sequence homologous to the chitinase genes C1 or C5, respectively.

3.4.6 Specific primers for differential display fragments

Primer designation	Length	Sequence	Tm (°C)
M2-F	20 nt	5′ CTCCTCGGGTTTCATTCACG 3′	62
M2-R	20 nt	5′ GGTGACTTGGTGATCGAGAC 3′	60
M2-5RACE	28 nt	5′ TCGCTGTACGTGAATGAAACCCGAGGAG 3′	74*
M2-3RACE	28 nt	5′ CTTGAGGAAGAGTCAACGCCGGAGTCAG 3′	74*
M3-F	20 nt	5′ AATTCTTGGTGGGTGGCCTC 3′	62
M3-R	20 nt	5′ TCAAAGGTGGAAGGATGACG 3′	60
M3-5RACE	28 nt	5′ AATCCTTCCGAACCCACAAACCCATTCC 3′	74*
M4-F	20 nt	5′ CAGCAGCACCTTCATTCGAC 3′	62
M4-R	21 nt	5′ CTCAACATCAGCAGCCCATTG 3′	64
M4-5RACE	28 nt	5′ ACTTGCCCAACAGGCACACCTTGGTACA 3′	74*
M6-F	20 nt	5′ ACAGGAGGTGGAACCATGTG 3′	62
M6-R	22 nt	5′ GTTTCCGACAATGACAGATTTC 3′	62
M6-5RACE	28 nt	5′ GAACAAAAGCCAGGCACACAACCAATGC 3′	74*
M6-3RACE	28 nt	5′ TTGGTTGTGTGCCTGGCTTTTGTTCCTC 3′	74*
M9-F	20 nt	5′ CGTCCATCTTTACCCGTTCC 3′	62
M9-R	20 nt	5′ GATTTGCTCTAACCGCGAAG 3′	60
M9-5RACE	28 nt	5′ TTGATTCGCACCCACAGCAGGGAGAATA 3′	74*
M9-3RACE	28 nt	5′ ATTCTCCCTGCTGTGGGTGCGAATCAAT 3′	74*
M10-F	22 nt	5′ GTAGGATGAAAAGAGGTGATAG 3′	62
M10-R	20 nt	5′ AGCAACCACCAATCCCATTC 3′	60
M10-5RACE	28 nt	5′ TGTTGTGATGGTCCGATTGCACTTCCAG 3′	74*
M13-F	20 nt	5′ TCGAAGGCGAAAACGACGGT 3′	62
M13-R	22 nt	5′ CATGAATCAACAGTGCCTTGTC 3′	64
M13-5RACE	28 nt	5′ CGTGATGATGACTAGGACCGGAGGATGC3′	74*
M13-3RACE	28 nt	5′ GCATCCTCCGGTCCTAGTCATCATCACG 3′	74*
M14-F	21 nt	5′ TCCCAGCAACACCTTCATTCA 3′	62
M14-R	19 nt	5′ GTCAACTTAGCCGCGCTGT 3′	60
M14-5RACE	28 nt	5′ CCGACAGTCGCACCTTGGTACAAAAACG 3′	74*
M14-3RACE	28 nt	5′ CAAGGTGCGACTGTCGGGCAAGTTTCTA 3′	74*

Primers used in RACE (rapid amplification of cDNA ends) experiments were designed with help of software Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

* Tm was calculated using the Primer3 software.

3.4.7 CAM primers

Specific primers were designed based on the sequence of a *Malus* x *domestica* calmodulin gene (Watillon *et al*., 1992).

Primer designation	Length	Sequence	Tm (°C)
CAM-F	22 nt	5′ TCAGCCTTTTCGACAAGGATGG 3′	66
CAM-R	21 nt	5′ GCCATCGCCATCAACATCAGC 3′	66

3.4.8 GUS primers

GUS-primers used had been designed with basis on the sequence from Jefferson *et al*. (1986).

Primer designation	Length	Sequence	Tm (°C)
5′GUSrev	20 nt	5′ TTTGATTTCACGGGTTGGGG 3′	60
GUSfwd	22 nt	5′ ATGTTACGTCCTGTAGAAACCC 3′	64
GUSrev	21 nt	5′ TCATTGTTTGCCTCCCTGCTG 3′	64

3.5 General molecular biology techniques

All DNA manipulations e.g. restriction endonuclease digestions, DNA modifications, ligations were performed according to Sambrook *et al*. (1989) or to the instructions of the supplier. DNA fragments were visualised on non-denaturing agarose gels prepared with 1x TAE buffer and 0.5µg/ml ethidium bromide. DNA samples were mixed with 1/6 vol of loading buffer. For separation of fragments smaller than 1000bp, 2% gels were used, larger DNA fragments were separated on 1.2% gels. The electrophoresis was carried out in 1x TAE as electrophoresis buffer. To estimate the size of the DNA fragments, 500ng of appropriate molecular weight standards (MBI) were loaded on the gels. RNA was visualised on 1.4% denaturing agarose gels prepared with 1x MOPS and formaldehyde as described in Fourney *et al*. (1988). For estimation of transcript size, a RNA ladder (GibcoBRL) ranging from 0.16-1.77kb was included. Gels were viewed and documented with an UVP White/Ultraviolet Transilluminator system.

3.6 Bacteria transformation

3.6.1 Transformation of *E. coli*

3.6.1.1 Electroporation

For preparation of electrocompetent bacterial cells 1000ml of prewarmed LB medium were inoculated with 10ml of an overnight culture of *E. coli* strain DH5α and grown to an OD_{600nm} of 0.5-0.8. Cells were immediately chilled on ice and centrifuged at 4°C 4500rpm for 7min. Pellets were gently resuspended in 400ml cold 1mM HEPES pH7.0 and recovered by centrifugation. Subsequent washes were performed with 300ml and 200ml HEPES,

respectively. The last wash was performed with 40ml 10%glycerol. After centrifugation, the bacterial pellet was resuspended in 2ml 10%glycerol and 100µl aliquots were shock-frozen and stored at –80°C. Solutions and plastic ware were prechilled and all the steps were carried out at 4°C.

For electroporation, ligation reactions were inactivated 10min at 75ºC and precipitated with $EtOH_{abs}$ in presence of 0.3M NaAc for 1h at –80°C. DNA was pelleted, washed with 70%EtOH and redissolved in 10µl A. dest.. Electroporation was performed with a Biorad Gene Pulser™ in precooled cuvettes at the following settings: capacitance 25µF, resistance 1000Ω, voltage 2.5V. Cells were immediately suspended in 250µl prewarmed SOC medium and incubated at 37°C for 1h. 2 x 150µl transformation mix was plated on LB_{agar} + 50µg/ml ampicillin or LB_{agar} + 50µg/ml kanamycin, respectively. For blue-white screening LB_{agar}-plates were coated with 20µl 0.1M IPTG + 35µl 50mg/ml X-Gal prior to streaking out the bacteria. Plates were incubated at 37°C overnight and, in case of blue-white screening, transferred to 4°C to accelerate the development of the blue color.

3.6.1.2 Heat shock transformation

Heat shock transformation was performed with competent cells from *E. coli* strain INVαF´ (provided in the TA Cloning® Kit Dual Promoter, Invitrogen™Life Technologies). Transformation was carried out as recommended in the supplier´s manual. 2 x 150µl transformation mix were plated in LB_{agar} + 50µg/ml ampicillin or LB_{agar} + 50µg/ml kanamycin, respectively. Blue-white screening and incubation was done as in III 3.6.1.1.

3.6.2 Transformation of *Agrobacterium tumefaciens*

3.6.2.1 Freeze-thaw method (An *et al.*,1988)

To frozen aliquots from competent *A. tumefaciens* strains LBA4404 or EHA105 3-5µg of purified plasmid were added and *Agrobacteria* were immediately incubated for 5min in a 37ºC water bath (heat shock). 1ml SOC was added and cells were further incubated at 28ºC for 3h with gentle shaking. The bacterial pellets were collected by centrifugation 30sec 13000rpm and resuspended in 200µl of SOC. 2x 100µl transformation mix were streaked out on LB_{agar} + 250µg/ml streptomycin+ 50µg/ml kanamycin. Plates were incubated at 28ºC until colonies appeared (48-72h).

3.7 Nucleic acid extractions

3.7.1 Bacteriophage DNA extraction

The large scale infection of 250ml liquid cultures of *E. coli* LE392 and purification of phage DNA was performed according to Sambrook *et al.* (1989) [Standard Method for Purification of Bacteriophage λ; Extraction of Bacteriophage λ DNA] with the following modifications in the DNA extraction procedure: RNase digestion between phenol and phenol:chloroform extractions and omission of the dialysis step.

3.7.2 Plant DNA extraction

Total genomic DNA was extracted from fresh leaves of *in vitro* propagated shoots following the procedure described by Doyle and Doyle (1990). 5ml 2xCTAB buffer supplemented with 0.3% ß-ME per gram of leaf material were used.

3.7.3 Plasmid DNA purification

Miniscale purification of plasmid DNA from overnight cultures either of *E. coli* grown at 37ºC in 2ml LB + 50µg/ml ampicillin or 50µg/ml kanamycin, respectively, or of *A. tumefaciens* cultivated at 28ºC in 2ml LB + 250µg/ml streptomycin + 50µg/ml kanamycin, was performed according to Sambrook *et al.* (1989). Precipitated DNA was dissolved in 30-50µl A. dest. (*E. coli*) or 15µl A. dest. (*A. tumefaciens*).

Midiscale purification of plasmid DNA from 25ml overnight cultures either of *E. coli* or of *A. tumefaciens*, cultivated as described for miniscale cultures, was performed using the plasmid purification kit Concert (GibcoBRL) following supplier´s instructions. DNA was dissolved in 40-50µl A. dest. (*E. coli*) or 10µl A. dest. (*A. tumefaciens*).

3.7.4 Further DNA purification

For the removal of enzymes or buffer components from previous enzymatic reactions, DNA was subjected to CI extraction and precipitation with $EtOH_{abs}$. DNA was recovered by centrifugation and redissolved in A. dest. or TE buffer. PCR products were purified using the QIAquick^{TM}PCR Purification Kit (QIAGEN) and eluted in 30µl A. dest.. Purification of DNA fragments from agarose gels was carried out using the QIAEX^{R}II Gel Extraction Kit (QIAGEN) or by agarase digestion, following supplier´s instructions. DNA was eluted in 30µl A. dest. (QIAEX^{R}II Gel Extraction Kit) or redissolved in 5-25µl A. dest. (agarase digestion).

3.7.5 RNA extraction

The following precautions were made to prevent RNase contamination: plastic ware was soaked 8h in A. dest. treated with 0.1%DEPC before autoclaving. All solutions, except of Tris-solutions (which cannot be treated with DEPC), were treated with 0.1%DEPC before autoclaving. Tris-solutions were prepared with autoclaved DEPC treated A. dest. in a RNase free-bottle. Glass material, mortars and pestles were heat-treated 8h at 180ºC.

Total RNA from leaves and flower organs from greenhouse grown plants, or from apple fruits was extracted following the procedure described by Chang *et al*. (1993). For RNA extraction from leaves and flower organs, 5ml Extraction buffer supplemented with 2% ß-ME were used per gram of material. For RNA extraction from apple fruits, 3ml Extraction buffer supplemented with 2%ß-ME were used per gram of fruit tissue (skin and flesh). RNA was dissolved in 50µl DEPC-treated sterile A. dest. and stored at –80°C.

3.7.6 Quantification of nucleic acids

Nucleic acids were quantified by UV-spectrophotometry, measuring the absorbance at 260nm. The amount of nucleic acids was calculated according to the following conversion formula:

1 A_{260} unit of double-stranded DNA = 50µg/ml

1 A_{260} unit of single-stranded RNA = 40µg/ml

Small DNA quantities were estimated on an ethidium bromide-stained agarose gel by comparing the fluorescent signal of the sample with known amounts of a DNA standard.

3.8 PCR standard conditions

Standard reactions were carried out in a volume of 25µl, containing 10mM Tris-HCl pH9.0, 50mM KCl, 0.1%Triton^{R}X-100, 2.5mM $MgCl_2$, 200µM each dNTPs, 0.2µM of each primer and 0.5U *Taq* DNA polymerase. Amplifications were performed in a Thermal cycler Biometra Trio with a Heated Lid (Germany). Cycling conditions included an initial denaturation step at 94ºC 2min, 35 cycles of denaturation at 94ºC 45sec, annealing at T_{ann} 1min and extension at 72ºC 45sec-2min, with a final extension step at 72ºC 5min. T_{ann} was chosen for the respective primer pair as Tm-2ºC, calculated for the primer with the lower Tm. Extension time varied upon the expected product, from 45sec for products until 500bp, 1min for products up to 1000bp to 2min for longer products.

3.9 DDRT-PCR

DNase I treatment of 40µg total RNA purified from water- and scab-inoculated ´Florina´ leaves was carried out with 40U RNase-free DNase I (Roche) in a final volume of 100µl containing 20mM Tris-HCl pH8.3, 50mM KCl, 2.5mM $MgCl_2$, 0.1mg/ml BSA and 120U RNase Inhibitor (MBI). The reaction was incubated at RT for 30min and stopped with 4mM EDTA. The DNase- treated RNA was purified with the RNeasy Plant Mini Kit (QIAGEN), following the protocol described for RNA Cleanup. RNA was recovered in 60µl of RNase-free water, quantified by UV-spectrophotometry and additionally controlled on a denaturing RNA gel.

First strand cDNA synthesis was performed in three independent reactions with 3µg total RNA, 2.5µM anchor primer dT_{GG}, dT_{AG} or dT_{CC}, respectively, 50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT (Gibco BRL), 1mM dNTPs, 24U RNase Inhibitor (MBI) and 150U reverse transcriptase Superscript^{TM}RT (Gibco BRL) in a total volume of 25µl. The reaction was incubated at 37°C for 1h and inactivated at 75°C for 10min. The second strand synthesis and amplification were carried out with 1µl cDNA, 10mM Tris-HCl pH8.3, 50mM KCl, 2mM $MgCl_2$, 200µM each dNTPs, 1µM anchor oligo(dT) primer (the same as for 1st strand synthesis), 1µM 10-mer arbitrary primer and 1.25U of *Taq* DNA polymerase (*AmpliTaq*^{R}DNA polymerase, Perkin Elmer Cetus) in a total of 25µl. The enzyme was added after a Hot Start at 95°C for 2min. Amplification conditions were: 40 cycles at 94°C 1min denaturation, 42°C 1min annealing, 72°C 1min extension and a final extension period of 10min at 72°C.

PCR products were mixed 2:1 with PAA loading buffer, denatured at 100°C for 5min and separated on a 4.5% polyacrylamide gel (38% acrylamide:2% methylenbisacrylamide Rotiphorese^{R}Gel 40, from Roth) in a Poker Face^{TM} SE1500 Sequencer (Hoefer Scientific Instruments). As DNA standard 500ng of 100bp Gene Ruler^{TM} (MBI) were loaded on the gel. 0.5x TBE was used as electrophoresis buffer and a pre-run step at 1500V was performed for 1h. The gel was run at 1650V for 4h.

Silver staining of the PAA gel was performed according to Bassam *et al.* (1991). The gel binding on the glass plate was subjected to 10% acetic acid for 20min, rinsed with A. dest. three times for 2min and then incubated 30min in a silver nitrate solution, gently shaking at room temperature. The staining reaction was performed with developer solution at 4°C for 2-5min and stopped with 10% acetic acid.

Before excision of fragments the gel was rinsed with A. dest.. Selected fragments were cut with a scalpel blade and stored in 100µl PAA Elution buffer. To elute the DNA, excised gel

bands were heated at 100°C for 15min, incubated at 37°C overnight and centrifuged. Eluted DNA from the supernatant was used either directly for PCR amplification or precipitated with $EtOH_{abs}$ at –80°C at least for 1h (or overnight). DNA was recovered by centrifugation and dissolved in 10µl A. dest.. Reamplification of differential display fragments was performed using the same primer combination, assay and cycling conditions as described for the corresponding display reaction, except that the $MgCl_2$ concentration was increased to 2.5mM. PCR products were reamplified either directly from the eluted DNA of the supernatant (2µl or 3µl in a total of 25µl or 40µl, respectively) or after precipitation of the DNA (4µl template in a total of 25µl). PCR products were separated on a 2% agarose gel, eluted with QIAEX^{R}II Gel Extraction Kit and cloned into the vectors pCR2.1 or pCRII.1 (TA Cloning^{R} Kit Dual Promoter, Invitrogen^{TM}Life Technologies). In cases where the reamplification was not very strong, the non-purified PCR product was used directly for cloning, otherwise PCR products were purified with PCR Purification Kit (QIAGEN) before cloning. For sequencing, inserts were amplified from the plasmid DNA with the vector-specific primers M13pUC-48 rev and T7SEQ as described in III 3.8.

3.10 RT-PCR reaction with fragment-specific primers

Reverse-transcription reactions were performed as described in III 3.9 using as anchor primer dT_{VN}. The amplification reactions were performed with 1µl cDNA and 1µM each sequence-specific primer (forward and reverse) under the same reaction conditions as described in III 3.9. Cycling conditions were as follows: 95°C 2min initial denaturation, Hot Start and 94°C 1min denaturation, 58°C 1min annealing, 72°C 1min extension for 35 cycles, with a final extension step at 72°C for 10min. PCR products were separated on a 2% agarose gel.

3.11 Elongation of display fragments using RACE technology

RACE experiments were carried out using the SMART^{TM} RACE cDNA Amplification Kit (Clontech). Primers were designed as recommended in SMART^{TM} RACE protocol, with 23-28nt and a high GC content, which allows annealing at a high temperature and enables the touchdown cycling program. The RACE reactions were performed as described in the user´s manual using 1µg DNase- treated total RNA from scab-infected sample for generation of the 5´and 3´RACE cDNA pools. RACE reactions were carried out in a Gene Amp PCR System 9600 thermal cycler (Perkin Elmer). After 27 cycles of amplification, 5µl of each sample were analysed on a 1.2% agarose gel. Samples with strong visible bands were stored at 4°C, samples without visible bands were subjected to 10 additional amplification cycles.

3.12 Sequencing and sequence analyses

Plasmid or PCR products were sequenced by the company IBL (Vienna) according to the method of Sanger *et al.* (1977). Sequence analyses (search for restriction sites, alignments, assembly of sequences, translations) were carried out using the softwares Lasergene (DNASTAR), PROSITE (http://www.expasy.org/prosite/) and CLUSTALW (http://www2.ebi.ac.uk/clustalw/). NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and TAIR (http://www.arabidopsis.org/Blast/index.html) databases were used for identification of sequence homologies, using the BLAST (basic local alignment search tool) algorithms (Altschul *et al*., 1990).

3.13 Non-radioactive hybridisation and detection

3.13.1 Synthesis of DIG-labeled probes

DNA probes were synthesised by PCR amplification with gene-specific primers using the PCR DIG Probe Synthesis Kit (Roche) following recommendations of the supplier. Standard cycling conditions (III 3.8) were used with a final extension step of 10min at 72°C. 2µl of synthesised probes were loaded on a 2% agarose gel to verify the size of the amplified product. For estimation of the labeling efficiency, 1µl of tenfold serial dilutions of the probe and of a labeled control DNA in DNA dilution buffer were spotted on a nylon membrane (Hybond N^+, Amersham Life Science), baked at 100°C 2h in an air incubator and detected as described (III 3.13.3). Amount of labeled probe was estimated by comparing signal intensities of the probe dilutions with those of the labeled control DNA with known amount.

For generation of antisense riboprobes, PCR products were ligated into the vector pCRII.1 and transformed into *E. coli* (see III 3.6.1.2). 1µg of purified and linearised plasmid was *in vitro* transcribed using either the T7 or SP6 RNA polymerase (Roche) to obtain antisense transcripts. The reaction was carried out as recommended by the supplier, precipitated overnight, and RNA was redissolved in 20µl A. dest.. 2µl transcribed RNA were electrophoresed on a denaturing agarose gel to control the size of the transcripts. Labeling efficiency was estimated as for DNA probes, except that labeled control RNA was diluted in RNA dilution buffer.

Synthesised probes are listed in Table 8.

Table 8: Length and primer combination used for synthesis of DIG-labeled probes.

Probe		Length (bp)	Primer combination
DNA	5´280bp pgip probe	280	5´PGIPdeg+Int PGIPdeg
	3´pgip	660	MDPGIP-F+PGIP-R
	chi	543	5´CHI+3´CHI
RNA	pgip riboprobe	729	MDPGIP-F+PGIP-R
	M9	416	M9-F+M9-R

3.13.2 Hybridisation

Membranes, sealed in an autoclaved plastic bag, were pre-hybridised 3h at 65ºC with either HBI for DNA-DNA hybridisation or Church buffer for RNA-RNA hybridisation. DNA probes were denatured 10min at 100°C and immediately chilled on ice. Riboprobes were denatured 15min at 65°C and immediately chilled on ice. Membranes were incubated with the respective hybridisation solution containing the denatured probe overnight at 65ºC, with gentle movement.

Hybridised membranes were washed at 65ºC with gentle shaking in 2x SSC+0.1%SDS for 15min and 30min and subsequently with 0.2x SSC+0.1%SDS for 15min and 30min. Membranes were shortly rinsed in Wash buffer before detection.

3.13.3 Chemiluminescent detection

Chemiluminescent detection was performed as described in The DIG system User´s Guide for Filter Hybridisation (Roche). CDP-Star^{TM} (Amersham Life Science) was used as substrate for the alkaline phosphatase conjugated anti-DIG antibody in dilutions varying between 1:2 and 1:10 in Detection buffer. Membranes were exposed to X-ray films (Cronex^{R}, Dupont or Kodak X-omat AR) for different durations, according to intensity of the expected signal. X-ray films were developed by immersion in developer solution (Ilford Multigrade), briefly immersed in tap water and fixed 3-5min in fixation solution (Ilford Hypam).

3.14 Screening of an apple genomic library

A genomic library of *Malus* x *domestica* cultivar McIntosh ´Wijcik´ (Watillon *et al*., 1992) was screened by plaque hybridisation. 500 000 recombinant phages in the bacterial strain *E. coli* KW251 suspended in the molten agar TA7 were plated on NZY medium at a density of 40 000 pfu/138mm-Petri dish. Preparation of bacteria, plating bacteriophage λ and

immobilisation of plaques onto Hybond-N (Amersham Life Science) filters were performed according to Sambrook *et al*. (1989). For DNA immobilisation, plaque lifts were baked 1h at 120°C in an air incubator. Hybridisation and chemiluminescent detection were carried out as described in III 3.13.2 and III 3.13.3.

In the screening for apple *pgip* genes and their upstream regulating sequences, filters were hybridised with HBI containing 2ng/ml of a 280bp DIG-labeled probe corresponding to the 5´end of *pgip* genes. In the screening for apple chitinase genes, filters were hybridised with HBI containing 1.5ng/ml of 543bp DIG-labeled probe binding to the intermediate region of known class III chitinases. Positive clones derived from the first screening underwent 4 (*pgip* genes) or 3 (chitinase genes) rounds of screening with the same probe. Finally, individual single bacteriophage λ clones were amplified in *E. coli* KW251, plaques were harvested in SM buffer and stored at 4°C. Large scale cultures for bacteriophage purification and extraction of bacteriophage DNA were done in *E. coli* LE392 (III 3.7.1).

3.15 Southern blotting

DNA was separated by electrophoresis on a 0.9% agarose gel. 30ng of a DIG-labeled molecular weight standard (λ*Eco*RI/*Hind*III) were loaded on the gel to estimate the size of the hybridising fragments. Gel was treated 10min in 0.25N HCl (light depurination to enhance transfer of large DNA fragments), 15min and 30min in Denaturation buffer and 2 x 15min in Neutralisation buffer. DNA was transferred on a nylon membrane (Hybond N^+, Amersham Life Science) by capillary blotting for 18h in presence of 10x SSC as described in Sambrook *et al*. (1989). DNA was fixed to the membrane by baking 2h at 80ºC in an air incubator. Southern blot was hybridised and detected as described in III 3.13.2 and III 3.13.3.

3.16 Northern blotting

For Northern blot analysis, total RNA was separated on a 1.4% agarose gel containing formaldehyde as described by Fourney *et al*. (1988). Equal loading of the gel was checked visually by ethidium bromide staining on the basis of the rRNA bands. RNA gel was treated 2 x 10min with 0.05N NaOH in 1x $SSC_{DEPC\ treated}$ and 2 x 20min in 10x $SSC_{DEPC\ treated}$. Capillary blotting was performed as described in III 3.15. Alkali fixation was used to immobilise the RNA to the nylon membrane: the membrane was placed RNA-side up on a blotting paper soaked with 0.05N NaOH for 5min and subsequently neutralised by transferring it to a blotting paper soaked with 2x $SSC_{DEPCtreated}$. The membrane was dried at RT and hybridised and detected as described in III 3.13.2 and III 3.13.3.

4. Biochemistry work

Composition of different used solutions is shown in Appendix 2.

4.1 Evaluation of polygalacturonase content by 2-cyanoacetamide assay

Polygalacturonase activity was correlated with the amount of GA (galacturonic acid) released from the enzymatic degradation of PGA (polygalacturonic acid) due to its activity. The reaction mixture contained 47µl Czapek-Dox salts+0.5% PGA and 3µl sample and was incubated at 25°C for 16h. A purified pectinase (Sigma) was dissolved in citrate buffer pH4.0 (Titrisol^{R}) and 0.15U were used as a positive control sample. The different fungal supernatants and their corresponding culture media only (III 2.3) were used as samples in this assay. The enzymatic reaction was diluted 1:250 in sterile A. dest. and its polygalacturonase activity was measured in the 2-cyanoacetamide assay.

Accumulated GA was quantified spectrophotometrically by measuring the absorbance at 274nm of the intermediate dienol produced in the reaction between GA and 2-cyanoacetamide under weak alkaline conditions (Bach and Schollmeyer, 1992). The reaction was performed with 300µl borate buffer pH9.0 (Titrisol^{R}), 150µl 2.5% 2-cyanoacetamide and 150µl sample. The mixture was incubated at 100°C for 10min and chilled on ice, prior to ultraviolet-measurement in a Biorad SmartSpec^{TM}3000 spectrophotometer. For generating a calibration curve defined concentrations of GA were measured.

4.2 Elicitation of tobacco explants with fungal supernatants

Young leaf explants from tobacco plants grown *in vitro* or in the greenhouse (3-5 weeks old) were incubated in 100µl of fungal supernatant or culture medium with pH adjusted to 5.5-5.7 (verified with pH paper). Leaves were cut through the midrib and one half of the leaf was incubated with the supernatant and the other with the control medium, to ensure equal developmental conditions in control and fungal elicitated explants. After 48h incubation at 22°C, leaf explants were rinsed in A. dest. and assayed for GUS activity.

4.3 GUS assays

4.3.1 Histochemical test (Martin *et al.*, 1992)

Leaf tissue was infiltrated with GUS solution (0.5% X-Gluc in GUS buffer) 5min at RT using a vacuum water pump. The tissue was incubated 24h at 37ºC, rinsed in A. dest. and bleached with 70%EtOH to remove the chlorophyll. The plant tissue was stored in 70%EtOH.

4.3.2 Fluorometric assay (Gallagher, 1992)

Leaf tissue was homogenised in 100µl GUS extraction buffer with freshly added 10mM ß-ME in a swing mill (Retsch MM2000). The extract was centrifuged at 4°C for 10min at 13000rpm and 20µl of the clear supernatant were combined with 180µl substrate solution (2mM 4-MUG in GUS extraction buffer). The enzymatic reaction was incubated 2h at 37°C and 25µl of it were stopped with 225µl 0.2M Na_2CO_3. Fluorescence was measured in a Fluorometer Spectra Max GeminiXS and converted to nmol 4-MU using a standard curve. 5µl of leaf extract were diluted 1:50 in Protein dilution buffer and total amount of protein was estimated by Biorad Protein Assay according to Bradford (1976) using bovine gamma globulin as standard. GUS activity was expressed as nmol 7-hydroxy-4-methylcoumarin (4-MU) accumulated at 37°C per mg total protein and per hour.

IV RESULTS

1. Establishment of a scab-infection system under controlled conditions

1.1 Culture of *Venturia inaequalis*

The growth and sporulation of 3 different scab isolates (kindly provided by ENFVN, Portugal) were monitored in Malt Extract Agar (MEA), in Malt Extract Agar supplemented with 0.24% $MgSO_4$ and 0.68% KH_2PO_4 (MEAm) and Potato Dextrose Agar (PDA). Stronger growth (measured as the size of the colonies) and more intense sporulation (evaluated microscopically) were observed in MEAm, than in MEA and PDA. Due to this observation, the fungal isolates were further cultivated in MEAm. Isolates 8 and 19 produced conidia in a high amount, whereas isolate 6 sporulated very poorly (Fig. 7).

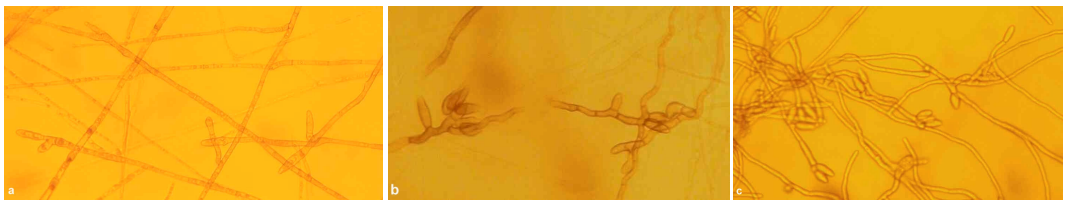


Fig. 7: Microscopical observation (250x magnification) of conidial production in the 3 fungal isolates cultivated in MEAm: sporulating margins of (a) isolate 6, (b) isolate 8 and (c) isolate 19.

1.2 Infection of plant material with a conidial suspension of *Venturia inaequalis*

As a source of inoculum for the infection experiments with apple scab, a conidial suspension combining the 3 different isolates was produced. The mycelial extract was prepared as described in III 2.2.1 and the spores were released into the liquid phase during 24h. In order to obtain a suspension with scab conidia free of mycelial debris, the extract was filtered through 3 layers of Miracloth (Fig. 8). This method proved to be most efficient and gave the best yield, compared to filtration through cotton, nylon mesh or filter paper.

To confirm the viability of the conidial suspension, an aliquot was inoculated on MEAm and incubated at 20°C. Microscopical observation of germinating conidia on MEAm (Fig. 9) indicated that the suspension preparation resulted in viable conidia.

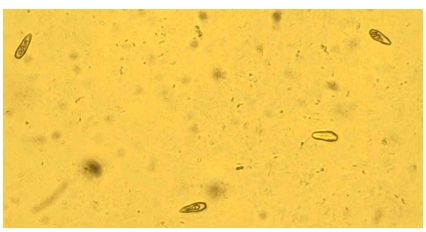


Fig. 8: Microscopical visualisation (250x magnification) of a suspension of *Venturia inaequalis* obtained by filtration through Miracloth tissue. Isolated non-germinated conidia are visible.

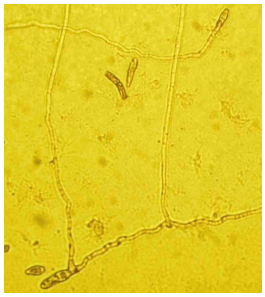


Fig. 9: Viability test of conidial suspension: germination of conidia in MEAm 5 days after inoculation (250x magnification).

Infection experiments using *in vitro* plant material were performed in Petri dishes in order to develop a functional infection system and to define towards which cultivars the scab inoculum was most agressive. The goal of these experiments was also to define which medium was most adequate in order to keep the leaf material under no stress conditions, but avoiding direct fungal growth. The experiments were carried out with detached leaves from *in vitro* cultivated shoots of different scab susceptible cultivars:´Maschanzker´, ´Elstar´, ´Golden Delicious´, ´Royal Gala´, ´McIntosh´, ´Jonagold´. The leaves were kept in basal MS medium with half of the concentration of macronutrients (½MS), in ½MS without sugar and myo-inositol (½MSm), in 1% or 0.6% water agar or in a moist filter paper. The suspension was pipetted to the leaves to simulate rain droplets, or sprayed trying to create a liquid film over the leaf surface. The Petri dishes were maintained at 20°C, 14h light/day.

These studies revealed that the growth of scab fungus was strongest in ´Jonagold´ and ´Golden Delicious´, where symptoms were most abundant and appeared earlier than in the cultivars ´Maschanzker´, ´Elstar´, ´Royal Gala´, and ´McIntosh´. ´Elstar´ was the cultivar that showed less susceptibility. The development of symptoms in the different cultivars showed that the inoculum prepared was generally virulent and not only infectious for a single cultivar. In ´Golden Delicious´ first chlorotic lesions were visible after 10dpi, in the other cultivars, clear chlorotic lesions were visible only after 14 dpi. Their intensity increased over the time of assay. Scab growth was microscopically observed after staining the fungal structures with periodic acid-basic fuchsin (Fig. 10).

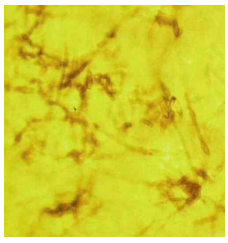


Fig. 10: Microscopical observation (160x magnification) of fungal development on *in vitro* leaf from ´Golden Delicious´ 17dpi, stained with periodic acid-basic fuchsin.

The medium ½MSm demonstrated to be the most promising, since it allowed the preservation of leaf material for long periods in a good vegetative condition (no senescence signs in the

leaf explants during longer than 6 weeks incubation) and it did not promote fungal growth. The spraying with a glass sprayer proved to be more effective in the distribution of the spores, albeit the inconvenience that, in the infection experiments in Petri dishes, also the surrounding medium was inoculated.

Due to these results ´Golden Delicious´ and ´Jonagold´ were selected as model susceptible cultivars and spraying as an infection method for further experiments.

As the extent and nature of mycelial development in plants grown *in vitro* differs from the growth pattern in plants grown under greenhouse conditions (Yepes and Aldwinckle, 1993a), assays with detached young leaves from greenhouse plants were carried out to reproduce the fungal development in *in vivo* tissues. As a first step, prior to the fungal inoculation, a surface desinfection of the leaves was required to inhibit growth of contaminant pathogens. The infected leaves survived in ½MSm for 6 weeks as described for assays with *in vitro* material. First symptoms of fungal growth were clearly visible after 18-20 days and developed further (Fig. 11).

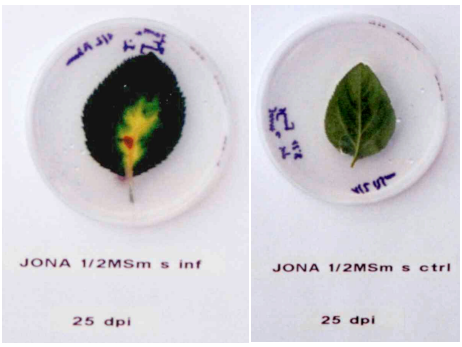


Fig. 11: Chlorotic lesions observed macroscopically on a greenhouse grown ´Jonagold´ leaf infected with *Venturia inaequalis* 25 dpi (left). Mock infection was performed with water only (right).

In addition, the efficiency of the infection system was confirmed by visual evaluation of the disease progression *in planta* in greenhouse acclimatised ´Jonagold´ plants that were inoculated with scab under controlled conditions (Fig. 12). Brown-olive green spots manifested 3 weeks after infection as first visible symptoms (Fig. 12a). These lesions further developed (Fig. 12b) and necrotic spots were clearly visible 5 weeks after infection (Fig. 12c). Lesions became visible also in the abaxial side (Fig. 12d). The number of necrotic lesions increased with the time (Fig. 12e) and leaves became senescent (Fig. 12f) 8 weeks after infection.

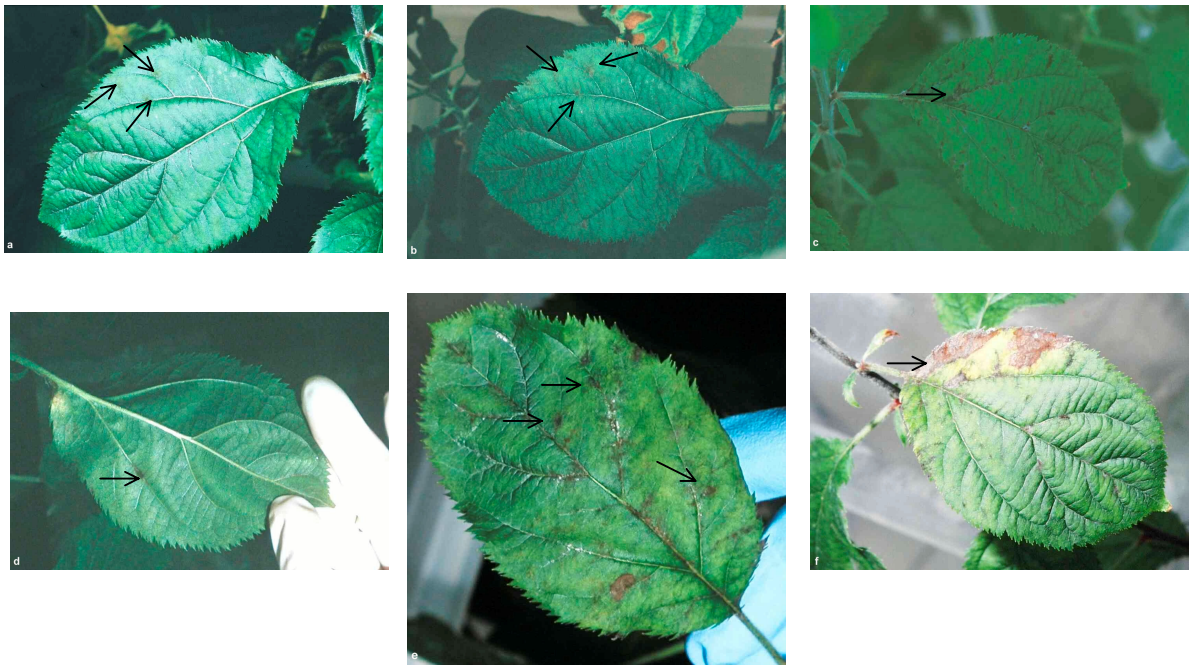


Fig. 12: Scab symptoms on leaves of ´Jonagold´ *in planta* (a) 3 weeks, (b) 4 weeks, (c) 5 weeks, (d) 6 weeks, (e) 7 weeks and (f) 8 weeks post infection.

2. Isolation of *pgip* promoters and characterisation of *pgip* family in apple

2.1 Cloning and identification of apple *pgip* genes and their regulating sequences

Apple *pgip* genes and their upstream regulating sequences were isolated from a genomic library of *Malus* x *domestica* cultivar 'McIntosh' (mutation 'Wijcik') in λGEM11 (Watillon *et al*., 1992). For the library screening, a PCR amplified probe hybridising with the 5´end of *pgip* genes, derived from genomic DNA of apricot (Pühringer *et al.*, unpublished) was used. After 4 rounds of subscreening, 10 single phage clones were isolated and amplified. 4 clones - N11, N17, Q8, M10 - were selected for DNA purification and clonable fragments were identified by restriction digestion and Southern blotting with the same probe. After subcloning into the Bluescript vector, primers flanking the cloning site and sequence-based primers were used to identify the sequences of the isolated clones. The characteristics of the 4 clones are summarised in Table 9.

Table 9: *pgip* clones derived from library screening and subcloned in Bluescript vector. Size of the fragments was calculated from sequences or estimated from agarose gels.

Clone	Restriction enzymes	Size of cloned fragment	upstream region from Start codon	Coding sequence	downstream region from Stop codon
N11	*Eco*RV	2.5 kb	2 kb	554 bp	-
N17	*Bam*HI/*Sac*I	4 kb	-	990 bp (ATG missing) intronless	3 kb
N17	*Pvu*II	4 kb	3.6 kb	380 bp	-
M10	*Pvu*II	3.8 kb	3.4 kb	380 bp	-
Q8	*Dra*II	2.1 kb	549 bp	1140 bp (complete) 147 bp intron	410 bp

Analyses of the obtained sequences revealed that N17 and N11, as well as Q8 and M10 were derived from the same bacteriophage clones. Thus, they were further referred as N17 and Q8. Sequences obtained from different subcloned fragments of the same clone (see Table 9) were assembled by software Lasergene (DNASTAR) and, in both cases, the complete coding sequence could be identified, as well as significant portions of the 5´regulating sequence upstream from the translation start (2.0kb in N17, 1.6kb in Q8).

The sequence from N17 encodes an intronless open reading frame with 330 amino acids, whereas the coding region of Q8 comprises 380 amino acids and is interrupted by an intron spanning 147bp at amino acid position 180. Alignment of the isolated 5´regulating sequences with a *pgip* promoter sequence from bean (Devoto *et al.*, 1998) revealed no significant stretches of homology. Analysis of the 5´ sequences flanking the coding region of the isolated *pgip* genes showed that in both clones, the 450 nucleotide sequence preceding the translational start codon contains several putative TATA boxes. In position –242 in N17 and –346 in Q8 (+1 corresponded to the A of the translation initiation codon, as the transcriptional start was not determined experimentally), a TATAA motif, described as functional TATA box in bean (Devoto *et al.*, 1998), could be identified. Putative CAAT boxes could be found at position –330 in N17 and at position –415 in Q8 (88 and 69bp upstream from TATAA, respectively).

A sequence AGTTGACC, similar to the consensus motif AN(T/C)TGACC, known to occur in promoters of different plant genes expressed in response to fungal infection and elicitor treatment (Raventós *et al.*, 1995) could be identified in Q8 in position –1057. In N17, a similar sequence (ATCTGACC) to this motif was found in position -202.

2.2 Characterisation of *pgip* family in apple

In order to determine how many *pgip* gene copies are present in the apple genome, Southern analyses were performed with different cultivars. A probe was hybridised under highly stringent conditions to the 3´end of the *pgip* gene. The genomic DNA was digested with restriction enzymes not cutting in this region. At least 3 copies were identified in the apple genome of all tested cultivars. Southern analysis of cultivar ´McIntosh´ digested with *Bam*HI and *Pst*I is shown in Fig. 13.

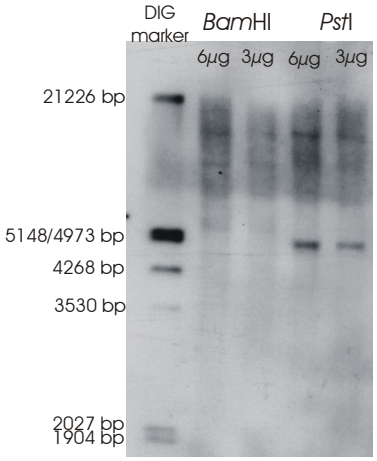


Fig. 13: A DIG-labeled 3´*pgip* DNA probe (10ng/ml) was hybridised to 6µg and 3µg of genomic DNA from ´McIntosh´ digested with *Bam*HI and *Pst*I. Southern blot was developed with CDP-Star diluted 1:5 and exposed to X-ray film overnight. Fragments of 30ng DIG-labeled DNA marker are visible in lane 1.

Northern analysis, using a DIG-labeled *pgip* antisense riboprobe, revealed the highest accumulation of 1.3kb- *pgip* transcripts in mature fruits when compared to old and young leaves or floral organs. Within the floral organs petals displayed the strongest *pgip* expression. In older leaves *pgip* transcripts were not detectable (Fig. 14).

Pgip gene expression in apple leaves infected with scab was investigated over a certain period of time. In detached leaves of cultivar ´Jonagold´ higher levels of *pgip* transcripts accumulated in scab-infected apple leaves compared to mock-infected material (Fig. 15a). *Pgip* expression level in scab-infected samples increased up to 48h after infection. In a similar experiment with detached leaves of cultivar ´Golden Delicious´, a higher accumulation of *pgip* transcripts in scab-infected apple leaves than in mock-infected material was also observed, with highest *pgip* expression observed 96h after infection (Fig. 15b). Comparable amounts of RNA were checked visually in a gel stained with ethidium bromide.

Fig. 14: Basal *pgip* mRNA expression in different organs. Northern blot of 10µg total RNA was hybridised with 10ng/ml antisense *pgip* probe. CDP-Star was diluted 1:5 and membrane was exposed to a X-ray film overnight.

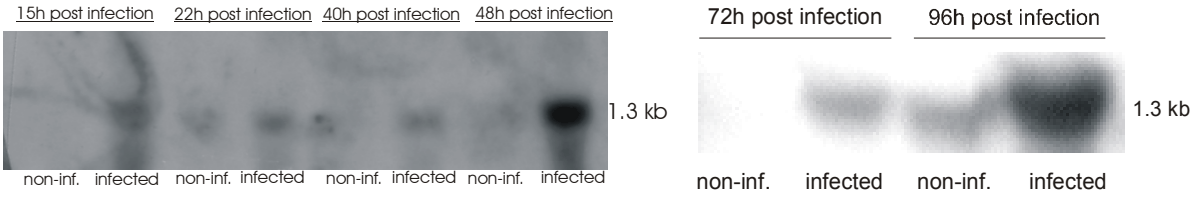


Fig. 15: Accumulation of *pgip* transcripts in leaves of a) ´Jonagold´ and b) ´Golden Delicious´ upon scab infection. Mock infection was performed with water only. Northern blots of 10µg total RNA were hybridised with 10ng/ml antisense *pgip* probe. CDP-Star was diluted 1:3 (a) or 1:5 (b) and membranes were exposed to X-ray films 3h (a) and 4h (b) .

2.3 Analysis of isolated *pgip* promoters in *Nicotiana tabacum*

2.3.1 Generation of *pgip* promoter-reporter gene constructs

Putative promoter sequences were set in front of the *uidA* gene encoding the ß-glucuronidase (GUS). The fusion with a promoterless reporter GUS-Intron cassette was achieved by cloning the isolated *pgip* regulating sequences into the *Sma*I site of the pBSGUS vector, thereby providing the construct with a transcription termination sequence. 3 different constructs – N, S and P – varying in length and sequence of the *pgip* promoter were generated. The N construct harbours a 2kb fragment derived from clone N17, excised with *Eco*RV and *Sac*I. The S construct harbours a 1.45kb fragment excised with *Nde*I and *Sph*I from clone Q8. The P construct harbours a 1.23kb fragment from Q8, excised with *Nde*I and *Pst*I, which is a 3´ truncated subfragment of S. As the TATA box could not be identified unambiguously, the goal of generating construct P was to evaluate whether the 3´truncation of the promoter sequence would have an influence on its activity and on pathogen inducibility. The translational fusion of the N construct, including the 5´untranslated region and the *pgip* start codon from N17, is presented schematically in Fig. 16. Thus, the entire 5‘non-translated region of the chimeric gene is identical to the *pgip* one. This was done in an attempt to mirror as much as possible the original expression pattern. The fusions of 5´upstream sequences from Q8 are presented in Fig. 17. The cassettes comprising the putative promoters set in front of the GUS gene were excised from Bluescript plasmids with *Not*I and *Sal*I. The fragments were blunted at the *Not*I site and cloned in the binary vector pBIN19 digested with *Sal*I and *Sma*I (Fig. 18).

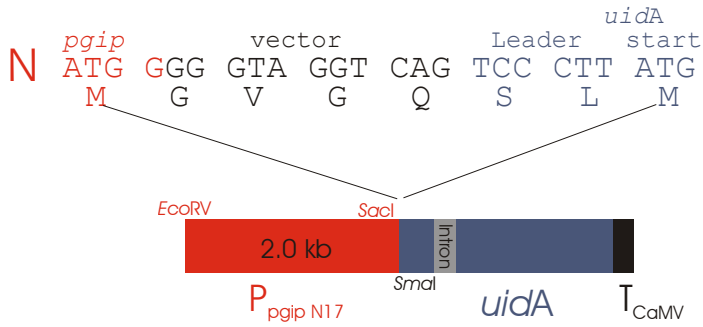


Fig. 16: Translational fusion of *pgip* promoter from clone N17 to the GUS-Intron gene (*uidA*), comprising the entire 5´untranslated region and the start codon of the *pgip* gene.

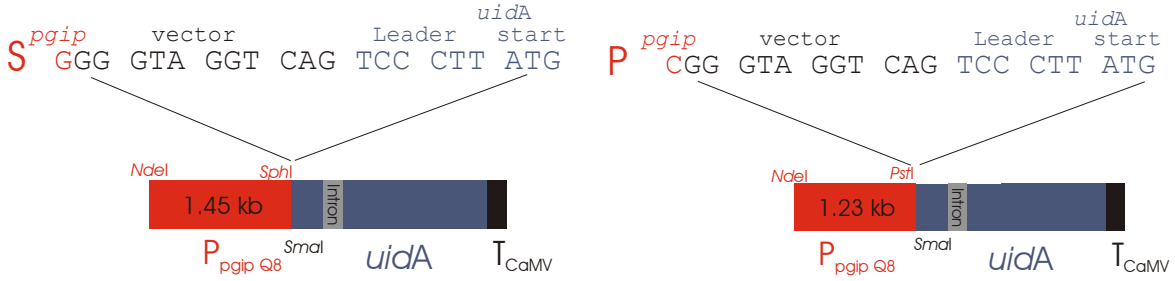


Fig. 17: Fusion of two different sizes of *pgip* promoter regions from clone Q8 to the GUS-Intron gene (*uidA*) in pBSGUS.

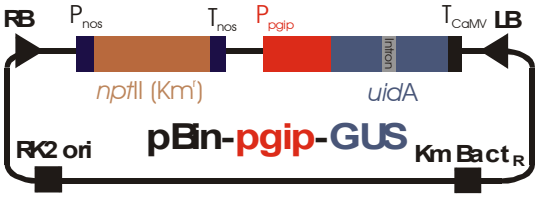


Fig. 18: Schematic representation of cassettes comprising the different *pgip*::GUS constructs in pBIN19.

2.3.2 Transformation of *Nicotiana tabacum* with *pgip*::GUS constructs

Leaf discs of *Nicotiana tabacum* were transformed with *Agrobacterium tumefaciens* (da Câmara Machado *et al.*, 1992) carrying the different *pgip*::GUS constructs. Transgenic shoots were regenerated under the selection pressure of 100µg/ml kanamycin in the culture medium. Regeneration of transgenic shoots turned out to be much more successful under dark conditions than when exposed to a regime of 16h light/day (Table 10). The prolonged growth of regenerated plantlets in medium supplemented with kanamycin was taken as criterium to select transgenic shoots, as no Southern analysis was performed to investigate the integration of the constructs.

Table 10: Transgenic lines, harbouring the different *pgip*::GUS constructs regenerated under darkness or 16h light/day, were selected after repeated subcultivation in regeneration medium supplemented with 100µg/ml kanamycin.

Construct	Lines regenerated in the dark	Lines regenerated under light conditions
N	N4, N6, N8, N12, N13, N64, N68	N16, N18, N21, N30
S	S1, S2, S11, S15, S21, S37, S38, S92, S95	S28, S30, S45, S119
P	P1, P8, P20, P62, P64, P77, P86, P87, P99	P32, P47

2.3.3 Synthesis and evaluation of fungal elicitors for elicitation assays

In elicitation assays the isolated *pgip*-regulating sequences should be investigated using elicitors produced from a phytopathogenic fungus. Promoter induction is assessed by measuring the accumulation of products derived from GUS reporter gene.

The filtrate from liquid cultures of a fungus pathogenic for tobacco plants, *Botrytis cinerea*, the causal agent of the grey mold disease, was chosen as eliciting substance. In this supernatant several enzymes secreted by the growing fungus, particularly endopolygalacturonases (Cruyssen and Kamoen, 1992; Johnston and Williamson, 1992a; Kusters-van Someren *et al.*, 1992), accumulate. These constitutively expressed enzymes (Johnston and Williamson, 1992a) are described to participate at the onset of plant-fungus interaction (Karr and Albersheim, 1970; Cervone *et al.*, 1989) and potentially activate *pgip* genes (Cervone *et al.*, 1992; Yao *et al.*, 1999). In order to produce different supernatants, the fungus was cultivated in liquid media with different carbon sources and salt/vitamins composition for various periods of time. The putative enzymatic activity in the collected supernatants was assessed by their polygalacturonase content.

The determination of polygalacturonase content of the different elicitors was done in an indirect way, using a biochemical assay in which the product, resulting from polygalacturonase activity on a pectic substrate, is quantified by spectrophotometry. The principle of this test is that the reaction of 2-cyanoacetamide with the reducing sugars, resulting from the degradation of polysaccharides due to the activity of the polygalacturonases, gives an intermediate substance (dienol) with an absorption peak at a wavelength of 274nm (Bach and Schollmeyer, 1992). The generation of a calibration curve, which correlates defined concentrations of galacturonic acid (reducing sugar) to their corresponding absorbances (Fig. 19), allows the estimation of the amount of galacturonic acid produced in a certain enzymatic reaction using polygalacturonic acid as substrate and, indirectly, the polygalacturonase activity responsible for this degradation.

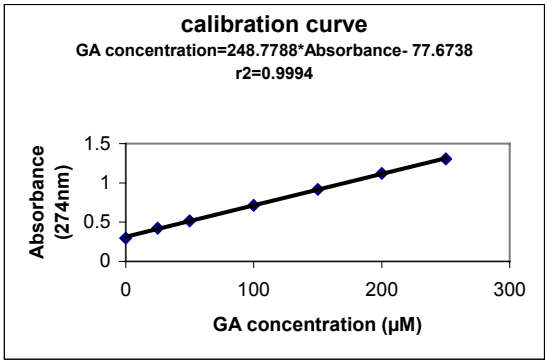


Fig. 19: Calibration curve generated with defined concentrations of galacturonic acid and the absorbance of dienol at 274nm. This intermediate product results from the reaction of galacturonic acid with 2-cyanoacetamide.

The putative capacity of the produced supernatants to degrade polygalacturonic acid due to their polygalacturonase activity was measured in this enzymatic assay. The amount of galacturonic acid was indirectly quantified in the spectrophotometer, and the values were correlated with the polygalacturonase activity of the different samples (the higher the absorbance, the higher the galacturonic acid amount, the higher the enzymatic activity).

The crude fungal supernatant was used directly in the enzymatic assay, instead of purified polygalacturonases. As the internal degradation of carbon sources present in the different culture media can also contribute to the synthesis of the uv-active dienol during the biochemical assay (Bach and Schollmeyer, 1992), this effect had to be considered. The control culture medium was also included in the enzymatic assay and subsequently in the biochemical assay with the 2-cyanoacetamide to evaluate how much galacturonic acid results from medium degradation. A purified pectinase was included in the enzymatic assay as a positive control and as a reference for the polygalacturonase activity, that can be maximally achieved. The goal of this assay was simply to make a broad screening of the different elicitors produced. Supernatants, collected from culture media differing in their carbon sources and in salt and vitamin composition, were assayed (Table 11). Different harvest times were considered, according to the reported time points of highest secretion of polygalacturonases for each carbon source (Cruyssen and Kamoen, 1992) or fungal growth.

In the culture media that showed galacturonic acid levels below or around background (citrate buffer, G, H, L, MSfpH5.7), the contribution from internal degradation was regarded as not relevant. All tested supernatants were much less active in terms of polygalacturonic acid degradation than the purified pectinase (see Table 11). Supernatants derived from culture media containing saccharose, tryptone or glucose displayed a higher polygalacturonase activity than supernatants derived from media with pectic substrates or with pectin combined

with glucose, the latter were suggested to confer a high polygalacturonase production (Johnston and Williamson, 1992b). According to the obtained results, supernatants were selected due to their high polygalacturonase activity and their low background contributed by medium degradation. Most active supernatants turned out to be those produced in a MS based medium with 2% sugar and pH5.0 ($SN_{MSfpH5.0}$) or pH5.7 ($SN_{MSfpH5.7}$) and those produced on a Czapek-Dox based medium with 2% glucose, pH4.8 (SN_J) or 2% tryptone, pH5.0 (SN_L).

Table 11: Polygalacturonase activity testing of different fungal supernatants. The amount of galacturonic acid produced in the enzymatic reaction due to polygalacturonase content of the supernatants was quantified by biochemical assay with 2-cyanoacetamide. Culture media were assayed in parallel, to evaluate contribution of internal degradation of carbon sources.

Samples		Original culture medium			Time point of harvest	Absorbance (274 nm)	GA Concentration (µM)
		Salts Vitamins	Carbon source	pH			
Purified pectinase		Na-citrate	-	4.0	-	0.5	46.716
Supernatants	SN_A	Czapek-Dox	1% glucose + 1% pectin non-esterif.	5.0	7days	0.338	6.413
	SN_B	Czapek-Dox	1% glucose + 1% PGA	5.0	4 days	0.311	0
	SN_D	Visser	1% glucose + 1% PGA	5.0	4 days	0.301	0
	SN_G	Czapek-Dox	0.5% PGA	5.0	5 days	0.286	0
	SN_H	Czapek-Dox	0.5% pectin esterif.	4.5	3 days	0.29	0
	SN_I	Czapek-Dox	2% GA	5.3	5 days	0.345	8.155
	SN_J	Czapek-Dox	2% glucose	4.8	3 days	0.377	16.116
	SN_L	Czapek-Dox	2% tryptone	5.0	3 days	0.369	14.126
	$SN_{MSfpH5.0}$	MSf	2% saccharose	5.0	7 days	0.401	22.086
	$SN_{MSfpH5.7}$	MSf	2% saccharose	5.7	7 days	0.405	23.082
citrate buffer		Na-citrate	-	4.0	-	0.285	0
Culture media	A	Czapek-Dox	1% glucose + 1% pectin non-esterif.	5.0	-	0.328	3.926
	B	Czapek-Dox	1% glucose + 1% PGA	5.0	-	0.329	4.174
	D	Visser	1% glucose + 1% PGA	5.0	-	0.333	5.170
	G	Czapek-Dox	0.5% PGA	5.0	-	0.28	0
	H	Czapek-Dox	0.5% pectin esterif.	4.5	-	0.291	0
	I	Czapek-Dox	2% GA	5.3	-	0.371	14.623
	J	Czapek-Dox	2% glucose	4.8	-	0.342	7.409
	L	Czapek-Dox	2% tryptone	5.0	-	0.28	0
	MSfpH5.0	MSf	2% saccharose	5.0	-	0.318	1.438
	MSfpH5.7	MSf	2% saccharose	5.7	-	0.288	0

For the analyses with the tobacco plants the fungus was cultivated in MSfpH5.7, as $SN_{MSfpH5.7}$ turned out to be the most promising in terms of elicitor composition. Additionally, due to the positive results obtained with SN_J and SN_L, a medium combining glucose and tryptone as carbon sources, MAMm, not included in the biochemical assay, was also used for fungal cultures.

2.3.4 Establishment of the elicitation assay

After the biochemical screening, the induction potential of the selected supernatants, that means their capacity to promote the expression of the reporter gene, had to be investigated *in planta*. To establish the elicitation assay, supernatants derived from MSfpH5.7 and MAMm were used in induction experiments with different leaf explants from the transgenic tobacco plants. Young leaves and older leaves, either derived from *in vitro* or from greenhouse material, were incubated for different periods. The qualitative evaluation of the reporter gene GUS was done in a histochemical assay (Martin *et al.*, 1992), using X-Gluc as substrate. The reaction product of GUS with this substrate is a blue indigo dye precipitate, which allows the localisation of the enzyme activity in the tissue. GUS accumulated in the vascular tissue of leaf explants from greenhouse plants of N12 and N21 elicited with $SN_{MSfpH5.7}$, during 48h and 24h, respectively, but not in explants incubated in MSfpH5.7 (Fig. 20).

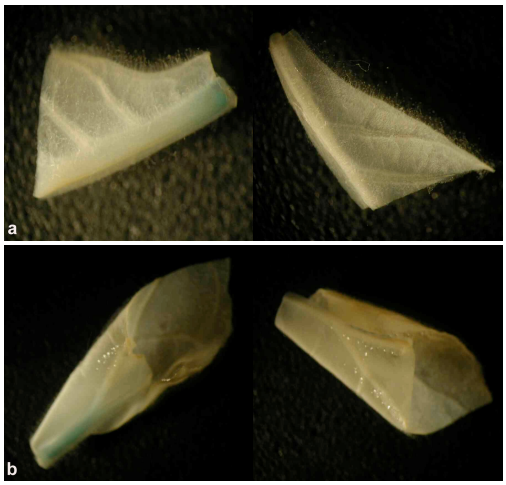


Fig. 20: Blue color precipitate, indicating GUS expression, was observed in the samples challenged with $SN_{MSfpH5.7}$ (left) but not in the control MSfpH5.7 (right): a) N12 (48h induction) and b) N21(24h induction).

The histochemical detection of GUS products revealed that the isolated N sequence directed the accumulation of GUS product essentially to the vascular tissues, in agreement with observations made with a bean *pgip* promoter (Devoto *et al.*, 1998) and with several genes involved in defense, that have been found to be constitutively expressed in the vascular tissues (Stanford *et al.*, 1990; Zhu *et al.*, 1993).

In previous histochemical assays (data not shown), it was observed that GUS accumulation increased upon 48 hours of induction and it was stronger in younger explants (youngest leaves from 3 weeks acclimatised plants) than in completely expanded older leaves. Due to the very acid pH (2.5-4.7) of the supernatants, it was necessary to adjust the pH to 5.5-5.7 prior to the use for induction, otherwise a severe degradation of leaf explants was observed. The pH of the culture medium was adjusted to the same values. Although the histochemical GUS detection has the advantage of permitting the localisation and direct visualisation of the transgene expression in the tissue, it does not permit an evaluation of quantitative GUS expression needed for the screening of the different lines and the elicitation assay.

In a fluorometrical assay GUS expression was measured quantitatively based on the cleavage of the substrate 4-methylumbelliferyl-ß-D-glucuronid (4-MUG) to the fluorigenic product 7-hydroxy-4-methylcoumarin (4-MU) due to ß-glucuronidase activity (Gallagher, 1992). In *in vitro* explants elicited with different supernatants and corresponding culture media, a clear GUS induction could be observed (Fig. 21). Explants elicited with the supernatants showed a much higher activity than those elicited with the corresponding culture media. However, a background fluorescence was observed in explants incubated in the culture media, as the measured 4-MU values were higher than in the non-elicited control. Supernatant $SN_{MSfpH.7}$ derived from MSfpH5.7 induced a stronger response in the leaf explant than the supernatant derived from MAMm (SN_{MAMm}).

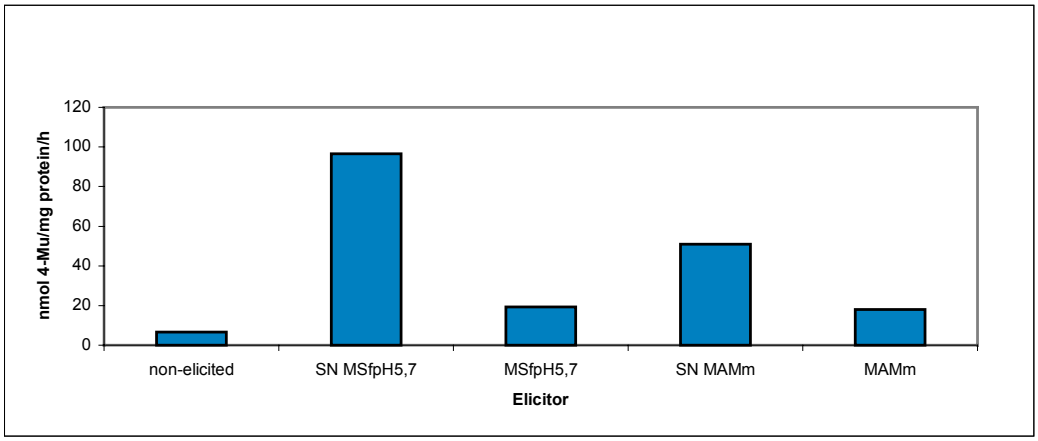


Fig. 21: GUS accumulation in an *in vitro* leaf explant from N6 upon elicitation with supernatants derived from different culture media compared to respective culture media and non-elicited tissue.

Due to the higher elicitation capacity of $SN_{MSfpH5.7}$ (see Fig. 21), derived from MSfpH5.7, (similar in composition to the medium where the plants were grown, and therefore adequate

to avoid physiological stress of the leaf explants) this supernatant was selected for the elicitation assays.

To evaluate promoter induction in transgenic tobacco plants, the assay was set up as follows: explants from young leaves of greenhouse grown plants were induced for 48h with non-diluted $SN_{MSfpH5.7}$ derived from a 14 days culture of *Botrytis cinerea*. As a control for each plant, similar explants were incubated during 48h with non-diluted MSfpH5.7. To ensure a similar physiological and developmental status of leaf explants used for control and fungal elicitation, the leaves were cut through the midrib and one half of the leaf was incubated with the supernatant and the other with the control medium.

Explants from greenhouse grown plants of seven randomly chosen lines were tested at the same time with a promoterless control line E19-3 containing the GUS gene only (Pühringer *et al.*, 2000) and a non-transgenic tobacco plant (Fig. 22). Threshold values measured in E19-3 and non-transgenic tobacco were considered as background values, not derived from promoter activity but rather from the fluorescence of the plant extract. Only transgenic plant lines showing GUS expression above threshold levels were considered to be positive. N4, N8, N12 and N21 expressed the transgene and were slightly inducible upon fungal elicitation, as a higher accumulation in the explant incubated in $SN_{MSfpH5.7}$ was observed than in the explant incubated in MSfpH5.7. In N12 and N21, GUS activity was significantly higher than in N4 and N8, indicating a clear variability among different independent lines of the same construct. S1, P32 and P47 were considered as not-expressing the transgene, as they displayed similar background levels as the negative control lines.

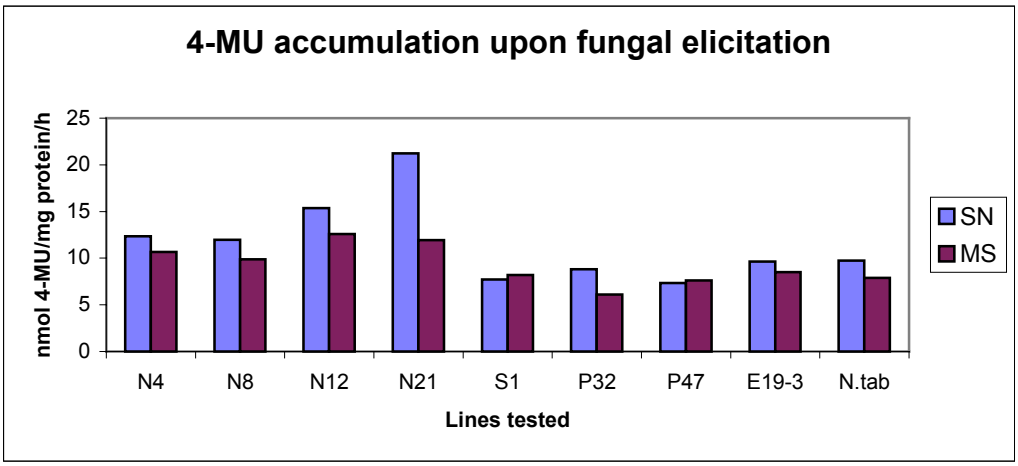
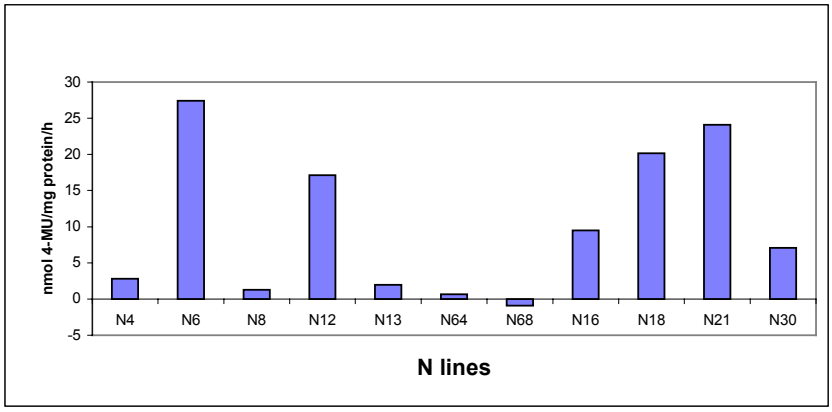


Fig. 22: 4-MU accumulation measured in fluorometrical GUS assay of explants from greenhouse grown plants upon elicitation with $SN_{MSfpH5.7}$ or MSfpH5.7.

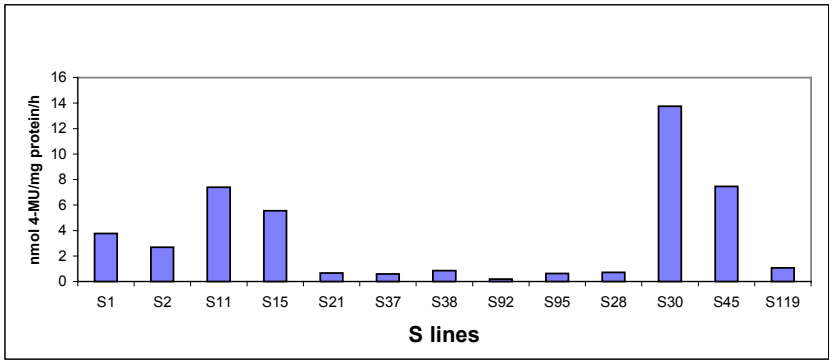
2.3.5 Selection of transgenic lines for generation of F1 progenies

In order to select the plant lines which show highest expression upon fungal elicitation, *in vitro* explants were incubated 48 hours in $SN_{MSfpH5.7}$ only and GUS activity was measured fluorometrically (Fig. 23). This assay was performed for all 3 constructs to evaluate the functionality of the promoters and the relative response of each line. For further promoter analysis, 4 plant lines showing the highest GUS expression levels were selected for constructs N and S (Fig. 23a,b). For construct P 3 high expressing lines and 1 very low expressing line (Fig. 23c) (to evaluate the behaviour of the progeny of a low-expressing line) were selected.

a)



b)



c)

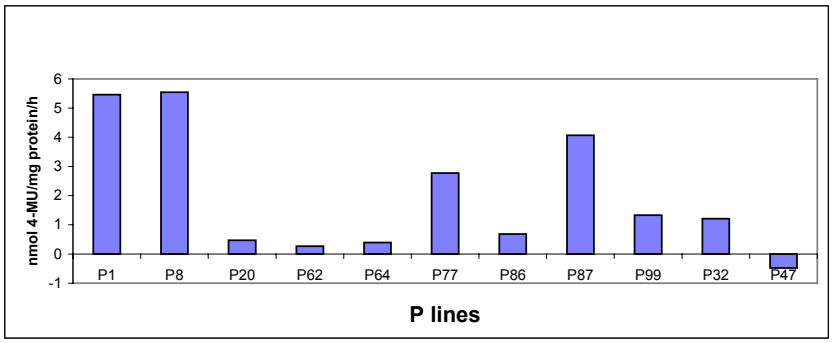


Fig. 23: GUS activity upon elicitation with $SN_{MSfpH5.7}$ of *in vitro* plant lines transformed with a) N b) S and c) P constructs.

Selected plant lines are listed in Table 12.

Table 12: Selected lines of primary transformants used to generate F1 progenies. GUS activity driven by the different promoter constructs upon elicitation with $SN_{MSfpH5.7}$ of young *in vitro* leaf explants is shown.

Construct	Promoter length	Lines selected	nmol 4-MU /mg protein/h
N	2kb	N6	27.4
		N12	17.1
		N18	20.2
		N21	24.1
S	1.45kb	S11	7.4
		S15	5.6
		S30	13.8
		S45	7.5
P	1.23kb	P1	5.5
		P62	0.3
		P77	2.8
		P87	4.1

Plant lines listed in Table 12 were acclimatised in the greenhouse and self pollinated. Seeds from these lines were germinated under sterile conditions on cotton+filter paper soaked in a solution of 350µg/ml kanamycin. Germinated green F1 plantlets were grown and clonally propagated *in vitro*. 10 plants/line were chosen and tested *in vitro* for their relative GUS expression under the conditions described for screening the mother plants. Again, an evident variability was seen among the F1 plants of the same line. For each construct 12 F1 plants, that showed the highest GUS expression (Table 13) and good growth were selected for subsequent promoter analysis.

Table 13: F1 lines derived from mother plants harbouring different promoter constructs were selected for promoter analysis. GUS activity induced in *in vitro* explants upon elicitation with $SN_{MSfpH5.7}$ could be measured in each progeny line.

N construct			**S construct**			**P construct**		
Mother plant	Selected progenies	nmol 4-MU/mg protein/h	Mother plant	Selected progenies	nmol 4-MU/mg protein/h	Mother plant	Selected progenies	nmol 4-MU/mg protein/h
N6	N6/1	23.6	S11	S11/2	10.8	P1	P1/1	7.2
	N6/9	29.8		S11/5	5.8		P1/5	9.4
	N6/10	88.1		S11/6	14.5		P1/10	7.6
N12	N12/5	28.7	S15	S15/2	10.3	P62	P62/1	4.1
	N12/9	27.1		S15/5	17.7		P62/3	3.6
	N12/10	40.8		S15/10	13.9		P62/9	5.3
N18	N18/1	105.0	S30	S30/3	14.8	P77	P77/1	5.4
	N18/5	43.8		S30/6	12.6		P77/6	6.2
N21	N21/3	70.7		S30/10	13.8		P77/9	5.9
	N21/4	71.8	S45	S45/2	8.6	P87	P87/2	10.7
	N21/6	40.0		S45/9	6.0		P87/4	8.0
	N21/8	31.6		S45/10	6.1		P87/9	8.7

2.3.6 Analysis of promoter features in tobacco plants upon elicitation with fungal products

Expression of the GUS reporter gene driven by the different *pgip* promoters was analysed in the selected F1 progenies of the transgenic lines. The promoter inducibility upon fungal elicitation was investigated in plants acclimatised to greenhouse conditions. The plant line E19-3 (IV 2.3.4) was included as a negative control in the elicitation assays. The significantly higher GUS activity from N, S and P lines, compared to the background level of E19-3, confirmed that all the promoter constructs were still active in the progeny lines. Obtained results show a clear promoter activation upon elicitation with $SN_{MSfpH5.7}$. The strong variability observed between independent tests is most likely due to different physiological conditions of the plants and due to the different lines.

Transgenic F1 lines harbouring construct N generally displayed high GUS expression levels. In addition, the fungal inducibility was rather low, due to a high basal expression. Only 5 (N6/9, N6/10, N18/1, N18/5 and N21/3) of the 12 lines tested showed significant inducibility (Fig. 24a).

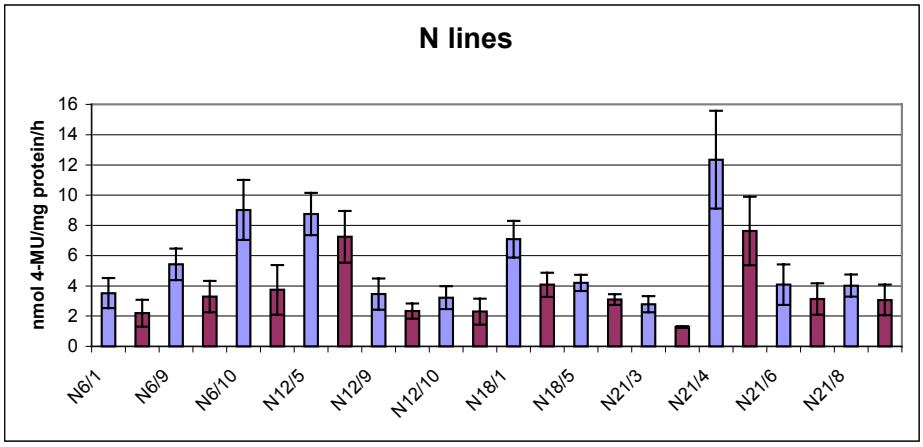
In contrast to construct N, all the progeny lines from construct S displayed a significant increase in GUS activity when the explant was induced with $SN_{MSfpH5.7}$, in comparison with the control induction with MSfpH5.7 (Fig. 24b).

Compared to constructs N and S, progeny lines from construct P displayed an intermediate response towards fungal elicitation. 10 from the 12 tested lines showed a significant increase of 4-MU accumulation in the fluorometrical assay (Fig. 24c). Only for P87/2 and P87/9 no clear inducibility could be seen.

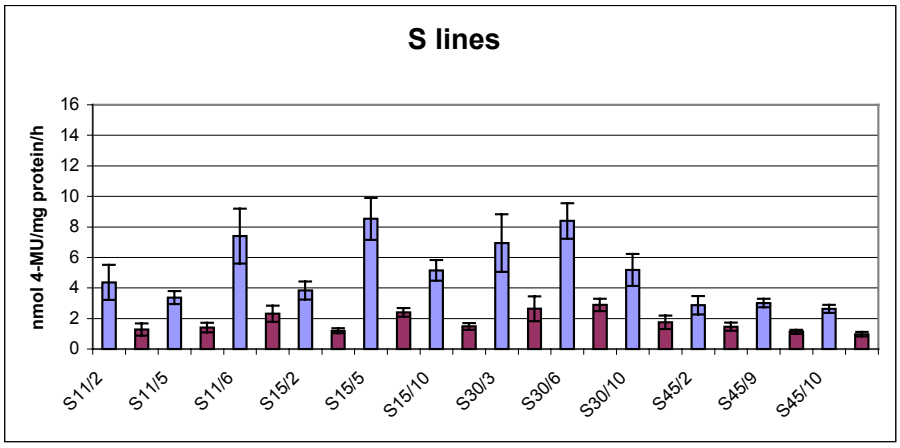
The N and the S constructs showed a high variability within the progenies from independent transformants. In contrast, the different lines from each P transformant showed a much more homogeneous expression pattern. Interestingly, no clear correlation of GUS expression between the independent transformants and their progenies could be observed. Evidence for this gave the result obtained with the progenies from plant line P62. Although this line was originally selected as a low expressing line, with 4-MU values comparable to negative control plants, its progenies showed a high expression of the transgene in response to fungal elicitation, even comparable to plants derived from previously considered stronger lines.

Summarising the results obtained, both isolated *pgip*-regulating sequences proved to be functional promoters. The S and P constructs, derived from clone Q8, responded clearly to the fungal elicitation and are more inducible than N, derived from clone N17. Lines derived from P construct seemed to be slightly weaker than S, but still clearly inducible upon fungal elicitation. These data support the suggestion that *pgip* genes are expressed in response to fungal endopolygalacturonases (Yao *et al.*, 1999). Most significant inducibility upon fungal elicitation was achieved with the construct S.

a)



b)



c)

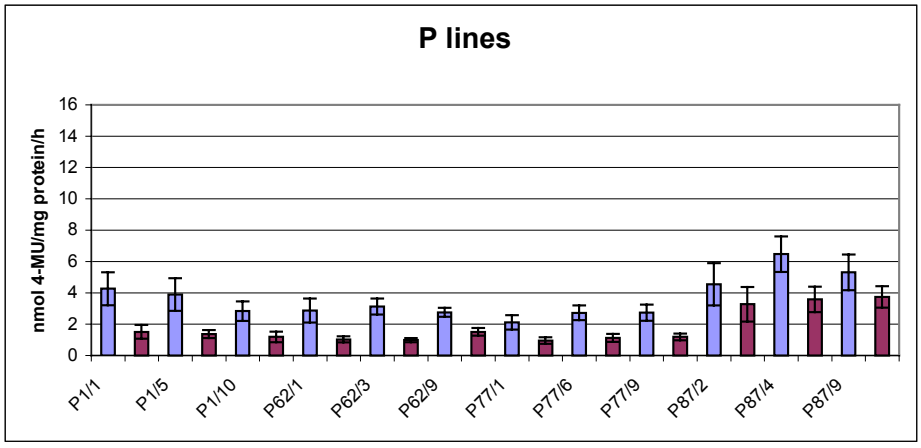


Fig. 24: 4-MU accumulation measured in the GUS assay of greenhouse explants from transgenic F1 lines harbouring construct N (a), construct S (b) and construct P (c) upon elicitation with $SN_{MSfpH5.7}$ (blue bars) and corresponding medium MSfpH5.7 (dark red bars). Each bar represents mean values of 5 independent experiments. Standard deviation of the calculated mean is presented as error bar.

3. Investigation of interaction ´Florina´- *Venturia inaequalis*

3.1 Analysis of altered gene expression by DDRT-PCR

DDRT-PCR is a method to identify and isolate genes that are differentially expressed at a mRNA level in different sets of eukaryotic cells. Using specifically designed 3´-anchor oligo(dT) primers, the mRNA populations of the different sets are divided into subpopulations and reverse-transcribed. Fragments of the generated cDNA populations are amplified using the same 3´-anchor primer in combination with 10-mer arbitrary primers and the PCR products are separated on denaturing polyacrylamide gel in order to identify differentially displayed bands. Reamplification and cloning of these bands allows the identification of the sequences of the corresponding genes (Liang *et al.*, 1995).

In order to identify genes that are activated or upregulated in the host leaves upon scab inoculation, DDRT-PCR was performed to compare the gene expression pattern between leaves of the resistant cultivar ´Florina´ either inoculated with a conidial suspension of *Venturia inaequalis* (infected) or with water (non-infected).

Total RNAs from non-infected and infected ´Florina´ leaves collected 11 days after inoculation were purified, DNase-treated and used as templates for independent reverse-transcription reactions using one of 3 oligo(dT)anchor primers (dT_{GG}, dT_{CC} or dT_{AG}) to generate different pools of cDNAs. These subsets of cDNAs were amplified with 10-mer arbitrary primers in combination with the anchor oligo(dT)primer used for reverse-transcription reaction. Several primer combinations were used, dT_{GG} x DD1-DD10, dT_{AG} x DD1, DD8, DD9 and dT_{CC} x DD1, DD4, DD6, DD8, DD9. Display amplification reactions included as template 1µl cDNA from non-infected sample (C), 1µl cDNA from infected sample (I) and an equivalent amount of DNase-treated RNA not reverse-transcribed from the infected sample (N) as a control for the DNase I treatment. Amplified cDNA fragments were separated on a 4.5% polyacrylamide (PAA) gel in Tris-borate buffer and silverstained (Fig. 25).

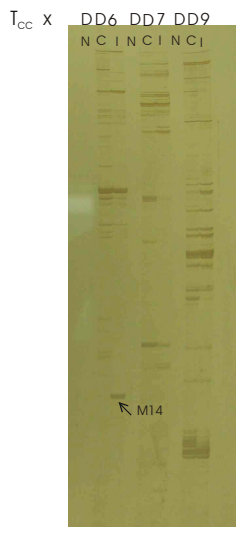


Fig. 25: Example of 4.5% polyacrylamide gel stained with silver nitrate. cDNA from non-infected (C) and infected (I) samples, as well as DNase-treated RNA not reverse-transcribed from the infected sample (N) were amplified with oligo dT_{CC} and either random primer DD6, DD7 or DD9. M14: example of differentially displayed band (see text).

The cDNA patterns of non-infected and infected samples were compared to identify differentially displayed cDNA bands with greater abundance in the infected sample. The appearance of bands in infected but not reverse-transcribed control sample (N) revealed that the DNase I treatment was not complete, but it was verified that the selected bands did not appear in this sample. With some primer combinations cDNA fragments appeared in the non-infected sample but not in the infected sample, suggesting that some genes could have been suppressed after infection. More promising fragments were considered to be those whose corresponding genes seemed to be exclusively expressed in the infected leaves (visible as cDNA bands in the lanes of infected sample, but not in the control lanes in the PAA gel).

17 cDNA fragments, whose corresponding genes seemed to be upregulated or specific in infected leaves, resulted from the 18 different primer combinations (Table 14). These cDNA fragments were excised from the gel and the DNA was purified from the polyacrylamide for reamplification. From the excised fragments, 11 fragments could be reamplified with the expected sizes, either directly from the eluate (M3, M6, M9, M10) or after precipitation (M2, M4, M5, M11, M12, M13, M14). Reamplified cDNAs were cloned into pCR2.1 or pCRII.1 and sequenced with vector-specific primers flanking the cloning site. All 17 excised differential display fragments are listed in Table 14.

Only in two of the reamplified fragments (M3, M4) a poly(A) tail was undoubtedly recognised. The complete sequences of the corresponding arbitrary primers could be identified in all fragments, except in M9.

Comparison of nucleotide sequences revealed a very high similarity (96.3%) between M5 and M6. The few mismatches could result either from sequence inaccuracies or eventually, from isoforms of the expressed gene. A complete identity between M11 and M12 was observed, which might result most probably from an inaccuracy during the excision of the band from the acrylamide gel. These fragments were further referred as M11.

Table 14: Isolated display fragments M1-M17, corresponding primer combinations and putative differential expression are presented. ´-/+´= present only in infected sample; ´+/++´= present in both, but upregulated in infected sample.

Display Fragment	Primer combination	Displayed bands in PAA gel		Estimated size on the PAA gel	Reamplification and cloning	Size of sequenced insert
		non-infected	infected			
M1	DD9 x dT_{GG}	-	+	800 nt	not	
M2	DD1 x dT_{GG}	-	+	600-700 nt	yes	662 nt
M3	DD4 x dT_{GG}	-	+	400 nt	yes	387 nt
M4	DD6 x dT_{GG}	-	+	500 nt	yes	503 nt
M5	DD7 x dT_{GG}	-	+	300-400 nt	yes	304 nt
M6	DD7 x dT_{GG}	-	+	300-400 nt	yes	297 nt
M7	DD1 x dT_{GG}	+	++	300-400nt	not	
M8	DD1 x dT_{AG}	-	+	800-900 nt	not	
M9	DD1 x dT_{AG}	-	+	400 nt	yes	411 nt
M10	DD1 x dT_{AG}	-	+	300-400 nt	yes	350 nt
M11	DD1 x dT_{CC}	+	++	300-400 nt	yes	342 nt
M12	DD1 x dT_{CC}	+	++	300-400 nt	yes	342 nt
M13	DD4 x dT_{CC}	+	++	300-400 nt	yes	318 nt
M14	DD6 x dT_{CC}	-	+	300 nt	yes	288 nt
M15	DD6 x dT_{CC}	-	+	900-1000 nt	not	
M16	DD7 x dT_{CC}	-	+	700-800 nt	not	
M17	DD7 x dT_{CC}	-	+	700 nt	not	

cDNA sequences and their deduced amino acid sequences were compared with sequences stored in NCBI and TAIR (The Arabidopsis Information Resources) databases, in order to identify their putative function. Homologies found on a protein level, summarised in Table 15, revealed similarity of some fragments with proteins involved in biosynthesis of cell walls (M9) and cuticle layer (M10), proteins involved in cell defense (M3, M5, M6), proteins participating in the synthesis of anthraquinones (M2), and with a protein involved in pre-rRNA processing (M11). For some fragments (M4, M13, M14) database searches revealed no significant homology to any gene with known function, neither at nucleotide nor at amino acid level.

Table 15: Comparison of deduced protein sequences from identified cDNA fragments with proteins stored in the TAIR database.

Fragment	Putative ORF	Homologous protein in *Arabidopsis thaliana*		
		% similarity / matching region	Size	Indicated function
M2	79 aa	76% / 50 aa	894 aa	probable menaquinone biosynthesis protein (Acc. no. AAF07354)
M3	39 aa	92% / 32 aa	242 aa	glutathione transferase (Acc. no. BAB11100)
M4	132 aa	57% / 123 aa	221 aa	hypothetical protein At2g46150 (Acc. no. AAC62891)
M5	101 aa	49% / 42 aa	1232 aa	disease resistance protein RPS4 (Acc. no. BAB11393)
M6	99 aa	52% / 50 aa	1232 aa	disease resistance protein RPS4 (Acc. no. BAB11393)
M9	59 aa	88% / 27 aa	1084 aa	cellulose synthase catalytic subunit (Acc. no. AAC39335)
M10	61 aa	89% / 61 aa	497 aa	very-long-chain fatty acid condensing enzyme CUT1 (Acc. no. AF129511)
M11	113 aa	94% / 109 aa	294 aa	putative U3 small nucleolar ribonucleoprotein protein (Acc. no. AAG52427)
M13	98 aa	77% / 32 aa	168 aa	hypothetical protein AAG29205.1 (Acc. no. AAG29205)
M14	95 aa	50% / 87 aa	235 aa	hypothetical protein F24B22.160 (Acc. no. CAB70994)

Due to the doubtless homology of M11 with a U3 small nucleolar ribonucleoprotein protein, involved in early processing of ribosomal RNA, which is a part of the basic metabolism of plant cells (Venema *et al.*, 2000), this clone was not further investigated, since no participation of these proteins in any defense mechanism was found in the literature. cDNA clones showing significant similarities in their deduced amino acid sequences with proteins involved in plant defense (M3, M5, M6) or participating in the formation of cell wall and cuticle layer (M9, M10) were considered to be most interesting, since it is known, that in the apple-scab interaction the fungus penetrates the cuticle and remains limited in the subcuticular space, where the resistance mechanism is expressed and the fungal development impaired (Chevalier and Lespinasse, 1994).

M2, M3, M6, M9, M10 and also those to which no putative function could be attributed (M4, M13, M14) were further investigated in order to confirm differential expression of the corresponding genes. M5 was excluded from further experiments due to the high homology between M5 and M6.

3.2 Verification of the differential expression of the corresponding genes

Due to the fact, that the low stringent conditions used for DDRT-PCR reaction can result in the identification of false positives, an important step after identification of the sequences of the isolated cDNA fragments was to confirm the differential expression of their corresponding genes by specific RT-PCR and Northern analysis.

3.2.1 RT-PCR reaction with fragment-specific primers

Using dT_{VN} as anchor primer, a general reverse-transcription reaction was performed with the same RNA pools, derived from non-infected and infected leaves, as for DDRT-PCR. An equal amount of RNA from the non-infected and infected sample was used in the reverse-transcription reaction, which was checked visually in a gel stained with EtBr on the basis of the intensity of rRNA bands. Specific primers derived from the sequences of the cloned cDNAs (M2, M3, M4, M6, M9, M10, M13 and M14) were used to amplify specific partial sequences from the genes corresponding to the original display fragments from the generated cDNA pools. The same amount of cDNA from both samples was used in the PCR amplification reactions with the specific primers and an exact amount of PCR product from both samples was loaded on a 2% agarose gel. The ubiquitously occurring gene calmodulin (CAM) turned out to be similarly expressed in both cDNA pools (Fig. 26) and was therefore used as an internal control for the cDNA amount, being amplified in parallel with the specific PCR reactions for the selected cDNAs.

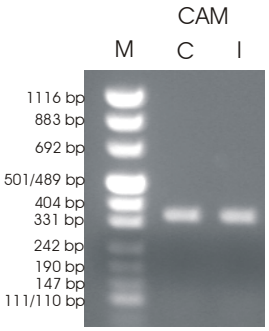


Fig. 26: Specific RT-PCR amplification of a 356 bp fragment of the CAM gene from cDNA pools of non-infected (C) and infected (I) samples as internal control for equal cDNA amount. PCR amplification was performed for 35 cycles and PCR products were separated on a 2% agarose gel stained with EtBr. pUCMix was used as DNA standard (M).

As the clones were unequally abundant, different numbers of PCR cycles were carried out. As standard approach, 35 cycles of amplification were performed. However, in cDNA clones where the amplification reached the plateau with 35 cycles (as for M9 and M10, data not shown), PCR amplifications were run for 25 cycles or 30 cycles. Differential amplification of cDNAs was observed for M9 after 25 cycles (Fig. 27), M10 after 30 cycles (Fig. 28) and M3, M4, M13, M14 after 35 cycles (Fig. 29), with the strongest upregulation of M4. As shown in

Fig. 27, 25 cycles of amplification were enough to display differential expression of M9 (more abundant expression in infected sample), however for M10 the product was merely visible. Therefore 5 additional cycles were performed to demonstrate the differential expression of M10 (Fig. 28). RT-PCR reactions to prove the differential expression for M2 and M6 were not sucessful, as either only unspecific smear appeared (M2) or no products (M6) could be amplified (Fig. 30).

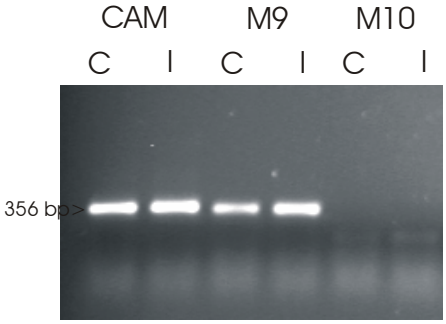


Fig. 27: Differential accumulation of fragments M9 (347bp) and M10 (234bp) upon specific RT-PCR amplification from cDNA pools of non-infected (C) and infected (I) samples for 25 cycles. CAM amplification was performed in parallel as internal control. PCR products were visualised on a 2% agarose gel stained with EtBr.

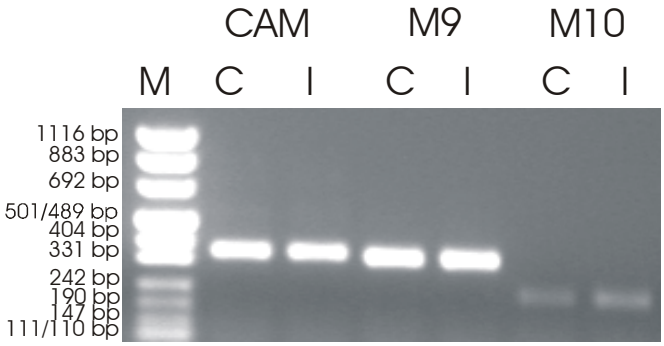


Fig. 28: Differential accumulation of fragments M9 (347bp) and M10 (234bp) upon specific RT-PCR amplification from cDNA pools of non-infected (C) and infected (I) samples for 30 cycles. CAM amplification was performed in parallel as internal control. PCR products were visualised on a 2% agarose gel stained with EtBr. pUCMix was used as DNA standard (M).

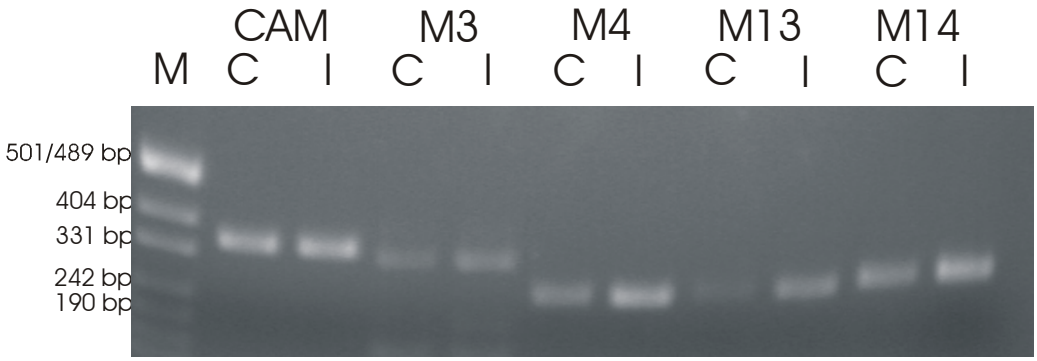


Fig. 29: Differential accumulation of fragments M3 (336bp), M4 (259bp), M13 (270bp) and M14 (277bp) upon specific RT-PCR amplification from cDNA pools of non-infected (C) and infected (I) samples for 35 cycles. CAM amplification was performed in parallel as internal control. PCR products were visualised on a 2% agarose gel stained with EtBr. pUCMix was used as DNA standard (M).

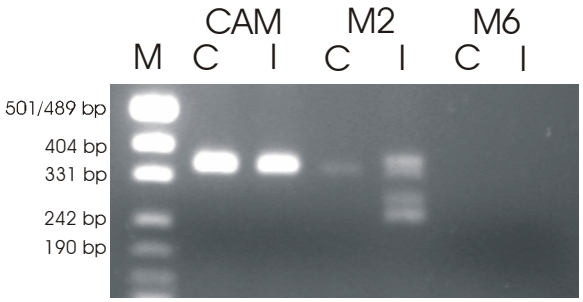


Fig. 30: RT-PCR amplification of M2 (348bp) and M6 (278bp) from cDNA pools of non-infected (C) and infected (I) samples after 35 cycles. CAM amplification was performed in parallel as internal control. PCR products were visualised on a 2% agarose gel stained with EtBr. pUCMix was used as DNA standard (M).

Differential gene expression, which was assumed due to the exclusive appearance of bands in the infected sample on the PAA gel, could not be confirmed in the specific RT-PCR, since it was possible to amplify the different fragments also from the non-infected cDNA pool. Nevertheless, the observed lower abundance in the non-infected sample indicated that the isolated cDNAs were upregulated in the infected sample.

3.2.2 Northern analysis

Due to the limitation of RT-PCR as a semiquantitative method, Northern analyses should be performed to confirm differential expression of the genes. In Northern analysis using 10µg total RNA isolated from non-infected and infected ´Florina´ leaves, the hybridisation with the DIG-labeled riboprobe of M9, whose transcript seemed to be the most abundant one (known from RT-PCR), did not give any signal, most likely due to the too low sensitivity of the non-radioactive detection system. For this reason, the analyses of the transcripts that correspond to the M3, M4 and M10 clones were not carried out.

3.3 Elongation of display fragments using RACE technology

In order to generate the corresponding full-length sequences of some selected partial cDNAs (M2, M3, M4, M6, M9, M10, M13, M14), the RACE (rapid amplification of cDNA ends) technique was performed. This method allows the amplification of unknown sequence regions from corresponding mRNAs, requiring only the knowledge of a single short sequence within the mRNA of interest and is therefore used for cloning of the remaining 5’ or 3’ end of an uncomplete cDNA (Frohman *et al.*, 1988). The key feature of this PCR method is the use of a specific primer designed from a known cDNA sequence in combination with a general primer complementary either to the mRNA poly(A) tail (for 3’RACE) or to a homopolymer tail that has been added to the 5’end of the mRNA (for 5’RACE).

Specific primers for elongation in each direction were designed based on the sequences obtained from differential display fragments. Primers were located at least 100 bases before

the cDNA end in order to guarantee sufficient overlapping regions to prove the specificity of the RACE products. Clones M3 and M4, containing a poly(A) tail, and clone M10, which displayed homology until the stop codon of the putative homologue protein, were only elongated in the 5´direction.

After 27 cycles of amplification, only 3´RACE elongations of M9 and M14 were clearly visible in an agarose gel. The remaining reactions were then subjected to 10 additional amplification cycles. The 5´RACE experiments were less successful than the 3´RACE as the amplification products in 5´ RACE were weaker, and did not always result in a single band (Fig. 31). In 3´ RACE, the amplification reactions were more specific, with exception of M2, in which only a smear was obtained and therefore excluded from cloning. In case of multiple bands, the largest well visible band was excised (see arrows). Resulting fragments were purified from agarose gel, cloned into pCRII.1 and sequenced with vector-specific primers flanking the cloning site.

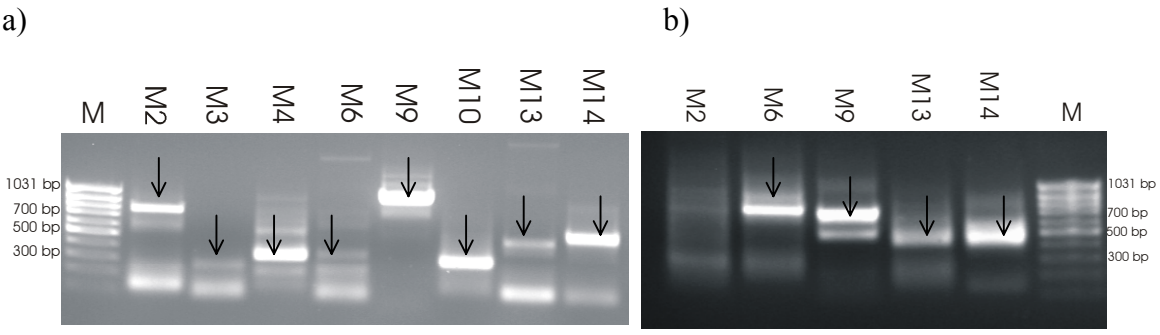


Fig. 31: a) 5´ and b) 3´ RACE fragments of indicated cDNA clones were separated on 1.2% agarose gel stained with EtBr. 100bp DNA Ladder was used as DNA standard (M).

5´RACE elongations of M4 and M10 cDNAs provided information about additional sequence of 154bp and 127bp, respectively. 3´RACE of M6, M9 and M13 cDNAs unraveled 272bp, 175bp and 31bp of additional sequence, respectively, including their poly(A) tails. Attempts of cloning 5´RACE fragments of M2, M9 and M14, as well as 3´RACE fragment from M14 failed, as no colonies with inserts were obtained. Sequenced 5´RACE clones of M3 and M6 only revealed homology in the primer region and the sequenced insert of M13 showed no homology with the overlapping part of the display sequence. Results derived from RACE are summarised in Table 16. The deduced amino acid sequences of the elongated cDNA clones were compared with sequences stored in TAIR database in order to identify their putative

function. Homologies that had been found with the original display fragments could be matched with those, found with the entire clones. However, no further information about the putative function of M4, M13 and M14, which displayed only homologies to hypothetical proteins of *Arabidopsis thaliana*, was gained.

Table 16: Summary of RACE experiments.

Fragment	5´RACE			3´RACE			Size of entire cDNA	
	Fragment cut (estimated)	Overlapping region	Elongation of unknown sequence	Fragment cut	Overlapping region	Elongation of unknown sequence	bases	amino acids
M2	700 bp	-	-	none	-	-		
M3	250 bp	28 bp	-					
M4	300 bp	138 bp	154 bp				583	181
M6	320 bp	28 bp	-	600 bp	162 bp	272 bp	571	122
M9	900 bp	-	-	700 bp	271 bp	175 bp	585	73
M10	300 bp	114 bp	127 bp				477	103
M13	400 bp	-	-	350 bp	191 bp	31 bp	349	98
M14	450 bp	-	-	500 bp	-	-		

4. Isolation of apple chitinase genes

4.1 Cloning and identification of chitinase genes

Degenerate primers were designed from highly conserved regions from known class III chitinase sequences (as indicated in Fig. 32) to synthesise a probe by PCR amplification using apple genomic DNA from cultivar ´McIntosh´ as template. A genomic library from *Malus* x *domestica* cultivar ´McIntosh´ ´Wijcik´(Watillon *et al*., 1992) was screened for putative full length clones with the DIG-labeled chitinase probe. After 3 rounds of subscreening, 6 phage clones were isolated and amplified. 3 clones were selected for DNA purification from bacteriophages, and subsequently DNA was digested with various enzymes. Clonable fragments were identified by Southern hybridisation with the same probe as for the library screening and subcloned into the Bluescript vector (Table 17). Vector primers flanking the cloning site and sequence-based primers were used to identify the entire sequences of the isolated clones. 3 different genes coding for chitinases (*chi*) could be isolated.

Table 17: Genomic *chi* clones derived from library screening. Size of the fragments was estimated from agarose gels, except for coding regions (calculated from sequences).

Clone	Restriction enzymes	Size of insert in pBS	upstream region from Start codon	Coding sequence	downstream region from Stop codon
C1	*Eco*RV	4.5 kb	1.3 kb	859 bp	-
C1	*Bam*HI/*Sac*I	2.4 kb	1.45 kb	859 bp	-
C5	*Bam*HI	3 kb	2.1 kb	900 bp (full length)	22 bp
C20	*Bam*HI/*Sac*I	3.2 kb	1.55 kb	897 bp (?)	700 bp

For C5, the entire coding region was identified. A *Sac*I site located at the 3´end of clone C1 marked the end of the insert in the original bacteriophage λ clone. Therefore, apparently 41 bases at the 3´end of the coding sequence are missing and could not be identified by further subcloning. C20 was not included in further work since a reading frame could not clearly be deduced from the sequences obtained. A 2-fold repetition of the motif CCGG at the 5´end of the putative coding sequence, unique for this clone, made any translational analysis impossible.

4.2 Characterisation of acidic chitinase genes in apple

4.2.1 Sequence analysis

The genomic clones C1 and C5 displayed a single open reading frame without introns. C5 encoded a polypeptide of 299 amino acid residues, with a calculated molecular mass of

31.6kDa and a pI of 4.6. Comparison between deduced amino acid sequences from C1 and C5 revealed 92.7% similarity. These sequences show high similarity with different acidic chitinases from class III (Fig. 32).

```
C1              ---MAPKSTMS-LALLSLVTLVLALGANAGGIAIYWGQNGNEGTLAETCATGNYQFVNVA 56
C5              ---MASKSTATFLALLSLVTLVLALGANAGGIAIYWGQNGNEGTLAETCASGNYQFVNVA 57
Fragaria        ---MASVSAAALLSLATL--LLLAVGSNAGGIAIYWGQNGNEGTLAQTCASGNYQFVNIA 55
Vitis           ---MARTPQSTPLLISLSV-LALLQTSYAGGIAIYWGQNGNEGTLTQTCNTGKYSYVNIA 56
Arabidopsis     MTNMTLRKHVIYFLFFISCSLSKPSDASRGGIAIYWGQNGNEGNLSATCATGRYAYVNVA 60
Nicotiana       -------MIKYSFLLTALVLFLRALKLEAGDIVIYWGQNGNEGSLADTCATNNYAIVNIA 53
Cicer           ------MEKCFNIIPSLLLISLLIKSSNAAGIAVYWGQNGNEGSLQDACNTNNYQFVNIA 54
Glycine         ---MKTPNKASLLLFPLLSLSLFINHSHAAGIAVYWGQNGGEGTLAEACNTGNYQYVNIA 57
                            :                ..*.:******.**.*  :* :..*  **:*

C1              FLTTFGNGRTPAINLAGHCDPTTDECTKLSPEIRSCQAKGIKVILSIGGASG-SYSLTSA 115
C5              FLTTFGNGQTPAINLAGHCDPTTEECTKLSPEIKSCQAKGIKVILSIGGASG-SYSLTSA 116
Fragaria        FHSSFGNGRTPTLNLAGHCDPSSNTCTKFSSQIKSCQAEGIKVILSVGGGWGGQYSLASS 115
Vitis           FLNKFGNGQTPEINLAGHCNPASNGCTSVSTGIRNCQNRGIKVMLSIGGGAG-SYSLSSS 115
Arabidopsis     FLVKFGNGQTPELNLAGHCNPAANTCTHFGSQVKDCQSRGIKVMLSLGGGIG-NYSIGSR 119
Nicotiana       FLVVFGNGQNPVLNLAGHCDPNAGACTGLSNDIRACQNQGIKVMLSLGGGAG-SYFLSSA 112
Cicer           FLSTFGNGQNPQINLAGHCDPSTNGCTKFSPEIQACQAKGIKVLLSLGGGAG-SYSLNSA 113
Glycine         FLSTFGNGQTPQLNLAGHCDPNNNGCTGLSSDINTCQDLGIKVLLSLGGGAG-SYSLSSA 116
                *   ****:.* :******:*    ** ..  :. **  ****:**:**. * .* : *

C1              ADARQVATYLWNNFLGGHSSSRPLGAAVLDGIDFDIEGGTDQYWDDLARYLS--GYSKRG 173
C5              DDARQVATYLWNNFLGGQSSSRPLGAAVLDGIDFDIEGGTDQHWDDLARYLS--GYSKRG 174
Fragaria        EDARQVGAYLWNNFLGGHSTSRPLGDAVLDGVDFDIEGGNDQYWDDLARYLS--AHSKKG 173
Vitis           NDAQNVANYLWNNFLGGQSSSRPLGDAVLDGIDFDIELGSTLHWDDLARALSRIEFQQER 175
Arabidopsis     EDAKVIADYLWNNFLGGKSSSRPLGDAVLDGIDFNIELGSPQHWDDLARSLSKFSH---R 176
Nicotiana       DDARNVANYLWNNYLGGQSNTRPLGDAVLDGIDFDIEGGTTQHWDELAKTLSQFSQQ--- 169
Cicer           EEATTLANYLWNNFLGGTSTSRPLGDAVLDGIDFDIESGG-QHYDELAKALNGFSQQ--- 169
Glycine         DDATQLANYLWENFLGGQTGSGPLGDVILDGIDFDIESGGSDHYDDLARALNSFSSQS-- 174
                 :*  :. ***:*:*** : : *** .:***:**:** *   ::*:**: *.

C1              -KKVYLTAAPQCPFPDAWIGNALKTGLFDNVWVQFYNNPPCQYTSGDVANLEDAWKQWTS 232
C5              -KKVYLTAAPQCPFPDAYVGNALKTGLFDNVWVQFYNNPPCQYASGDVTNLEDAWKQWTS 233
Fragaria        GKKVYLTAAPQCPFPDANIGNALKTGLFDNVWVQFYNNPPCQYTSGNVTNLEDAWKQWTS 233
Vitis           GRKVYLTAAPQCPFPDKVPGTALNTGLFDYVWVQFYNNPPCQYSSGNTNNLLNSWNRWTS 235
Arabidopsis     GRKVYLTGAPQCPFPDRLMGSALNTKRFDYVWIQFYNNPPCSYTSGNTQNLFDSWNKWTT 236
Nicotiana       -RKVYLTAAPQCPFPDTWLNGALSTGLFDYVWVQFYNNPPCQYSGGSADNLKNYWNQWN- 227
Cicer           --KVYLSAAPQCPYPDAHLDSAIQTGLFDYVWVQFYNNPQCQYSNGNINNLVNAWNQWT- 226
Glycine         --KVYLSAAPQCIIPDAHLDAAIQTGLFDYVWVQFYNNPSCQYSSGNTNDLINSWNQWI- 231
                  ****:.****  **   . *:.*  ** **:****** *.*:.*.  :* : *::*

C1              AIPAHKIFLGLPAAPQAAGSG-FIPASDLNSQVLPAIKNSGKYGGVMLWSKYYDD----- 286
C5              AIPADKIFLGLPAAPQAAGSG-FIPATDLSSQVLPAIKSSAKYGGVMLWSKYYDDPDGYS 292
Fragaria        AIPAQQVFLGLPAAPEAAGSG-FIPAAALTTTVLPGIKTSDKYGGVMLWSKYYDDLYGYS 292
Vitis           SINSTGSFMGLPASSAAAGRG-FIPANVLTSQILPVIKRSPKYGGVMLWSKYYDDQSGYS 294
Arabidopsis     SIAAQKLFLGLPAAPEAAGSG-YIPPDVLTSQILPTLKKSRKYGGVMLWSKFWDDKNGYS 295
Nicotiana       AIQAGKIFLGLPAAQGAAGSG-FIPSDVLVSQVLPLINGSPKYGGVMLWSKFYDN--GYS 284
Cicer           SSQAKQVFLGVPASDAAAPSGGLIPADVLTSQVLPAIKTSPKYGGVMIWDRFNDAQSGYS 286
Glycine         TVPASLVFMGLPASEAAAPSGGFVPADVLTSQILPVIKQSSNYGGVMLWDRFNDVQNGYS 291
                :  :   *:*:**:  **  *  :*.  * : :** :: * :*****:*.:: *

C1              --------
C5              SSIKNDV- 299
Fragaria        SSIKNHV- 299
Vitis           SSIKSSV- 301
Arabidopsis     SSILASV- 302
Nicotiana       SAIKANV- 291
Cicer           NAIKGSV- 293
Glycine         NAIIGSVN 299
```

Fig. 32: Alignment of deduced amino acid sequences from C1 and C5 with different plant class III chitinases: *Fragaria* x *ananassa* (Accession no. AF134347), *Vitis vinifera* (Accession no. Z68123), *Arabidopsis thaliana* (Accession no. AB006069), *Nicotiana tabacum* (Accession no. Z11563), *Cicer arietinum* (Accession no. X70660) and *Glycine max* (Accession no. AB007127). Degenerate primers were designed from yellow shadowed regions. Region of putative active site for class III chitinases is shadowed in blue. Glutamic and aspartic residues are red. Conserved 6 cysteine residues are blue. * = identical residues; : = conserved residues; . = semi-conserved residues.

Comparison of the deduced amino acid sequences from C1 and C5 with class III chitinases of *Fragaria* sp. (Accession no. AF134347) and *Vitis* sp. (Busam *et al*., 1997) showed between 67% and 78% identity. Significant stretches of homology were also identified in the deduced amino acid sequences from C1 and C5 and an antifungal chitinase from *Rhizopus oligosporus* (Terakawa *et al.*, 1997). The similarity regions between deduced amino acid sequence from C5 and the chitinase I from *Rhizopus oligosporus* are presented in Fig. 33.

```
C5   : 32  YWGQNG-----NEGTLAETCASGNYQFVNVAFLTTFGNGQTPAINLAGHCD----PTTE- 81
           YWGQN       + +L   C SG    V ++FL  F  G TP INL+  C     P T+
CHI1 : 34  YWGQNSAGGSNTQASLGTYCESGQVDAVLLSFLHVFNVGGTPEINLSSACAGTYFPNTQL 93

C5   : 82  -ECTKLSPEIKSCQAKGIKVILSIGGASGSYSLTSADDARQVATYLWNNFLGGQSSSRPL 140
             C  +  +IK CQ KG+KVILS+GGA+G Y  TS    +Q A  +WN F GG S +RP
CHI1 : 94  LSCPAVGADIKKCQDKGVKVILSLGGAAGVYGFTSDAQGQQFAQTIWNLFGGGSSDTRPF 153

C5   : 141 GAAVLDGIDFDIEGGTDQHWDDLARYLSGYSKRGKKVYLTAAPQCPFPDAYVGNALKTGL 200
           G AV+DG+D DIEGG    +      L    K      + AAPQCPFPDA +G+ L +
CHI1 : 154 GDAVIDGVDLDIEGGASTGYAAFVNALR--QKFSSNFLIGAAPQCPFPDAILGSVLNSAS 211

C5   : 201 FDNVWVQFYNNPPCQYASGDVTNLEDAWKQWTSAIPADKIFLGLPAAPQAAGSGFIPATD 260
           FD V VQFYNN      S    +  D W + TS     KI   +P +P AAGSG++P +
CHI1 : 212 FDYVNVQFYNNYCSATGSSFNFDTWDNWAKTTSPNKNVKIMFTIPGSPTAAGSGYVPMST 271

C5   : 261 LSSQVLPAIKSSAKYGGVMLW 281
           L + V       + YGGV +W
CHI1 : 272 LQTIVPSLASEYSSYGGVSVW 292
```

Fig. 33: Comparison of deduced amino acid sequence from C5 with the CHI1 from *Rhizopus oligosporus* (Acc. no. P29026). --- = indicates gaps in sequence; + = conserved amino acid substitutions.

By computer analysis, the deduced amino acid sequences from both clones revealed the presence of a sequence – LDGIDFDIE - characteristic for the active site of chitinase family 18 (family 18 glycosyl hydrolases, PR-8), where the class III chitinases are included (Neuhaus *et al.*, 1996). In this region, glutamic acid and aspartic acid residues in positions identical with Asp-125 and the catalytic Glu-127 of *Hevea* class III chitinase, were also identified in C1 and C5, as shown in Fig. 32. These amino acids are proposed to be involved

in the catalytic site of plant class III chitinases (Yeboah *et al.*, 1998) and therefore conserved in the enzymatically active proteins. The deduced amino acid sequences of both isolated clones showed also six cysteine residues, which are characteristic for class III chitinases (Lawton *et al.*, 1992). These sequences did not show any putative N-glycosylation sites.

In the identified chitinase genes, amino-terminal sequences with characteristics of signal peptides could be deduced. According to the computer analysis (Nielsen *et al.*, 1997), C1 and C5 encode a putative hydrophobic signal peptide of 25 and 21 amino acid residues, respectively, indicating that the deduced proteins are secretory proteins. The vacuolar or extracellular localisation of each isoform of chitinase is a fixed property dependent upon its amino acid sequence. The C-terminal sequence GLLVDTM has been shown to be necessary and sufficient for targeting chitinases to the vacuole (Neuhaus *et al.*, 1991a). The lack of a hydrophobic sequence similar to this motif at the C-terminus of the deduced amino acid sequences suggests that both chitinases are located extracellularly.

4.2.2 Genomic organisation

Copy number of chitinase genes was estimated by Southern analysis using genomic DNA from apple cultivars ´Jonagold´ and ´ Royal Gala´. DNA was digested with *Bam*HI and *Eco*RI, enzymes that do not cut within the probe region, and blotted DNA was hybridised under high stringent conditions with the same probe used for library screening. Results (shown in Fig. 34) obtained with the enzyme *Eco*RI indicated the presence of 3 copies in the genome. The stronger intensity of the signal (see arrows) observed in the *Bam*HI lanes may result from overlaying bands, which could explain that only two bands are visible in these lanes.

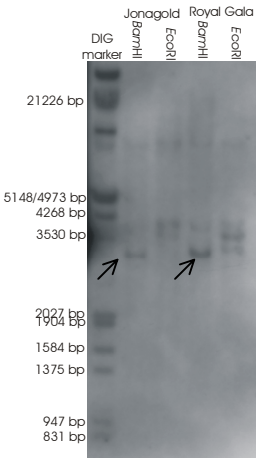


Fig. 34: Southern analysis of genomic DNA (6µg) from ´Jonagold´ and ´Royal Gala´ digested with *Bam*HI and *Eco*RI. Blot was hybridised with HBI containing 10ng/ml of *chi* probe. CDP-Star was diluted 1:5 and membrane was exposed to a X-ray film overnight. Fragments of 30ng DIG-labeled DNA marker are visible in lane 1.

4.2.3 Inducibility upon fungal infection

Gene-specific PCR amplification of cDNA pools generated by reverse-transcription using dT_{VN} as an anchor primer revealed an increased accumulation of *chitinase* transcripts in the sample derived from scab-infected ´Florina´ leaves compared to the water control (non-infected) sample. In the internal control CAM, a comparable accumulation was observed in both samples (Fig. 35).

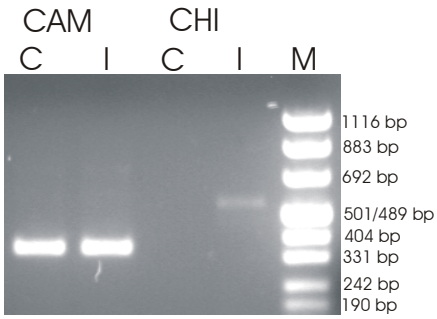


Fig. 35: RT-PCR of *chitinase* transcripts amplified with 5´CHI and 3´CHI primers in cDNA pools derived from non-infected (C) and infected sample (I). CAM amplification was performed as internal control for cDNA amount. PCR products accumulated after 35 cycles were separated on a 2% agarose gel stained with EtBr. pUCMix was used as DNA standard (M).

4.3 Overexpression of the isolated chitinase gene in apple

In order to evaluate the antifungal potential of the isolated chitinase gene C5, its expression in transgenic apples under the regulation of the CaMV 35S promoter was tried.

4.3.1 Generation of a *CaMV 35S*::Chitinase construct

The coding sequence of chitinase gene C5 was cloned into the pRT104 vector, providing the 35S promoter (a viral promoter provoking a strong constitutive expression in plants) and the polyadenylation site of CaMV.

C5 coding sequence was amplified by PCR with a forward primer comprising an *Eco*RI site at the 5´end and the vector primer downstream from 3´ end of the coding sequence, thus including the *Bam*HI site. The product was cut with *Eco*RI/*Bam*HI and cloned directly into the *Eco*RI/*Bam*HI site of pRT104 (Fig. 36). The whole cassette was excised from pRT104 with *Sph*I, blunted and inserted into the *Sma*I site of pBin19 (Fig. 37). This construct (pBin-35S-*chiC5*) was further transformed into *Agrobacterium tumefaciens* strain EHA105.

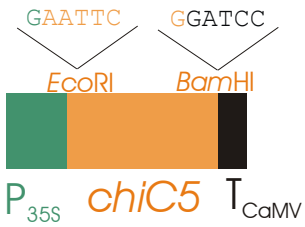


Fig. 36: Directional cloning of the *Eco*RI/*Bam*HI cut C5 coding sequence into the *Eco*RI/*Bam*HI cut pRT104, thus providing the chitinase gene with the 35S promoter (P_{35S}) and the CaMV termination sequence (T_{CaMV}).

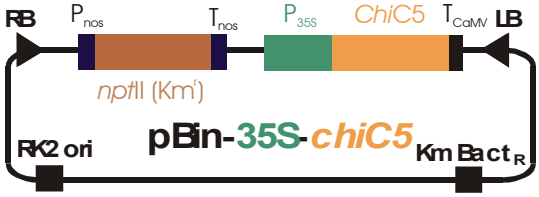


Fig. 37: Schematic representation of the *CaMV 35S*:: Chitinase cassette in pBIN19.

4.3.2 Transformation of apple leaf discs with a *CaMV 35S*::Chitinase construct

4.3.2.1 Control regeneration experiment

Regeneration of plants from single cells and complex explants by organogenesis or somatic embryogenesis is a prerequisite for any program aiming at improvement of crop quality by *Agrobacterium*–mediated transformation (Laimer *et al.*, 1989). The effect of the media J3 (containing 3mg/l BAP and 3mg/l IAA) and TE3 (containing 2mg/l TDZ and 3mg/l IAA) on the regeneration of shoots from apple leaf discs was evaluated in the cultivar ´Jonagold´. First shoots appeared after 21 days in TE3 and the number of leaf discs in which shoots developed increased strongly and continuously until 4 weeks. After 4 weeks, new shoot induction was still observed, but was less numerous. The number of shoots per leaf disc, as well as the number of leaf discs with shoots was lower in J3 than in TE3, indicating that TE3 induced a better shoot production than J3. However, shoots formed in J3 were less vitrified and more elongated than those derived from TE3 (Fig. 38) and the rate of growth in the eprouvettes was much higher in shoots derived from J3 (75%) than in TE3 (20%).

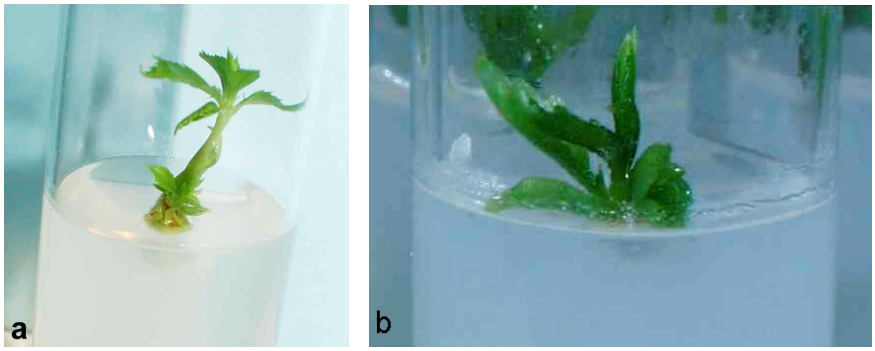


Fig. 38: Regenerated shoots from ´Jonagold´ leaf discs on a) J3 and b) TE3.

Results of this experiment are shown in Table 18.

Table 18: Summary of the regeneration experiment.

Cultivar	Regeneration conditions	Leaf discs	Leaf discs with shoots (7 weeks on regeneration medium)	Formed shoots	Shoots/leaf disc
´Jonagold´	J3, dark, 23°C	21	7	24	1.14
	TE3,dark, 23°C	28	27	102	3.64

4.3.2.2 Transformation experiment

Agrobacterium-mediated transformation has the advantage of conferring mostly single or low-copy integration of complete constructs (van der Fits *et al.*, 2000), in contrast to direct DNA transfer, in which transgenes tend to integrate as multiple copies, often fragmented and rearranged (Register *et al.*, 1994). Therefore *Agrobacterium*-mediated transformation was the method of choice to integrate the designed construct in an apple cultivar susceptible to scab disease. The construct was introduced in *Agrobacterium tumefaciens* strain EHA105, a kanamycin-sensitive derivative from the supervirulent strain EHA101 (de Bondt *et al.*, 1996). As plant target material apple leaf discs were chosen, trying to regenerate shoots directly from the leaf discs and avoiding an intermediate callus phase, that could give rise to somaclonal variation (James *et al.*, 1990a). The wounded surface should be an ideal target for *Agrobacterium*-mediated transformation as the cells undergoing organogenesis are those most readily accessed by the bacteria (Sharma and Anjaiah, 2000).

The standard protocol, successfully used in the transformation of *Nicotiana tabacum*, with the addition of acetosyringone to increase the virulence of the *Agrobacteria* (James *et al.*, 1993), was used (III 1.4.2). The selection pressure was only introduced after the shoot formation had occurred, in agreement with Laimer *et al*. (1992).

First shoots in infected leaf discs appeared after 24 days of cultivation on TE3 and one week later on J3. The total number of shoots formed (see Table 19) was much lower than in the control regeneration experiments (IV 4.3.2.1), suggesting that cocultivation with bacteria impairs shoot formation, making more difficult the regeneration process even in the absence of selecting antibiotic. After 4 weeks of cultivation on regeneration medium supplemented with 250µg/ml cefotaxime to eliminate the bacteria, leaf discs were passaged to regeneration medium supplemented with 100µg/ml kanamycin and 250µg/ml cefotaxime. Under the long-term effect of the kanamycin, formed shoots became progressively more chlorotic and died. No shoot survived more than 5 weeks under selection.

The method of infection caused a very strong bacterial growth during the 48h cocultivation period, which was difficult to eliminate even with successive passages to fresh medium containing 250µg/ml cefotaxime. In spite of a lower efficiency of the regeneration process, compared to control experiments, TE3 turned out to be more effective than J3, since the leaf discs in TE3 formed shoots earlier and in a higher number.
This experiment is summarised in Table 19.

Table 19: Summary of the transformation experiment.

Cultivar	**Regen. medium**	**Leaf discs**	**Infection method**	**Cocultivation conditions**	**Shoots/leaf disc**	**Results after selection**
´Jonagold´	J3	51	standard	48h, dark, 23°C	0.39	chlorosis death
	TE3	62			1.56	

V DISCUSSION

1. Establishment of an infection system under controlled conditions

To have a better understanding about the molecular mechanisms involved in the interaction between apple scab and its host, the establishment of an infection system under controlled conditions was a first necessary step. Different isolates of *Venturia inaequalis* maintained *in vitro* were chosen as source of inoculum, instead of leaf lesions, used in several scab studies (Lamb and Hamilton, 1969; Valsangiacomo and Gessler, 1988; Gessler, 1992, King *et al.*, 1998). This should ensure that only apple scab was participating in the plant-pathogen interaction, and therefore, plant responses would result from this interaction, which in case of field inoculum could not be assumed entirely. An additional benefit was the availability of the inoculum at any time needed.

As conidia formation depends on the isolate (Palmiter, 1934), but also on the culture medium where the fungus is grown (Lopes, 1993), different culture media were tested and evaluated concerning the growth and, essentially, the conidia formation they permitted. The higher sporulation observed in malt extract medium than in potato dextrose agrees with the results reported by Lopes.

It is known that isolates produced most lesions on the cultivar genotypes from which they were isolated, suggesting that specific pathogen genotypes showed specialisation towards particular host genotypes (Sierotzki *et al.*, 1994a,b). To overcome this eventual limitation and to obtain a more aggressive inoculum, that would be virulent towards different cultivars, a conidial suspension was produced from a mycelial extract prepared from different isolates. Confirmation of the aggressivity of this inoculum was obtained by the development of symptoms in the infection assays with different apple cultivars described as scab susceptible.

The infection system was established in steps. The first approach used plant material from *in vitro* origin, since the evaluation of fungal growth with light microscopy is easier in these conditions, as the fungus grows apparently above the cuticle on the leaf surface (Yepes and Aldwinckle, 1993a). This pattern of colonisation of the host tissue is due to the specific leaf structure of *in vitro* grown tissues. In contrast to greenhouse grown leaves, i*n vitro* leaves have no or sparse trichomes and have a thinner cuticle, formed only by a polylamellate layer and no inner reticulate zone. It was suggested that the thin, undifferentiated cuticular membrane of *in vitro* plantlets may allow leakage of nutrients to the surface, permitting the fungal proliferation above the cuticle (Yepes and Aldwinckle, 1993a). Additionally, *in vitro*

cultivated leaves produce a low amount of polyphenolic compounds, enabling an easy maintenance of the assay (Yepes and Aldwinckle, 1993b).

However, as the pattern of colonisation in *in vitro* leaves does not reproduce exactly the disease development in *in vivo* tissues, in the next step, detached leaves from apple plants grown under greenhouse conditions were used to evaluate the disease establishment and development of symptoms in a small-scale assay. At last, infections *in planta* were performed with greenhouse plants kept under controlled low pathogenic pressure, as an attempt to reproduce the fungal interaction that occurs in the orchard between apple plants and *Venturia inaequalis*. The maintenance under low phytopathogenic conditions was a prerequisite to omit any spraying with pesticides during the growing season. During infection experiments, the plants were kept in a closed system to assure, as far as possible, that variations in gene expression of the plants resulted from fungal interaction.

The periodic acid-Schiff staining method used in medical mycology has been found effective on plant material, particularly for the early detection of apple scab lesions (Preece, 1959). Although leaf hair and cut or injured parts also take up some stain, fungal chitin is among the substances that are most stained, leading therefore to a doubtless identification of fungal structures, prior to the development of macroscopically visible symptoms.

Evaluation of symptoms and microscopical observations proved that a functional infection system was established.

2. Isolation and characterisation of apple *pgip* promoters

2.1 Advantage of a fungus-inducible promoter

By fusion with defense genes, pathogen- or elicitor-specific promoters would be good candidates for engineering an artificial defense response, as they can be induced upon infection, thus activating the defense response. They provide an alternative to constitutive promoters that permanently express the engineered defense gene.

A fungus-inducible promoter, activated in response to scab infection would be of particular interest in an engineered protection mechanism against scab disease. *Pgip* promoters, supposed to be activated by fungal attack and activated in a very early stage of infection (Nuss *et al*., 1996; Favaron *et al*., 2000) might be promising candidates as one of the enzymes that is described to participate in the beginning of the fungal interaction is a endopolygalacturonase (Kollar, 1998). Fungal endopolygalacturonases are known to play a role in activation of *pgip* genes (Cervone *et al.*, 1992; Yao *et al.*, 1999). The finding, that PGIP is an extracellular protein accumulating to high levels particularly in those epidermal

cells immediately surrounding infection sites (Bergmann *et al*., 1994; Yao *et al.*, 1999), underlines a possible beneficial use of a *pgip* promoter combating apple scab, due to the special type of parasitism of the scab fungus, that remains in the subcuticular space (Corlett *et al.*, 1976; Kollar, 1998).

The plant origin of such a promoter would be also a benefit, overcoming the concerns driven by the use of viral promoters in genetically modified plants for human consumption.

2.2 Characteristics of *pgip* genes in apple

Two different 5´regulating sequences from *pgip* genes of apple were isolated, both containing several hypothetical TATA boxes in the region up to 450bp of the translational start codon. However, as observed in a *pgip* gene isolated in tomato (Stotz *et al.*, 1994), no unambiguous TATA box could be identified. The TATA box is a strongly conserved motif among eukaryotic promoters that is normally located circa 25 to 30bp from the transcription start. A motif TATAA, indicated as TATA box in a bean *pgip* promoter (Devoto *et al.*, 1998), could be encountered in both sequences, but as the initiation of the transcription was not determined, the function of this motif as TATA box remains speculative. A CAAT box, also a highly conserved motif among eukaryotic promoters frequently found in a region 80-120bp upstream from the transcription start, could be identified in both sequences upstream from the putative TATA box motif. The isolated 5' regulating sequences show no significant homology with the published *pgip* promoter sequence from bean. Analyses of the sequences were performed in order to identify putative regulatory elements characteristic for pathogen-responsive genes. Sequences similar to the consensus motif identified in different plant promoters, that are activated in response to fungal infection and treatment with elicitors (Raventós *et al.*, 1995), could be identified in both clones but no clear data concerning the function of these sequences were yet obtained.

The Southern data indicate that a small multigene family with three copies encodes PGIP in the apple genome. Similar results had been described by Yao *et al*. (1999) and agree with analysis performed in bean, where a family with at least five members was reported (Frediani *et al.*, 1993), in pear, where the presence of two genes was suggested (Stotz *et al.*, 1993) or in soybean, where Southern analysis revealed a single copy of the isolated *pgip* gene, together with the presence of some related genes (Favaron *et al.*, 1994).

Apple *pgip* genes seem to be developmentally regulated, with higher accumulation of transcripts in fruits, particularly in mature fruits, and lower in old leaves. These results agree partially with those obtained by Yao *et al*. (1999), where lowest accumulation in immature

fruits and highest accumulation in ripe fruits after storage was observed. In pear, *pgip* mRNA accumulation was also much higher in fruits than in flowers or leaves (Stotz *et al.*, 1993). These data are, however, in contradiction with those obtained by Abu-Goukh *et al.* (1983) and Powell *et al.* (2000), who report that PGIP declines in ripening pear or tomato fruits. In bean, *pgip* genes are also regulated during development, with higher mRNA levels in cell suspension cultures and much lower levels in leaves, hypocotyls and flowers (Toubart *et al.*, 1992). Low transcription levels were also detected in roots and in stems, except in their basal region. *pgip* mRNA analysis in whole flowers, pods and seeds revealed high levels of transcripts only in pods (Devoto *et al.*, 1997). In soybean, highest PGIP activity was found in cotyledons, lowest in roots (Favaron *et al.*, 1994). These findings suggest that the promoters of *pgip* genes may differ and confer specific expression patterns.

The detection of only one transcript size (1.3kb) may indicate that either only one gene is expressed under the conditions examined or that different *pgip* genes might have similar transcript size. Similar results were reported in bean, where a 1.2kb transcript was identified (Toubart *et al.*, 1992; Nuss *et al.*, 1996; Devoto *et al.*, 1997), in tomato, where a 1.35kb transcript was identified (Stotz *et al.*, 1994), in soybean, where 1.3kb (Favaron *et al.*, 2000) or 1.35kb (Favaron *et al.*, 1994) transcripts were identified, or in pear, where only a single mRNA species was detected in Northern analysis, with 1.35-1.4kb (Stotz *et al.*, 1993). However, it is noteworthy that in soybean two (Favaron *et al.*, 2000) and in apple three hybridising transcripts were observed upon fungal infection (Yao *et al.*, 1999).

As described in other plant species, e.g. bean and soybean, upon fungal challenge (Nuss *et al*., 1996; Devoto *et al.*, 1997; Favaron *et al*., 2000), an accumulation of *pgip* transcripts in ´Golden Delicious´ and ´Jonagold´ within the first days after scab infection was observed. This supports the potential value of *pgip* promoters as being fungus-inducible. Specially interesting seems the activation of the promoter(s) at the onset of the disease process, suggesting the possible use to regulate an early activation of the defense mechanism. However, PGIPs encoded by different members of the bean *pgip* gene family, differ not only in specificity but also in regulation (Devoto *et al.*, 1998). Moreover, in pear and tomato fruits *pgip* genes were reported to be constitutively expressed and not induced by wounding or pathogen challenge (Powell *et al.*, 1994). These results show that plant PGIPs may have multiple functions both in normal plant development and in response to biotic stresses and reinforce the importance of analysing the characteristics of the isolated regulative sequences individually. As Northern analysis in apple did not discriminate between the contribution of different *pgip* genes, the observed expression pattern likely reflects the sum of differential

expression of the individual genes, and does not allow any conclusion about single characteristics. A differential regulation has been reported to occur within many defense-related gene families (Stanford *et al.*, 1989; Harker *et al.*, 1990). The need for a differential regulation and specificity may explain the presence of multiple *pgip* genes and implies a divergence of either the promoter sequences, where each promoter comprises different complements of regulatory elements and/or the coding sequences (Devoto *et al.*, 1998). In order to characterise the features of each isolated 5´regulating sequence, their activity in transgenic tobaccos was analysed.

2.3 Characterisation of the isolated *pgip* promoter sequences in transgenic tobaccos

In order to investigate the 5´upstream regulating sequences of the isolated *pgip* genes, they were fused with the ß-glucuronidase reporter gene and the constructs were introduced in tobacco plants. The use of herbaceous plants as model permits the analysis of the transgene expression in a shorter time and easier way. The life cycle is accomplished in a short period and transformation protocols to integrate the transgene are very well established, permitting the analysis of different sequences. The combination of the different sequences with the reporter gene allows the evaluation of the individual characteristics of each sequence. This fact is particularly important, because although fungal inducibility of *pgip* genes was described in different species, it has been shown that a bean *pgip* promoter did not respond upon fungal attack in transgenic tobaccos (Devoto *et al*., 1998).

As a reporter gene, the engineered GUS gene containing an intron from the potato ST-LS1 gene was used (Vancanneyt *et al.*, 1990). In contrast to *Agrobacterium*, plants are able to splice out this intron, thereby providing a marker for T-DNA transfer to plant cells.

The different constructs designed should give information about the putative fungal inducibility of the isolated sequences and should elucidate in which manner the length and the diversity of the different sequences can influence the expression of the reporter gene.

To analyse the fungal inducibility of the isolated *pgip* regulating sequences in tobacco it was necessary to select a phytopathogen for which tobacco plants are a host, as it is not the case for *Venturia inaequalis*. The infection of the different tobacco plants in the greenhouse under homogenous, controlled and reproducible conditions would be difficult, therefore fungal elicitors derived from an ubiquitous fungus, also phytopathogenic for tobacco plants, were produced and used as challenging substances.

During pathogenesis cell walls act as the first line of defense, that phytopathogens encounter to colonise the plant tissue and obtain nutrition. Plant pathogenic bacteria and fungi have

evolved a battery of pectic enzymes to hydrolyse the plant cell walls during the early stages of pathogenesis (Hahn *et al.*, 1989). Endopolygalacturonases are among the first enzymes secreted by the pathogen in plant tissues that cleave polysaccharides and produce a pathogenic effect on cells (Karr and Albersheim, 1970) and are suggested to be one of the signals released from fungal pathogens that could activate *pgip* genes (Yao *et al.*, 1999). Thus, elicitation with a culture supernatant in which fungal polygalacturonases had been secreted, seemed a simplified but acceptable method to induce promoter activity in transgenic tobacco. Hence, the supernatants of liquid cultures, where the ubiquitous fungus *Botrytis cinerea* was grown, were used in induction experiments.

The supernatants derived from different culture media were prepared, in order to select an active substance that could be used for elicitation assays with the tobacco plants. As an approach to estimate the biochemical activity of differently produced fungal supernatants, their polygalacturonase content was evaluated in an enzymatic assay. This test allowed a qualitative screening of the available inducers, thus removing those without or low polygalacturonase activity. It was also an alternative to a Western blot, that would require an adequate, specific antibody and that would indicate only the amount of produced polygalacturonase but not its activity.

The finding that in supernatants derived from media containing saccharose, tryptone or glucose a higher polygalacturonase content was measured, might be due to the stronger fungal growth, that could be visually observed, particularly when the carbon source was saccharose. An impairment of fungal growth, when polygalacturonic or galacturonic acid were used as unique source of carbon, was also observed in *Fusarium oxysporum* f. sp. *radicis* (Patino *et al.*, 1997).

In preliminary assays, supernatants that could induce a stronger response in the leaf explants from transgenic tobaccos, were considered to have a higher biological activity and selected for use as challenge substances.

An important prerequisite for the establishment of the elicitation assay was the non-degradation of the leaf explant during the induction period. In this view, and due to the more active elicitors, a supernatant derived from a culture medium, similar to the medium where tobacco plants are usually grown in tissue culture, was chosen.

The very low pH observed in the fungal supernatants was probably derived from the secretion of organic compounds like oxalic, citric, malic or succinic acid (Johnston and Williamson, 1992b) and could not be circumvented. Therefore the correction of the pH to 5.5-5.7 prior to the induction assay was indispensable to prevent tissue degradation.

The functionality of the 3 *pgip*::GUS constructs and the response of the different transformants upon fungal elicitation was investigated with *in vitro* leaf explants. The observed GUS accumulation in several lines of each construct was a doubtless proof for the functionality of the integrated constructs in the transgenic tobacco plants. This fact was of particular relevance since no clear TATA box could be identified in isolated promoter sequences. It is likely that the suggested TATA motifs are responsible for the behaviour of the isolated sequences as a functional promoter in transformed tobacco plants. The generation of progressive promoter deletion constructs enabling the analysis of the influence of regulating sequences could bring more relevant information about the promoter characteristics and their activation potential.

A very high variability in GUS expression was observed among the different independent lines within a construct. As all the plants grew very good under kanamycin pressure, it can be deduced that the selection was successful and that all plants are transgenic. The non-expression of the reporter gene in some lines might be due to a positional effect of the inserted transgene in the genome or due to a silencing phenomenon. Promoter analysis was made in F1 plants to ensure that the construct is stably integrated.

In the elicitation assays, a rather strong variability among different tests within the same line was observed. This variability may be caused by differences in the physiological and developmental condition of the plants. Although it was ensured that the explants for induction were always collected from plants in the same age, the growth rate varied from plant to plant, even when they were acclimatised exactly at the same time. To overcome this variability, 5 test series with acclimatised plants were performed.

The basal value, caused by induction with culture medium alone, was similar among the different lines, particularly between progenies from P and S constructs. Progenies from N lines obviously expressed slightly higher basal values and showed a much higher variability. The already higher basal values might have reduced the potential for further upregulation upon treatment.

The results obtained from transgenic tobacco plants indicated that both isolated apple *pgip* promoters were inducible upon fungal challenge, whereas one (Q8) of the two responds better to fungal elicitation, since nearly all the different lines from the 2 constructs derived from this sequence showed a significantly increased GUS expression in explants elicited with $SN_{MSfpH5.7}$. These data also supported the results obtained from Northern analysis of apple. They confirm the potential value of *pgip* promoter sequences in the regulation of genes whose products might confer fungal resistance. However, the investigation of a heterologues

promoter in tobacco still has to be regarded as a model and the results obtained do not have to mirror exactly the situation in the apple. The promoter activity might be influenced by interactions with nearby genes, those cis-acting elements (short sequence motifs on promoters that can strongly stimulate transcription) and trans-acting factors (proteins that influence the transcription of the activated gene) vary from apple to tobacco (Chen *et al.*, 1986; Kuhlemeier, 1992).

The demonstrated fungal inducibility of both promoters contrasts the results reported from bean, where expression of a *pgip*-1-b-GUS fusion in *Nicotiana tabacum* revealed that the promoter responded to wounding but not to oligogalacturonides, fungal glucan, salicylic acid, cryptogein or fungal infection (Devoto *et al.*, 1998).

The lower level of expression driven by the S and P constructs might be related with the shorter size of the promoter sequence. The increase in GUS expression upon elicitation in lines derived from N construct was considered less significant, since it was only observed in less than half of the lines tested. The 5´regulating sequence derived from Q8 and used to create the S construct seems to act as a functional promoter inducible upon fungal elicitation and would be a good candidate to use as a fungus-inducible promoter. Since only limited studies were performed, the weak inducibility of the N sequence cannot be explained yet. Within the 2kb fragment there might be sequence variations to Q8 and regulating elements that could modulate the response upon fungal elicitation.

3. Identification of putative defense genes

Interesting genes to be used under the regulation of a fungus-inducible *pgip* promoter in an engineered protection mechanism would be those involved in resistance shown by wild genotypes as well as genes encoding proteins participating in fungal cell wall degradation. A first approach to identify such genes was based on the investigation of the molecular mechanisms occurring during the interaction between a resistant apple cultivar and scab fungus. A second approach consisted of the isolation of genes encoding a chitinase, a chitin-degrading enzyme, which is a major component of the cell wall of different fungi, including the ascomycetes.

3.1 Isolation of putative defense genes from a scab-resistant apple cultivar

As an approach towards the unravelling of the defense mechanism developed in scab-resistant cultivars, the gene expression pattern following scab infection in resistant cultivar ´Florina´ was analysed. Differential display of mRNA, a technique that allows the identification of

genes differentially expressed in infected tissue (Benito *et al.*, 1996), is thus a powerful tool to analyse this interaction.

3.1.1 Differential display as a tool to analyse the interaction ´Florina´- *V. inaequalis*

Microscopical observations were made after performing the artificial infections to accompany the fungal development and disease process. They were the basis for the determination of the time point for the harvest of the leaves. As the goal was to detect any changes occurring in genetic expression upon scab invasion, the leaf material for the differential display analysis was collected when the fungus showed strong development and the disease was spreading in the plants of the susceptible cultivar infected at the same time.

The exclusion of cDNA of *Venturia inaequalis* in the comparison of gene expression patterns was a limitation of the whole procedure, with the associated risk that the isolated fragments derive from fungal genes and not from the plant. However, the omission of the fungal cDNA in the display analysis was considered as an acceptable simplification, as there exists no method permitting a clear evaluation in which moment the conidial suspension developing on the MEAm is expressing the same gene pattern as in the plant tissue. The amount of fungal suspension in the leaves is quite reduced, therefore the abundance of fungal genes in the pool of total RNA should also be rather low. Furthermore, the plant origin of putatively differential clones can be confirmed by Southern analysis.

A first hint that omission of the fungal cDNA in the differential display analyses was an acceptable simplification was given by analysing the isolated fragments and searching for homologies in the databases. Homologies found with plant genes let suppose that the isolated fragments derived from the apple tissue. In cases where no meaningful homology was found or was not significant, the possibility still remains that the analysed genes derived from the apple scab and demands further investigation.

3.1.2 Methodological aspects of the differential display

A too low amount of DNA or inhibition of the reaction due to the presence of traces of acetic acid could explain the difficulties to reamplify some fragments. The latter could be circumvented by using the precipitated DNA as template. Another problem encountered during the reamplification of the displayed fragments was the unexpected size of some reamplified products. These fragments were considered to be PCR artefacts (M1, M8) due to the low stringency of the differential display conditions, which might also explain the appearance of some mismatches in the primer regions (e.g. M9).

An analysis of the cloned cDNA sequences showed that only M3 and M4 contained the anchor oligo(dT) primer, whereas in all other only the sequence of the corresponding arbitrary primer could be identified. Amplification of internal fragments by arbitrary primers only is an often occurring event, likely due to the low stringency of the differential display conditions or due to partial RNA degradation. In the cases where a poly(A)tail was identified, homologies with the 3´end of proteins were expected to be found. It was not the case for M3, where a possible open reading frame showed very high homology with the intermediate part (between amino acid 148 and 179, in a total of 242) of the coding region of the putative homologue. This result can derive from a sequence inaccuracy, as it was observed that the removal of one nucleotide from the presumed first stop codon would provide a much larger (66 amino acids) open reading frame that shows homology with the same protein. The isolation of a cDNA clone comprising the full coding region would be required in order to prove this assumption.

The differential expression of the genes corresponding to the isolated cDNAs can be investigated by common end-point RT-PCR only semi-quantitatively. However, this method is suitable to exclude false positive clones, which originate from non-differentially expressed genes. For six of the eight cloned cDNAs the enhanced gene expression in the infected sample, suggested from the differential display experiments, could be confirmed by specific RT-PCR.

Despite the relatively abundant expression of M9, compared to the other cloned cDNAs, the use of the chemiluminescent detection of this transcript in a Northern analysis was not successful. An alternative to overcome this problem would be the use of a radioactive detection method to increase sensitivity in Northern analysis or the use of RealTimePCR. Both methods would allow the quantitative measurement of low abundant transcripts and thus allow confirmation of differentially expressed genes in infected and non-infected leaves.

5´RACE and 3´RACE technique was applied to elongate cloned cDNA fragments in both directions. Only clones M4 and M10 could be elongated in their 5´direction and clones M6, M9 and M13 could be extended to their 3´direction. However, for none of the elongated clones the full-length coding region could be identified. The small product length obtained for all clones in both, 5´and 3´RACE, did not provide enough additional sequence information in terms of homology searches.

In order to obtain full-length clones further RACE experiments or the screening of a cDNA library, established from scab-infected leaves, should be carried out.

3.1.3 Putative function of the genes corresponding to the isolated fragments

For M4, M13 or M14 no clear function could be attributed, as the deduced amino acid sequences did not show any homology with proteins with known function.

For the remaining fragments, significant homologies were found that demonstrate a strong indication for the function and emphasise the plant origin of the isolated cDNAs. The homology of fragments M9 and M10 with proteins involved in biosynthesis of cuticle and cell walls and of fragments M3, M5 and M6 with proteins involved in plant defense constitute a first step towards the characterisation of the interaction mechanism between resistant cultivar 'Florina' and the fungus *Venturia inaequalis*. Nevertheless, further investigation is required, as the display amplifications performed were only a small fraction of all the possible combinations that would be necessary to analyse the complete bulk of expressed genes.

3.1.3.1 Homologies observed with proteins involved in biosynthesis of plant cell walls and cuticle layer

Significant homologies on a protein level of M9 and M10 were encountered with a protein participating in the synthesis of a major component of the cell walls, the cellulose synthase, and to a very important protein for the cuticle formation, the very-long-chain fatty acid condensing enzyme CUT1, respectively.

The homologue in cotton of M9 has a basic function in the synthesis of cellulose, a plant polysaccharide built of ß-1,4 linked glucose units that constitutes the main component of cell walls (existing in primary to tertiary cell walls, with exception of the middle lamella). Cellulose synthases from cotton were localised in the plasma membrane and expressed at high levels in cotton fibers at the onset of the secondary wall synthesis (Pear *et al.*, 1996; Richmond and Somerville, 2000). A greater activity of a cellulose synthase after scab inoculation would fit to the presence of cell wall appositions, microscopically observed in resistant plants upon fungal treatment (Chevalier and Lespinasse, 1994) and could therefore contribute to unravel the molecular events behind these observations. These cell wall appositions are suggested to be involved with the host response mechanism that restricts the stroma to the outer surface of the epidermis. They could provide a physical and chemical barrier, impairing the hyphal growth or the release of host nutrients needed for the development of the fungus (Chevalier and Lespinasse, 1994; MacHardy, 1996).

M10 seems to encode a epidermis-specific very-long-chain fatty acid condensing enzyme required for cuticular wax production (Millar *et al.*, 1999). The observed activation of a gene encoding such an enzyme in apple leaves upon scab inoculation might be related with a plant

response towards the fungal penetration through the cuticle and subcuticular growth, supposed to occur with the help of a cutinase (Köller *et al.*, 1991).
Waxes are major constituents of the cuticle, a hydrophobic protective layer above the cell wall of the epidermis that can help plants resist bacterial and fungal pathogens (Jenks *et al.*, 1994). Condensing enzymes like CUT1 can be useful for manipulating wax accumulation and composition in plants and thus, play an important role in engineered mechanisms to increase resistance to insects and pathogens (Millar *et al.*, 1999). In this context, the isolation of a CUT1 homologue from the apple cultivar ´Florina´ appears to be a promising step in a possible strategy for an engineered protection mechanism.

3.1.3.2 Homology observed with a probable menaquinone biosynthesis protein

A putative open reading frame from fragment M2 showed homology with a protein involved in the biosynthesis of menaquinone, probably a isochorismate synthase, that regulates the conversion of chorismate to isochorismate (Daruwala *et al.*, 1997). This result is in agreement with other studies that revealed that a similar enzyme was purified from cell cultures of *Catharanthus roseus* elicited with a fungal extract (Moreno *et al.*, 1994). Little is known about the roles of isochorismate synthase in plants; this enzyme competes for the chorismate substrate together with the anthranilate synthase and chorismate mutase, enzymes that lead to the synthesis of phenylalanine/tyrosine (precursors of secondary phenylpropane like lignanes) and tryptophan (precursor of indole alkaloids), respectively. One of its known functions is to catalyse the first step towards the synthesis of anthraquinones, most abundant natural plant quinones (Simantiras and Leistner, 1989; Poulsen and Verpoorte, 1991; van Tegelen *et al.*, 1999), that can catalyse delignification reactions.
In certain bacteria (e.g. *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*) salicylic acid, a key signalling compound in the activation of certain defense responses against invading pathogens (Dempsey *et al.*, 1999), is formed from chorismate via isochorismate by isochorismate synthase and isochorismate pyruvate-lyase. A similar pathway was engineered in *Arabidopsis*, in order to manipulate the content of salicylic acid in the plants (Mauch *et al.*, 2001). This finding supports the putative application of the isochorismate synthase as a modulating enzyme in the salicylic acid synthesis in plants, thereby altering the defense response.

3.1.3.3 Proteins involved in plant cell defense

Clone M3 showed significant homology with a glutathione S-transferase in *Arabidopsis thaliana*. This protein is encoded by a multigene family, whose members are transcriptionally induced by multiple stimuli, including fungal infection, heat shock, heavy metals or hormone treatment (Dudler *et al.*, 1991; Marrs, 1996) and therefore considered to have protective functions (Edwards *et al.*, 2000). Plant glutathione S-transferases are thought to be involved in the detoxification of products created by oxidative damage (e.g. certain herbicides; Gronwald and Plaisance, 1998) and thereby protecting cells against such a stress (Dixon *et al.*,1998; Subramaniam *et al.*, 1999). Enhancement of glutathione S-transferases has become a marker for plant response to stress (Edwards *et al.*, 2000). The application of DDRT-PCR to identify differentially expressed genes during oxidative stress induced by paraquat in *C. elegans* revealed the upregulation of cDNA products homologous to a glutathione S-transferase, together with a potential zinc finger protein (Tawe *et al.*, 1998). In this context, the identification of a transcript that is putatively induced in cultivar ´Florina´ upon fungal attack as a glutathione S-transferase would constitute a first step towards the unraveling of this interaction mechanism.

Deduced amino acid sequences from the almost identical clones M5 and M6 show a 50% homology in a short amino acid stretch with RPS4 protein from *Arabidopsis thaliana*. However, the question whether these clones represent a gene similar to the disease resistance gene encoding RPS4 remains open. RPS4, a member of the R gene family in *Arabidopsis*, reported to determine resistance to viral and fungal pathogens (Gassmann *et al.*, 1999), confers resistance to strains of *Pseudomonas syringae* pv. *tomat*o. Elongation attempts with RACE did not provide more conclusive information about the putative function of the gene corresponding to M6.

3.2 Identification of apple genes encoding class III chitinases

3.2.1 Use of chitinases as putative factor for protection against apple scab

The causal agent of apple scab disease, *Venturia inaequalis*, belongs to the ascomycetes, whose cell wall contains chitin. It shows a subcuticular growth and is restricted to the intercellular space. Genes encoding chitinases, enzymes capable of hydrolysing chitin, with extracellular expression and antifungal properties could therefore constitute an interesting factor towards impairment of fungal development. The hypothesis about the potential interest of such a chitinase to inhibit apple scab was supported by results published recently, in which extracellularly expressed chitinases from *Trichoderma harzianum*, a fungus commonly used

in biocontrol, proved to be active against the apple scab fungus in *in vitro* tests (Wong *et al.*, 1999). In addition transgenic apples expressing a chitinase isolated from the same fungus showed increased resistance against apple scab (Wong *et al.*, 1999; Bolar *et al.*, 2000). A plant gene encoding a chitinase with similar properties would be a helpful contribution in a genetically engineered approach, as the safety concerns derived from integration of a gene isolated from a fungus into the apple genome could be circumvented.

3.2.2 Isolation from genes encoding class III chitinases from apple

The deduced amino acid sequences from C1 and C5 show a high degree of similarity to class III chitinases of different plants, particularly grapevine and strawberry, and also to the consensus sequence of the active site of these enzymes (Neuhaus *et al.*, 1996). The calculated isoelectric point, indicating an acidic protein, and the absence of an intron, are also characteristic features of the identified sequences that were observed for other class III chitinases (Nielsen *et al.*, 1993; Lawton *et al.*, 1994; Robert *et al.*, 2002).

For clone C20 it was not possible to deduce a protein sequence as an open reading frame could not be identified unambiguously. The possibility that the repeated sequence CCGG is a cloning artefact that could have been introduced during construction and/or screening of the genomic library cannot be completely excluded.

Class III chitinases have an unclear antifungal potential (Melchers *et al.*, 1994). It has been demonstrated, that a class III chitinase from chickpea does not display any inhibitory activity against several test fungi, although it has almost similar specific activity on purified chitin than a class I chitinase with clear antifungal properties (Vogelsang and Barz, 1993). However, tobacco plants displayed SAR (systemic acquired resistance) upon fungal inoculation and the systemic response was tightly correlated with the induced expression of class III chitinases (Ward *et al.*, 1991; Ryals *et al.*, 1996). It was shown, that a class III chitinase is upregulated upon fungal challenge in grapevine, with highest transcript accumulation at the onset of disease resistance (Busam *et al.*, 1997). Furthermore, a chitinase isolated from *Rhizopus oligosporus*, homologous to plant class III chitinases in its primary sequence, possessed antifungal activity in transgenic tobacco (Terakawa *et al.*, 1997), suggesting the potential use of such enzymes in breeding against fungi.

Both isolated sequences, C1 and C5, encoded a N-terminal hydrophobic signal peptide indicating the secretory nature of the corresponding proteins. These findings are in agreement with results obtained in grapevine, where a hydrophobic signal peptide was also identified in VCH3 cDNA and described as required for the Golgi passage and cellular excretion of the

enzyme (Busam *et al.*, 1997). The absence of a C-terminal vacuolar targeting extension suggests the extracellular localisation of the deduced proteins, since it was reported that vacuolar targeting requires a positive sorting signal and proteins lacking this specific targeting information are secreted by plant cells (Neuhaus *et al.*, 1991a; Büchter *et al.*, 1997; Esaka and Teramoto, 1998).

The influence of the localisation of the chitinases has to be evaluated with respect to the growth pattern of the invading fungi (Collinge *et al.*, 1993). Due to the specific type of parasitism of the apple scab, which does not penetrate into the cells but remains in the intercellular space between epidermis and cuticle, antifungal proteins showing apoplastic activity would be of particular importance. Furthermore, with respect to their localisation and implication in defense reaction, it has been proposed that extracellular chitinases are part of an early induced response (Mauch and Staehelin, 1989). They may act directly, by blocking the growth of the hyphae invading the intercellular space, and possibly indirectly, by releasing fungal elicitors which then induce additional chitinase activity and other defense reactions in the host (Neuhaus *et al.*, 1991b). According to this view, vacuolar chitinase acts later in the infection process when cell colapse releases the vacuolar content into the extracellular compartment (Mauch and Staehelin, 1989; Samac and Shah, 1994). From these results it can be deduced that a class III chitinase isolated from apple, with features strongly pointing to extracellular expression, could be regarded as a potential factor in scab inhibition.

Results obtained by Southern hybridisation suggested, that class III chitinases in apple are encoded by a small gene family consisting of three members in each cultivar, which is consistent with results obtained in grapevine (Busam *et al.*, 1997; Robert *et al.*, 2002) or in cucumber (Lawton *et al.*, 1994). In the deduced amino acid sequences from C1 and C5 no putative glycosylation sites were identified, although examples of glycosylated class III chitinases were already reported (Heitz *et al.*, 1994).

RT-PCR was used to demonstrate that class III chitinase genes are inducible by fungal treatment. Enhanced accumulation of *chitinase* transcripts in ´Florina´ leaves upon inoculation with *Venturia inaequalis* appears in agreement with results obtained in grapevine upon infection with *Plasmopara viticola* (Robert *et al.*, 2002). Similar results were also observed in the incompatible interactions between *Vitis rupestris* and *Plasmopara viticola* (Busam *et al.*, 1997) and between *Vitis vinifera* and P*seudomonas syringae* pv. *pisi*, which is characterised by a rapid development of a hypersensitive response together with the elicitation of several defense-related mechanisms (Robert *et al.*, 2002). These findings support the suggestion that the class III *chitinase* transcripts can be considered as a reliable indicator for

the SAR response (Busam *et al.*, 1997). In RT-PCR the absence of an amplification product of the chitinase gene in the non-infected sample agrees with the fact that basal expression of these chitinases in leaves is very low (Yeboah *et al.*, 1998), but increases upon fungal inoculation (Lin *et al.*, 1995).

3.2.3 Overexpression of genes encoding class III chitinases in transgenic apples

Several examples of resistance against fungi derived from overexpression of chitinases in transgenic plants were reported (Broglie *et al.*, 1991; Lin *et al.*, 1995; Grison *et al.*, 1996; Tabaeizadeh *et al.*, 1999; Yamamoto *et al.*, 2000). Nevertheless, the observation that some chitinases possess no antifungal activity (Neuhaus *et al.*, 1991b; Nielsen *et al.*, 1993) demands a characterisation of each gene and an evaluation of its antifungal potential. The presence of multigene families can complicate the analysis of gene expression because different members of the family can differ in their tissue specificity and/or regulation by external factors. A transgenic approach to evaluate the real potential of each individual gene could overcome this problem (Wu *et al.*, 1997) and would allow to evaluate the effective antifungal potential of the isolated chitinase genes in the impairment of scab development. In order to evaluate the properties of the isolated genes, they were tried to be expressed in transgenic apples under the regulation of the CaMV 35S promoter.

The use of an *Agrobacterium*-mediated gene transfer system is dependent on the target plant species, as they need to be a host for *Agrobacterium*, on the plant genotype and on the regeneration capacity of the target cells (Moloney *et al.*, 1989). Several reports exist about the successful application of this method in fruit species (Nehra *et al.*, 1990; James *et al.*, 1990b; Laimer *et al.*, 1992; Gao *et al.*, 2001), particularly in apple (Norelli and Aldwinckle, 1993; Holefors *et al.*, 1998; Zhu *et al.*, 2001) but with low efficiency rates (James *et al.*, 1989; Schaart *et al.*, 1995), even in cultivars that are very responsive to regeneration under non-selecting conditions (James *et al.*, 1990a; Maximova *et al.*, 1998).

Although it was possible to regenerate a high number of shoots, the transformation experiment was not successful, since none of the shoots, regenerated from the infected leaf discs, survived the prolonged selection on kanamycin. The supplementation of the bacterial culture with acetosyringone, reported to increase the virulence of the bacteria (James *et al.*, 1993) did not contribute to an increased gene transfer efficiency. Furthermore, it was clearly seen that the shoot formation was much lower in infected leaf discs than in the comparable controls, suggesting that the bacterial infection inhibited plant regeneration. On the contrary, James *et al.* (1990a) reported that the rate of shoot regeneration without selection pressure is

similar to that in non-infected control regeneration experiments. An inhibition caused by the presence of cefotaxime used to kill the surviving *Agrobacterium* after infection, seems less likely, as a stimulatory effect of cefotaxime on regeneration had been reported for different species (Mathias and Boyd, 1986), including fruit trees like apple (James *et al.*, 1990a) or pear (Predieri *et al.*, 1989).

The precultivation for 24h, performed to enhance the number of dedifferentiating cells along the cut edge at the time of infection, did not seem to confer any benefit. However, preculture of explants has been found to be effective in the transformation of different species (McHughen *et al.*, 1989; Sangwan *et al.*, 1992).

The age and physiological state of the mother plants at the time when the leaf discs are excised, as well as the handling procedure play also an important role in the regeneration of shoots. For instance, a too long time elapsing from cutting the leaf discs to putting them on regeneration medium can result in a very high accumulation of polyphenolic compounds at the cut site, that will prevent the shoot induction. In accordance with de Bondt *et al.* (1994), leaf discs were excised from shoots propagated between 4 and 7 weeks.

In the transformation experiment, the clear difference in the potential of shoot induction among the different media seen in the regeneration experiment, could be reproduced. In both regeneration and transformation experiments, it became clear that the TDZ contributes in a decisive way to shoot formation. The obtained results agree with other observations made in experiments with apple, where the replacement of BAP by TDZ increased significantly the regeneration rate (James *et al.*, 1990a) or a very low concentration of TDZ resulted in a similar effect than a much higher concentration of BAP (Theiler-Hedtrich and Theiler-Hedtrich, 1990). However, it is noteworthy that a very high concentration of this hormone leads to many shoot forming units but less good-growing and elongating shoots, as this growth regulator inhibits shoot elongation (Theiler-Hedtrich and Theiler-Hedtrich, 1990; Dufour, 1990).

However, these results have to be considered as preliminary due to the limited number of leaf discs cut in the experiment, and only a first approach towards the identification of some conditions that can influence the efficiency of a transformation protocol.

In the transformation experiment performed, the selecting agent (kanamycin) was only introduced after the shoot formation had occurred, opposite to what was done in *Nicotiana tabacum* in agreement with da Câmara Machado *et al.* (1992) or is described in other protocols (Schaart *et al.*, 1995; Norelli *et al.*, 1996; Maximova *et al.*, 1998; Holefors *et al.*, 1998; Zhu *et al.*, 2001). The reason for this was to avoid an additional stress to the

transformed cells caused by the early exposure to the selecting pressure. This would permit the shoots to acquire a good physiological status, and by this enhance the transformation efficiency (Laimer *et al*., 1992; Sharma and Anjaiah, 2000). However, under the applied conditions, a competition between transformed and non-transformed cells could have occurred and favoured the non-transformed cells, that regenerated the obtained shoots. Infection of apple leaf discs without immediate selection was already considered to be inefficient, since no transgenic shoot could be obtained, despite a high number of explants (James *et al.*, 1990a). Experiments in strawberry revealed that the absence of selection at initial stages might result in very low recovery of transformants (Mathews *et al.*, 1995). The immediate selection with kanamycin would have the effect that the transgenic shoot derives only from a transformed single cell, which forms first a disorganised callus tissue that then must organise into a meristematic tissue (Maximova *et al.*, 1998).

The use of sorbitol as carbon source, instead of sucrose, and gelrite as solidifying agent was suggested to contribute to a higher transformation efficiency in apple (Zhu *et al.*, 2001) although also opposite results are described (de Bondt *et al.*, 1994). A two-step protocol, in which the leaf discs would be submitted to a callus induction phase following a shoot induction phase was suggested to be very efficient (Holefors *et al.*, 1998; Zhu *et al.*, 2001) and could constitute the basis for another approach in the future. The efficiency of the transformation was suggested to be higher than in the case of direct regeneration from the leaf tissue (Schaart *et al.*, 1995; Holefors *et al.*, 1998; Puite and Schaart, 1999). However, a disadvantage of this method is the higher risk of somaclonal variation and of chimeric plants, in contrast to direct regeneration (plant formation from one cell) (Dufour, 1990).

The incubation of the bacteria in a virulence induction medium with low pH (James *et al.*, 1993; Tao *et al.*, 1997; Holefors *et al.*, 1998) as well as the use of acetosyringone during the cocultivation phase, and not only during bacterial growth might also contribute for an increase of the gene transfer efficiency (Dandekar *et al.*, 1990).

VI GENERAL DISCUSSION

Apple scab disease

Apple scab is the most important disease of apple orchards in all temperate regions. This disease, first described in Sweden in 1819, affects most of the commercially important apple cultivars. This disease is caused by the fungus *Venturia inaequalis*, that attacks all the growing organs of the plants, like leaves, petioles, blossoms, sepals, fruits, pedicels and, less frequently young shoots and buds. Nevertheless, most significant losses occur on actively growing leaves and fruits. In the leaves, brownish-olive green spots with a velvety surface appear, causing a decrease of photosynthetic activity and repeated defoliation. In the fruits, darker scabby lesions appear, sometimes the fruit cracks and in most cases it becomes unsellable.

Control of apple scab disease

Chemical control is still the most widespread form of fighting apple scab. Fungicide spray applications in apple growing account for most of the annual routine spray programme, increasing costs and complicating cultural practices. However, the public pressure and new regulations are pushing for a significant reduction in pesticide use, due to the effects of pesticide residues on the environment and on human health. These facts contributed, together with the appearance of fungicide resistance (e.g. benomyl), to the reduction of the repeated fungicide treatment as control method. Breeding of resistant plants is an alternative method for fungal pathogen control, which is safer for the environment and yet effective against the fungus. However, apple growers will only make use of scab-resistant cultivars if the cultivars match market requirements and the resistance is durable. Therefore, breeding durable disease-resistant apple cultivars that incorporate good pomological characters is now one of the most important objectives for apple fruit breeding worldwide.

Interest of resistance breeding for durable protection

As mentioned above, increasing scab resistance of commercial apple cultivars in a durable way could significantly reduce the use of chemical fungicides and provide economical and environmental benefits for apple growers and consumers. Historically, early stages for breeding of fungal resistance were based on single genes that were inherited in a mendelian way. However, the observed overcome of the resistance trait conferred by Vf-system introgression by a race of *Venturia inaequalis* (Parisi *et al.*, 1993) showed the limitations of a

mechanism based on a dominant oligogenic source of resistance with characteristics of vertical resistance, concerning the achievement of durable protection. The expression of plant genes that would impair basic mechanisms of fungal development and the identification of proteins involved in the onset of protection reactions of resistant cultivars might be seen as an alternative to a resistance mechanism based on a gene-for-gene response. The regulation of such a defense gene by a fungus-inducible promoter which is active in the early stage of the fungal infection and the introduction of the engineered protection construct in commercially successful apple cultivars by transformation via *Agrobacterium* could constitute a promising strategy towards the strengthening of the endogenous defense capability of susceptible apple plants.

Proposed strategy and objectives

In this respect, this work focused on the establishment of the basis for an alternative control mechanism against apple scab, in which the effect of a fungus- and early inducible promoter would be combined with different putative defense genes conferring protection.

The main objectives of this work were the identification and characterisation of different 5´regulating sequences from *pgip* (polygalacturonase-inhibiting proteins) genes of apple, in order to analyse their potential value as fungus- and early-inducible promoters, and the isolation of genes with potential defense properties. Two different strategies were followed to identify putative genes participating in a protection mechanism:

a) isolation of genes encoding an acidic class III chitinase from apple, supposed to show antifungal activity and to be expressed extracellularly

b) identification of genes activated upon fungal attack that might participate in the defense reaction of a resistant cultivar.

Summary of the obtained results

Isolation of apple pgip regulating sequences

Pgip genes were identified in different species (e.g. tomato, pear, bean, apple) and are supposed to participate in plant defense activated by fungal endopolygalacturonases (Cervone *et al.*, 1992). In scab disease, an endopolygalacturonase from *Venturia inaequalis* is considered to participate in the initiation of the infection. This constitutively expressed enzyme disintegrated apple cell walls and was suggested to be one of the first enzymes involved in the infection process (Kollar, 1998). The accumulation of *pgip* transcripts upon fungal infection in early stages was reported in bean and soybean (Nuss *et al.*, 1996; Favaron

et al., 2000), which let suggest that a *pgip* promoter would be a good candidate to use as scab-inducible promoter activated at the onset of infection. Detached leaves from apple cultivars ´Golden Delicious´ and ´Jonagold´ infected with a scab conidial suspension revealed the accumulation of *pgip* transcripts in Northern analysis (IV 2.2) thus supporting the above mentioned assumption. However, a differential regulation has been reported to occur within the *pgip* family (Devoto *et al.*, 1998), emphasising the need of characterising the properties of the different apple *pgip* promoters, in order to select the most appropriate for an engineered protection mechanism.

In order to elucidate the properties of different apple *pgip* promoters and their putative induction upon fungal elicitation, two different *pgip* genes and part of their 5´regulating sequence (Q8 and N17) were isolated from an apple genomic library. Fragments from the *pgip* upstream regulating sequences were fused with the reporter gene GUS (IV 2.3.1) and 3 different constructs, differing in sequence and length, were transformed into *Nicotiana tabacum* via *Agrobacterium tumefaciens* (IV 2.3.2). Elicitation assays with transformed tobacco plants were performed in order to evaluate the inducibility of the isolated *pgip*-promoters. Supernatants of liquid cultures of *Botrytis cinerea* in different media were produced and tested for their polygalacturonase activity (IV 2.3.3). Culture conditions leading to a higher accumulation of polygalacturonases in the supernatant were selected to synthesise the fungal products being used in elicitation assays with transgenic tobacco plants harbouring the *pgip*::GUS constructs. In quantitative GUS assays with leaf explants of primary transformants, an increased accumulation of 4-MU could be measured when leaves were treated with fungal elicitors compared to mock treated leaves. Also the F1 generation revealed a clear promoter activation upon elicitation with fungal supernatants in all three constructs, suggesting the potential use of both isolated 5´upstream regulating sequences as fungus-inducible promoters (IV 2.3.6). However, the promoter response varied with the construct. Lines carrying the N construct (2kb promoter of clone N17) showed high basal GUS expression values but a weak response towards fungal elicitation. In contrast, GUS activity in lines carrying promoter constructs S (1.45kb) and P (1.23kb) of clone Q8 increased strongly upon treatment of leaf explants with the fungal supernatant, with slightly higher accumulation in S lines than in P lines. From these results, it can be deduced that the sequence from Q8 integrated in construct S would be a promising sequence to use as a fungus-inducible promoter.

Isolation of putative defense genes

The interaction between apple scab and the resistant cultivar ´Florina´ was investigated to look for genes that could be used in an engineered protection mechanism. The aim of this analysis was the identification of genes involved in pathways that are activated in the resistant cultivar upon scab inoculation, and therefore are likely to participate in the defense response.

Differential display reverse-transcription PCR (DDRT-PCR) is a PCR based technique that allows the comparison of pools of genes expressed under different conditions. In plant pathology it was first used to isolate genes participating in the interaction between tomato and the fungus *Botrytis cinerea* (Benito *et al.*, 1996) to identify fungal and host genes involved in the interaction process. In this work, DDRT-PCR was applied to analyse the gene expression pattern in resistant cultivar ´Florina´ upon inoculation with a scab conidial suspension and several differently expressed cDNAs could be identified.

Three isolated cDNAs (M3, M5, M6) showed significant homologies with defense genes from *Arabidopsis thaliana*. 2 isolated cDNAs (M9, M10) displayed significant homology with genes involved in synthesis of cell wall and the cuticular layer. For the other cDNAs isolated by DDRT-PCR, database searches revealed homologies with proteins of unknown function (M4, M13, M14) or to a protein whose function in plants is not completely unravelled (M2) (IV 3.1). In order to confirm differential expression observed in polyacrylamide gel, RT-PCR with specific primers and Northern analysis were performed. The non-radioactive detection in Northern analysis with a DIG labeled riboprobe did not provide positive results, suggesting a too low sensitivity of this method. Specific RT-PCR remained the single confirmation for the more abundant expression of clones M3, M4, M9, M10, M13 and M14 in cDNA pool derived from infected leaves (IV 3.2.1).

The attempt to obtain full-length clones by RACE was not completely successful, nevertheless 5 cDNA clones could be extended in their size (IV 3.3). Further RACE experiments or the screening of a cDNA library generated from scab-infected leaves would be required in order to isolate full-length clones.

Another option for protection could be provided by genes encoding enzymes capable of degrading fungal cell walls. Since cell walls of some fungi are rich in chitin, the use of genes encoding chitinases could provide an useful contribution in genetically engineered control of fungal pathogens. This strategy was successfully applied, e.g. in rapeseed and in grapevine, where fungal development was inhibited upon overexpression of chitinase genes in genetically modified plants (Broglie *et al.*, 1991; Grison *et al.*, 1996; Yamamoto *et al.*, 2000).

In this respect, an apple gene encoding a class III chitinase could be considered as possible defense gene. Although class III chitinases have an unclear antifungal potential, they show strong similarity in their primary structure with a fungal chitinase from *Rhizopus oligosporus*, which proved to have antifungal properties (Terakawa *et al.*, 1997). In addition, they show extracellular expression. An apoplastic chitinase may directly inhibit early growth of invading hyphae, as well as release fungal cell wall fragments which act as elicitors of additional chitinase activity and induce expression of other defense-related genes (Samac and Shah, 1994). Extracellular expression would be a major advantage in an engineered protection system against apple scab, as *Venturia inaequalis* does not penetrate into the cell, but remains in the subcuticular space above the epidermal cells. Two chitinase genes (C1, C5) were isolated from an apple genomic library (IV 4.1). In C5 the complete coding region could be identified, but in C1 the 3´end is missing. Characteristic features of class III chitinases, as pI, predicted molecular weight, copy number in the genome, absence of intron (Busam *et al.*, 1997; Robert *et al.*, 2002) were also encountered for the isolated clones C1 and C5. *In vitro* inhibition of fungal growth due to chitinase activity was also found to be dependent on the pathogen (Mauch *et al.*, 1988; Verburg and Huynh, 1991), suggesting that the effect of plant chitinases on pathogenic fungi is largely dependent upon the amount and specific distribution of chitin within the cell walls (Benhamou *et al.*, 1993). These findings underline the need of verifying the putative antifungal activity of the proteins encoded by these genes towards *Venturia inaequalis*.

The development of gene transfer techniques to crop plants has facilitated testing constitutive overexpression of proteins involved in active defense mechanisms. In an approach to evaluate the antifungal properties of the chitinase gene C5 in apple, this gene was cloned behind the viral promoter 35S and an *Agrobacterium*-mediated transformation experiment was performed with apple leaf discs (IV 4.3). The attempt to overexpress the isolated chitinase gene in the scab-susceptible apple cultivar ´Jonagold´ failed (IV 4.3.2.2) and thus, it was not possible to evaluate *in planta* its potential use as resistance factor against apple scab. The positive results obtained in the control regeneration assay (IV 4.3.2.1) suggested that the transfer of the constructs into the plant cells constituted the limiting factor in the transformation experiment.

Outlook

All plant cells can regenerate a complete organism under adequate conditions (totipotency). Due to this property, the transfer of genes into a single cell and regeneration of complete

plants from this cell (Gautier, 1993) became a possible strategy for breeding fruit trees. The generation and transfer of constructs combining a pathogen-inducible promoter and a putative defense gene into the plant genome by genetic engineering do not only accelerate breeding programs by avoiding several generations of crossings, but also prevent the introduction of undesired, non-selected genes that are introgressed during backcrossings (Korban and Chen, 1992). Only a small portion of DNA is integrated in the apple genome, while the main characteristics of the genotype remain unchanged, an important factor particularly in fruit trees, where the cultivar identification is very strong (Mehlenbacher, 1995; Bolar *et al.*, 2000). However, optimisation of transformation protocol would be required since first approach was not successful and a stable engineered resistance requires the production of numerous independent transformants to allow the selection of those with adequate level of gene expression (Mehlenbacher, 1995). *Agrobacterium*-mediated transformation would still be the method of choice, as it provides the integration of single or low transgene copies, which is more favourable than lines carrying multiple copies (van der Fits *et al.*, 2000).

Although public concerns about health and environmental impact limit the future application of transgenic plants, the use of an apple promoter in the regulation of defense genes obtained from the same genus would be a benefit. In contrast to the use of fungal or viral sequences, this might reduce the safety concerns about the transgene integration or "gene pollutioning".

In this frame, a more comprehensive characterisation of the isolated promoters, including the identification of essential and inducible elements involved in gene regulation, would be interesting.

The extracellular expression and antifungal properties of the isolated chitinase genes should be confirmed. The biochemical analysis of the enzymatic composition and activity of the intercellular fluid of fungus-infected plants could elucidate the localisation of the expressed chitinase. Another approach to evaluate the antifungal properties of chitinases encoded by the isolated genes could be the expression in a heterologous system. The chitinolytic activity of the purified protein could be then assayed directly in a fungal inhibition assay.

A possible strategy to achieve a high degree of resistance could be to pyramide chitinase genes with other defense genes for complementary activities. A synergistic effect between chitinases and ß-1,3-glucanases in impairment of fungal growth was observed (Zhu *et al.*, 1994; Jach *et al.*, 1995; Jongedijk *et al.*, 1995), which may be derived from a combined effect of hydrolases on the pathogen cell wall.

Differential display experiments revealed the upregulation of some genes involved in plant defense and in cuticle and cell wall metabolism. It would be of great relevance to confirm this

upregulation by Northern analysis and to identify the time points after fungal inoculation in which the upregulation occurs, as well as to prove the plant origin of the identified cDNAs. Investigations on the activation of similar or other genes, in susceptible cultivars upon scab inoculation might provide some information about the genetic mechanism involved in the differential response developed by susceptible and resistant cultivars. In this context, further information could be achieved by combining the concept of bulk segregant analysis (Michelmore *et al.*, 1991), successfully adapted to the analysis of molecular markers related with resistance genes (Yang *et al.*, 1997b), with the differential display RT-PCR technique. Inoculation experiments of different susceptible cultivars in one bulk, and of plants from a resistant cultivar in another bulk might be a convenient approach. From the comparison between the expression pattern of cDNA populations generated from non-infected and infected RNA pools, activated genes upon scab attack shall be identified in the susceptible and in the resistant bulks. These findings shall provide information about the differences existing between the molecular mechanisms that lead to disease establishment (susceptible cultivar) or to fungal impairment (resistant cultivar).

VII REFERENCES

Abu-Goukh A.A., Strand L.L., Labavitch J.M. 1983- Development-related changes in decay susceptibility and polygalacturonase inhibitor content of ´Bartlett´ pear fruit. *Physiol. Plant Pathol.* **23**:101-109.

Agrios G.N. 1997- Plant Pathology, 4th ed., Acad. Press, San Diego.

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. 1990- Basic local alignment search tool. *J. Mol Biol.* **215**: 403-410.

Amaro P. and Baggiolini M. 1982- Introdução à protecção integrada. DGPPA, Lisboa.

Amaro P. 1993- A evolução das pragas e doenças da macieira e pereira em Portugal. *Revista de Ciências Agrárias* **XVI**: 280-300.

An G., Ebert P.R., Mitra A., Ha S.B. 1988- Binary vectors. In: *Plant Molecular Biology Manual*. Gelvin S.B., Schilperoort R.A., Verma D.P.S. (eds). Kluwer Acad. Pub..

Andersen M.D., Jensen A., Robertus J.D., Leah R., Skriver K. 1997- Heterologous expression and characterization of wild-type and mutant forms of a 26 kDa endochitinase from barley (*Hordeum vulgare* L.). *Biochem. J.* **322**: 815-322.

Andrews J.H., Berbee F.M., Nordheim E.V. 1982- Microbial antagonism to the imperfect stage of the apple scab pathogen, *Venturia inaequalis*. *Phytopathology* **73**: 228-234.

Anonyme 2001- Europe du Nord: quelles résistantes contre la tavelure? *L´arboriculture fruitière* **550**: 15.

Anuratha C.S., Zen K.C., Cole K.C., Mew T., Muthukrishnan S. 1996- Induction of chitinases and beta-1,3-glucanases in *Rhizoctonia solani*- infected rice plants: Isolation of an infection-related chitinase cDNA clone. *Physiol. Plantarum* **97**: 39-46.

Asao H., Nishizawa Y., Arai S., Sato T., Hirai M., Yoshida K., Shinmyo A., Hibi T. 1997- Enhanced resistance against a fungal pathogen *Sphaeroteca humuli* in transgenic strawberry expressing a rice chitinase gene. *Plant Biotech.* **14**: 145-149.

Bach E. and Schollmeyer E. 1992- An ultraviolet-spectrophotometric method with 2-cyanoacetamide for the determination of the enzymatic degradation of reducing polysaccharides. *Anal. Biochem.* **203**: 335-339.

Bassam B.J., Caetano-Anolles G., Gresshoff P.M. 1991- Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **196**: 80-83.

Bénaouf G. and Parisi L. 1997- Pathogenicity of *Venturia inaequalis* strains from *Malus floribunda* 821: comparison with race 6 on apple clones. In: *Integrated control of pome fruit diseases*. Proc. of the 4th Workshop, Croydon, England, 19-23 August 1996. IOBC/WPRS Bulletin Vol. **20**: 8-11.

Bénaouf G. and Parisi L. 2000- Genetics of host-pathogen relationships between *Venturia inaequalis* races 6 and 7 and *Malus* species. *Phytopathology* **90**: 236-242.

Benhamou N., Broglie K., Broglie R., Chet I. 1993- Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultrastructural changes and cytochemical aspects of chitin breakdown. *Can. J. Microbiol.* **39**: 318-328.

Benito E.P., Prins T., van Kan J.A.L. 1996- Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Mol. Biol.* **32**: 947-957.

Bergamini A. and Giongo L. 2000a- Three new scab resistant apples: Golden Lasa, Summerfree and Golden Orange. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Bergamini A. and Giongo L. 2000b- Two promising selections from the apple scab breeding program at ISF:TN: Netta and Ciosa. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Bergamini A. and Giongo L. 2000c- Red Earlib: a new Red Delicious type. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Bergamini A. and Giongo L. 2000d- Golden Mira: a new variety resistant to apple scab and powdery mildew. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Bergamini A. and Giongo L. 2000e- Brina, a new variety resistant to apple scab and powdery mildew. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Bergmann C.W., Ito Y., Singer D., Albersheim P., Darvill A.G., Benhamou N., Nuss L., Salvi G., Cervone F., De Lorenzo G. 1994- Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. *Plant J.* **5**: 625-634.

Bevan M. 1984- Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* **12**: 8711-8721.

Biggs A.R. 1990- Apple scab. In: *Compendium of apple and pear diseases*. Jones A.L. and Aldwinckle H.S. (eds). APS Press, 6-9.

Bolar J.P., Norelli J.L., Wong K.W., Hayes C.K., Harman G. E., Aldwinckle H.S. 2000- Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. *Phytopathology* **90**: 72-77.

Boller T. 1988- Ethylene and the regulation of antifungal hydrolases in plants. *Oxford Surv. Plant Mol. Cell Biol.* **5**: 145-174.

Boone D. 1971- Genetics of *Venturia inaequalis*. *Annu. Rev. Phytopathol.* **9**: 297-318.

Bradford M.M. 1976- A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**: 248-254.

Broekaert W.F., Van Parijs J., Allen A.K., Peumans W.J. 1988- Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. *Physiol. Mol. Plant. Pathol*. **33**: 319-331.

Broglie K., Chet I., Holliday M., Cressman R., Biddle P., Knowlton S., Jeffry-Mauvais C., Broglie R. 1991- Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**: 1194-1197.

Büchter R., Stromberg A., Schmelzer E., Kombrink E. 1997- Primary structure and expression of acidic (class II) chitinase in potato. *Plant Mol. Biol.* **35**: 749-61.

Burr T.J., Matteson M.C., Smith C.A., Corral-Garcia M.R., Huang T.C. 1996- Effectiveness of Bacteria and Yeasts from Apple Orchards as Biological Control Agents of Apple Scab. *Biol. Control* **6**: 151-157.

Busam G., Kassemeyer H., Matern U. 1997- Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. *Plant Physiol.* **115**: 1029-1038.

Carisse O., Philion V., Rolland D., Bernier J. 2000- Effect of fall application of fungal antagonists on spring ascospore production of the apple scab pathogen, *Venturia inaequalis*. *Phytopathology* **90**: 31-37.

Cervone F., De Lorenzo G., Salvi G., Camardella L. 1986- Molecular evolution of fungal polygalacturonases. In: *Biology and molecular biology of plant pathogen interactions.* Bailey J. (ed). Plenum Press, New York, 385-392.

Cervone F., Hahn M.G., De Lorenzo G., Darvill A., Albersheim P. 1989- Host-pathogen interactions: XXXIII A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiol*. **90**: 542-548.

Cervone F., De Lorenzo G., Pressey R., Darvill A.G., Albersheim P. 1990- Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? *Phytochemistry* **29**: 447-449.

Cervone F., De Lorenzo G., Caprari C., Clark A.J., Desiderio A., Devoto A., Leckie F., Nuss L., Salvi G., Toubart P. 1992- The interaction between fungal endopolygalacturonase and plant cell wall pgip (polygalacturonase-inhibiting protein). In: *Mechanisms of plant defence responses*. Fritig B., Legrand M. (eds). Kluwer Acad. Pub., Dordrecht, 64-67.

Chang S., Puryear J., Cairney J. 1993- A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* **11**: 113-116.

Chang M.M., Horovitz D., Culley D., Hadwiger L.A. 1995- Molecular cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. *Plant. Mol. Biol.* **28**: 105-111.

Chen Z.-L., Schuler M.A., Beachy R.N. 1986- Functional analysis of regulatory elements in a plant embryo-specific gene. *Proc. Natl. Acad. Sci. USA* **83**: 8560-8564.

Chen C. and Chen Z. 2000- Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. *Plant. Mol. Biol.* **42**: 387-396.

Cheng F.S., Weeden N.F., Brown S.K., Aldwinckle H.S., Gardiner S.E., Bus V.G. 1998- Development of a DNA marker for V_m, a gene conferring resistance to apple scab. *Genome* **41**: 208-214.

Chevalier M., Lespinasse Y., Renaudin S. 1991- A microscopic study of the different classes of symptoms coded by the Vf gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathol.* **40**: 249-256.

Chevalier M. and Lespinasse Y. 1994- Ultrastructural study of host-parasite interaction: Early phases of *Venturia inaequalis* installation on susceptible and resistant apple leaves. *Bulletin de l'Association des Anatomistes* **78**: 33-37.

Cohen E. 1987- Chitin biochemistry: synthesis and inhibition. *Annu. Rev. Entomol*. **32**: 71-93.

Collinge D.B., Kragh K.M., Mikkelsen J.D., Nielsen K.K., Rasmussen U., Vad K. 1993- Plant chitinases. *Plant J.* **3**: 31-40.

Collinge M. and Boller T. 2001- Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by *Phytophthora infestans* and to wounding. *Plant. Mol. Biol.* **46**: 521-529.

Cooper R.M. 1984- The role of cell wall-degrading enzymes in infection and damage. In: *Plant disease: infection, damage and loss*. Wood R.K.S. and Jellis G.J. (eds). Blackwell Scientific Publications, Oxford, 13-27.

Corlett M., Chong, J., Kokko E.G. 1976- The ultrastructure of the *Spilocaea* state of *Venturia inaequalis* in vivo. *Can. J. Microbiol.* **22**: 1144-1152.

Crosby J.A., Janick J., Pecknold P.C., Korban S.S., O'Connor P.A., Ries S.M., Goffreda J., Voordeckers A. 1992- Breeding apples for scab resistance: 1945-1990. *Acta Hort.* **317**: 43-70.

Cruyssen G. and Kamoen O. 1992- Polygalacturonases of *Botrytis cinerea*. In: *Recent advances in Botrytis research*. Proceedings of the 10th International *Botrytis* Symposium, Heraklion, Crete, Greece, 5-10 April 1992. Verhoeff K., Malathrakis N.E., Williamson B. (eds), 69-75.

Cullen D. and Andrews J.H. 1984- Evidence for the role of antibiosis in the antagonism of *Chaetomium globosum* to the apple scab pathogen, *Venturia inaequalis*. *Can. J. Bot*. **62**: 1819-1823.

Cullen D., Berbee F.M., Andrews J.H. 1984- *Chaetomium globosum* antagonizes the apple scab pathogen, *Venturia inaequalis*, under field conditions. *Can. J. Bot*. **62**: 1814-1818.

da Câmara Machado A., Regner F., Steinkellner H., Mattanovich D., Hanzer V., Weiss H., Laimer da Câmara Machado M., Katinger H. 1992- Coat protein-mediated protection against plum pox virus. *Acta Hort.* **309**: 203-210.

da Câmara Machado A., Brazda M., Katinger H., Laimer da Câmara Machado M. 1995- Influence of different light qualities on the regeneration from leaf explants of *Malus domestica*. In: *Proceedings 50th Anniversary of the Institute of Applied Microbiology*. 19-21 April 1995, Vienna.

Dandekar A.M., Uratsu S.L., Matsuta N. 1990- Factors influencing virulence in *Agrobacterium*-mediated transformation of apple. *Acta Hort*. **280**: 483-494.

Daruwala R., Bhattacharyya D.K., Kwon O., Meganathan R. 1997- Menaquinone (vitamin K_2) biosynthesis:overexpression, purification, and characterization of a new isochorismate synthase from *Escherichia coli*. *J. Bacteriol.* **179**: 3133-3138.

Datta K., Tu J., Oliva N., Ona I., Velazhahan R., Mew T.W., Muthukrishnan S., Datta S.K. 2001- Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Sci.* **160**: 405-414.

Dayton D.F. and Williams E.B. 1970- Additional allelic genes in *Malus* for scab resistance of two reaction types. *J. Am. Soc. Hort. Sci*. **95**: 735-736.

de Bondt A., Eggermont K., Druart P., de Vil M., Goderis I., Vanderleyden J., Broekaert W.F. 1994- *Agrobacterium*-mediated transformation of apple (*Malus* x *domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Rep.* **13**: 587-593.

de Bondt A., Eggermont K., Penninckx I., Goderis I., Broekaert W.F. 1996- *Agrobacterium*-mediated transformation of apple (*Malus* x *domestica* Borkh.): an assessment of factors affecting regeneration of transgenic plants. *Plant Cell Rep.* **15**:549-554.

de Coster J. 1998- European trends in the development of apple varieties. http://www.idfta.org/cft/1998/vol31no4/interpomapapers.html.

Dempsey D.M.A., Shah J., Klessig D.F. 1999- Salicylic acid and disease resistance in plants. *Crit. Rev. Plant. Sci.* **18**: 547-575.

Derckel J.P., Legendre L., Audran J.-C., Haye B., Lambert B. 1996- Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. *Plant Sci.* **119**: 31-37.

Derckel J.P., Audran J.C., Haye B., Lambert B., Legendre L. 1998- Characterization, induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and beta-1,3-glucanases of ripening grape berries. *Physiol. Plantarum* **104**: 56-64.

Devoto A., Clark A.J., Nuss L., Cervone F., De Lorenzo G. 1997- Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. *Planta* **202**: 284-292.

Devoto A., Leckie F., Lupotto E., Cervone F., De Lorenzo G. 1998- The promoter of a gene encoding a polygalacturonase-inhibiting protein of *Phaseolus vulgaris* L. is activated by wounding but not by elicitors or pathogen infection. *Planta* **205**: 165-174.

Dixon D., Cummins I., Cole D., Edwards R. 1998- Glutathione-mediated detoxification systems in plants. *Curr. Opin. Plant Biol.* **1**: 258-266.

Doyle J.I. and Doyle J.L. 1990- Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Dudler R., Hertig G., Rebmann G., Bull J., Mauch F. 1991- A pathogen-induced wheat gene encodes a protein homologous to glutathione S-transferase. *Mol. Plant Microbe Interact*. **4**: 14-18.

Dufour M. 1990- Improving yield of adventitious shoots in apple. *Acta Hort*. **280**: 51-60.

Edwards R., Dixon D.P., Walbot V. 2000- Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant. Sci*. **5**: 193-198.

Enochs N.J. 1964- Histological studies on the development of *Venturia inaequalis* (Cke.) Wint. in susceptible and resistant selections of *Malus*. Master´s Thesis, Purdue Univ., Lafayette, Indiana.

Esaka M. and Teramoto T. 1998- cDNA cloning, gene expression and secretion of chitinase in winged bean. *Plant Cell Physiol.* **39**: 349-356.

Evans K., Lespinasse Y., Parisi L., Laurens F., Durel C.-E. 2000- Durable resistance to scab and mildew in apple- a european project. *Pesticide Outlook* **June:** 84-87.

FAO Database 2001- http://apps.fao.org.

Favaron F., D'Ovidio R., Porceddu E., Alghisi P. 1994- Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. *Planta* **195**: 80-87.

Favaron F., Destro T., D´Ovidio R. 2000- Transcript accumulation of polygalacturonase-inhibiting protein (PGIP) following pathogen infections in soybean. *J. Plant Pathol.* **82**: 103-109.

Fideghelli C. 2000- The activity on apple scab resistance within the national research programs "Fruit growing" and "List of recommended fruit varieties" supported by the ministry for agricultural policy. In: *ISHS symposium on apple breeding for scab resistance*, 14-16 September 2000, Trento. *Acta Hort*. (in press).

Flor H.H. 1956- The complementary genic systems in flax and flax rust. *Adv. Genet.* **8**: 29-54.

Fourney, M.R., Miyakoshi J., Day III R.S., Paterson M.C. 1988- Northern blotting: efficient RNA staining and transfer. *Focus* **10**: 5-7.

Franke W. 1961- Ectodesmata and foliar adsorption. *Am. J. Bot.* **48**: 683-691.

Frediani M., Cremonini R., Salvi G., Caprari C., Desiderio A., D´Ovidio R., Cervone F., De Lorenzo G. 1993- Cytological localization of the PGIP genes in the embryo suspensor cells of *Phaseolus vulgaris* L.. *Theor. Appl. Genet*. **87**: 369-373.

Frohman M.A., Dush M.K., Martin G.R. 1988- Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**: 8998-9002.

Gadoury D.M., MacHardy W.E., Rosenberger D.A. 1989- Integration of pesticide application schedules for disease and insect control in apple orchards of the northeastern United States. *Plant Dis.* **73**: 98-105.

Gallagher S.R. 1992- Quantitation of GUS activity by fluorometry. In: *GUS protocols. Using the GUS gene as a reporter of gene expression.* Gallagher S.R. (ed). Acad. Press, 47-59.

Gao M., Tao R., Miura K., Dandekar A.M., Sugiura A. 2001- Transformation of Japanese persimmon (*Diospyros kaki* Thunb.) with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. *Plant Sci.* **160**: 837-845.

Garcia M.E., Berkett L.P., Clements J.F., Clements J., Neff G. 2000- Productivity and fruit quality evaluation of ´Liberty´ apple (*Malus* x *domestica* Borkh) under a reduced fungicide program. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Gardiner S.E., Bassett H.C.M., Noiton D.A.M., Bus V.G., Hofstee M.E., White A.G., Ball R.D., Foster R.L.S., Rikkerink E.H.A. 1996- A detailed linkage map around an apple scab resistance gene demonstrates that two disease resistance classes both carry the V-f gene. *Theor. Appl. Genet.* **93**: 485-493.

Gassmann W., Hinsch M.E., Staskawicz B.J. 1999- The *Arabidopsis* RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. *Plant J.* **20**: 265-277.

Gautier M. 1988- Les productions fruitières. Agriculture d'aujourd'hui, Paris.

Gautier M. 1993- L'arbre fruitier, 2ème edition. Agriculture d'aujourd'hui, Paris.

Geri C., Cecchini E., Giannakou M.E., Covey S.N., Milner J.J. 1999- Altered patterns of gene expression in *Arabidopsis* elicited by cauliflower mosaic virus (CaMV) infection and by a CaMV gene VI transgene. *Mol. Plant Microbe Interact.* **12**: 377-84.

Gessler C. 1992- Scab resistance in apple: the minor genes in the Vf-resistance. *Acta Phytopatologica et Entomologica Hungarica* **27**: 257-263.

Gessler C. 1994- Biology and biotechnology in strategies to control apple scab. *Norwegian J. Agric. Sci.* **Supplement 17**: 337-353.

Gianfranceschi L., Mc Dermott J.M., Seglias N., Koller B., Kellerhals M., Gessler C. 1994- Towards a marker assisted breeding for resistance against apple scab. *Euphytica* **77**: 93-96.

Gianfranceschi L., Koller B., Seglias N., Kellerhals M., Gessler C. 1996- Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. *Theor. Appl. Genet.* **93**: 199-204.

Gomes V.M., Oliveira A.E.A., Xavier-Filho J. 1996- A chitinase and a ß-1,3-glucanase isolated from the seeds of cowpea (*Vigna unguiculata* L Walp) inhibit the growth of fungi and insect pests of the seed. *J. Sci. Food Agric*. **72**: 86-90.

Grison R., Grezes-Besset B., Schneider M., Lucante N., Olsen L., Leguay J., Toppan A. 1996- Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. *Nature Biotech.* **14**: 643-646.

Gronwald J.W. and Plaisance K.L. 1998- Isolation and characterization of glutathione S- transferase isozymes from sorghum. *Plant Physiol*. **117**: 877-892.

Hahn M.G., Buchell P., Cervone F., Doares S.H., O´Neill R.A., Darvill A.G., Albersheim P. 1989- The role of cell wall constituents in plant-pathogen interactions. In: *Plant-Microbe Interactions* Vol. 3 Nester E. and Kosuge T. (eds). MacMillan Press, New York, 131-181.

Harker C.L., Ellis T.H., Coen E.S. 1990- Identification and genetic regulation of the chalcone synthase multigene family in pea. *Plant Cell* **2**: 185-194.

Heitz T., Segond S., Kauffmann S., Geoffroy P., Prasad V., Brunner F., Fritig B., Legrand M. 1994- Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. *Mol. Gen. Genet.* **245**: 246-254.

Hering O., Zinkernagel V., Bartscherer H.C. 1993- SEM investigations of infection patterns of *Venturia inaequalis* (Cooke) Aderh. on leaves of differently resistant apple cultivars. *Zeitschrift für Pflanzenkrankenheiten und Pflanzenschutz* **100**: 379-388.

Hermsmeier D., Mazarei M., Baum T.J. 1998- Differential display analysis of the early compatible interaction between soybean and the soybean cyst nematode. *Mol. Plant Microbe Interact.* **11**: 1258-1263.

Hermsmeier D., Hart J.K., Byzova M., Rodermel S.R., Baum T.J. 2000- Changes in mRNA abundance within *Heterodera schachtii*-infected roots of *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **13**: 309-315.

Hermsmeier D., Schittko U., Baldwin I.T. 2001- Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiol.* **125**: 683-700.

Holefors A., Xue Z.-T., Welander M. 1998- Transformation of the apple rootstock M26 with the *rol*A gene and its influence on growth. *Plant Sci.* **136**: 69-78.

Holowczak J., Kuc J., Williams E.B. 1962- Metabolism in vitro of phloridzin and other compounds by *Venturia inaequalis*. *Phytopathology* **52**: 1019-1023.

Hough L.F. 1944- A survey of the scab resistance of the foliage on seedlings in selected apple progenies. *Proc. Am. Soc. Hort. Sci.* **44**: 260-271.

Hough L.F., Shay J.R., Dayton D.F. 1953- Apple scab resistance from *Malus floribunda* Sieb. *Proc. Amer. Hort. Sci.* **62**: 341-347.

Hough L.F., Williams E.B., Goonewardene, Janick J., Korban S.S. 1988- Progress in developing environmentally and commercially acceptable apple cultivars. *International Symposium on Horticultural Germplasm Cultivated and Wild*. Part I, Beijing, China, 205-208.

IAM Plant Pathology Webpages 2001- http://www.boku.ac.at/iam/pbiotech/phytopath/.

INE Database 2001- http://www.ine.pt.

Ishige F., Mori H., Yamazaki K., Imaseki H. 1993- Cloning of a complementary DNA that encodes an acidic chitinase which is induced by ethylene and expression of the corresponding gene. *Plant Cell Physiol.* **34**: 103-111.

Ivanicka J., Kellerhals M., Theiler R. 1996- Evaluation of scab (*Venturia inaequalis* (Cooke) G. Wint.) on shoots and detached leaves from in vitro and greenhouse grown plants of the apple (*Malus domestica* Mill.) cultivars Golden Delicious and Florina. *Gartenbauwissenschaft* **61**: 242-248.

Jach G., Gornhardt B., Mundy J., Logemann J., Pinsdorf E., Leah R., Schell J., Maas C. 1995- Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J.* **8**: 97-109.

Jagoueix-Eveillard S., Tarendeau F., Guolter K., Danet J.-L., Bové J., Garnier M. 2001- *Catharanthus roseus* genes regulated differentially by mollicute infections. *Mol. Plant Microbe Interact.* **14**: 225-233.

James D.J., Passey A.J., Barbara D.J., Bevan M. 1989- Genetic transformation of apple (*Malus pumila* Mill.) using a disarmed Ti-binary vector. *Plant Cell Rep*. **7**: 658-661.

James D.J., Passey A.J., Barbara D.J. 1990a- *Agrobacterium*-mediated transformation of apple and strawberry using Ti-binary vectors. *Acta Hort*. **280**: 495-502.

James D.J., Passey A.J., Barbara D.J. 1990b- *Agrobacterium*-mediated transformation of the cultivated strawberry (*Fragaria* x *ananassa* Duch.) using disarmed binary vectors. *Plant Sci*. **69**: 79-94.

James D.J., Uratsu S., Cheng J., Negri P., Viss P., Dandekar A.M. 1993- Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Rep.* **12**: 559-563.

Janick J. 2000- History of the PRI apple breeding program. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Jarvis M.C. 1984- Structure and properties of pectin gels in plant cell walls. *Plant Cell and Environment* **7**: 153-164.

Jefferson R.A., Burgess S.M., Hirsh D. 1986- beta-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**: 8447-8451.

Jenks M.A., Joly R.J., Peters P.J., Rich P.J., Axtell J.D., Ashworth E.A. 1994- Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. *Plant Physiol*. **105**: 1239-1245.

Johnston D.J. and Williamson B. 1992a- Production in vitro of polygalacturonases by *Botrytis cinerea*. In: *Recent advances in Botrytis research.* Proceedings of the 10th International *Botrytis* Symposium, Heraklion, Crete, Greece, 5-10 April 1992 Verhoeff K., Malathrakis N.E., Williamson B. (eds), 59-62.

Johnston D.J. and Williamson B. 1992b- Purification and characterization of four polygalacturonases from *Botrytis cinerea*. *Mycol. Res.* **96**: 343-349.

Johnston D.J., Williamson B., McMillan G.P. 1994- The interaction *in planta* of polygalacturonases from *Botrytis cinerea* with a cell wall-bound polygalacturonase-inhibiting protein (PGIP) in raspberry fruits. *J. Exp. Bot.* **45**: 1837-1843.

Jones T.M., Anderson A.J., Albersheim P. 1972- Host-pathogen interactions. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. *Physiol. Plant Pathol*. **2**: 153-166.

Jones J.D.G. 1988- Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. *Mol. Gen. Genet*. **212**: 536-542.

Jones A.L. and Aldwinckle H.S. 1990- Compendium of apple and pear diseases. APS Press.

Jongedijk E., Tigelaar H., van Roekel J.S.C., Bres-Vloemans S.A., Dekker I., van der Elzen P.J.M., Cornelissen B.J.C. and Melchers L.S. 1995- Synergistic activity of chitinases and ß-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85**: 173-180.

Karr A.L. and Albersheim P. 1970- Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by a "wall-modifying enzyme". *Plant Physiol*. **46**: 69-80.

Kellerhals M., Fouillet A., Lespinasse Y. 1993- Effect of the scab inoculum and the susceptible parent on resistance to apple scab (*Venturia inaequalis*) in the progenies of crosses to the scab resistant cv ´Florina´. *Agronomie* **13**: 631-636.

Kellerhals M. and Meyer M. 1994- Combining stable disease resistance with high fruit quality and good yielding capacity in apple. *Norwegian J. Agric. Sci.* **Supplement 17**: 39-48.

Kim C.Y., Gal S.W., Choe M.S., Jeong S.Y., Lee S.I., Cheong Y.H., Lee S.H., Choi Y.J., Han C.D., Kang K.Y., Cho M.J. 1998- A new class II rice chitinase, Rcht2, whose induction by fungal elicitor is abolished by protein phosphatase 1 and 2A inhibitor. *Plant Mol. Biol.* **37**: 523-534.

King G.J., Alston F.H., Battle I., Chevreau E., Gessler C., Janse J., Lindhout P., Manganaris A.G., Sansavini S., Schmidt H., Tobutt K. 1991- The "European apple genome mapping project"- developing a strategy for mapping genes coding for agronomic characters in tree species. *Euphytica* **56**: 89-94.

King G.J., Alston F.H., Brown L.M., Chevreau E., Evans K.M., Dunemann F., Janse J., Laurens F., Lynn J.R., Maliepaard C., Manganaris A.G., Roche P., Schmidt H., Tartarini S., Verhaegh J., Vrielink R. 1998- Multiple field and glasshouse assessments increase the reliability of linkage mapping of the Vf source of scab resistance in apple. *Theor. Appl. Genet*. **96**: 699-708.

Koch T., Kellerhals M., Gessler C. 2000- Virulence pattern of *Venturia inaequalis* field isolates and corresponding differential resistance in *Malus* x *domestica*. *J. Phytopathol.* **148**: 357-364.

Kollar A. 1994- Characterization of specific induction, activity and isozyme polymorphisms of extracellular cellulases from *Venturia inaequalis* detected in vitro and on the host plant. *Mol. Plant Microbe Interact.* **7**: 603-611.

Kollar A. 1998- Characterization of an endopolygalacturonase produced by the apple scab fungus, *Venturia inaequalis*. *Mycological Res.* **102**: 313-319.

Koller B., Müller M., Valsangiacomo C., Gessler C. 1992- Cell wall degrading enzymes and inhibitors involved in the interaction between *Venturia inaequalis* and *Malus domestica*. *Acta Phytopatologica et Entomologica Hungarica* **27**: 353-359.

Koller B., Gianfranceschi L., Seglias N., McDermott J., Gessler C. 1994- DNA Markers linked to *Malus floribunda* 821 scab resistance. *Plant Mol. Biol.* **26**: 597-602.

Köller W. and Parker D.M. 1989- Purification of cutinase from *Venturia inaequalis*. *Phytopathology* **79**: 278-283.

Köller W., Parker D.M., Becker C.M. 1991- Role of cutinase in the penetration of apple leaves by *Venturia inaequalis*. *Phytopathology* **81**: 1375-1379.

Komjanc M., Festi S., Rizzotti L., Cattivelli L., Cervone F., De Lorenzo G. 1999- A leucine-rich repeat receptor-like protein kinase (LRPKm1) gene is induced in *Malus* x *domestica* by *Venturia inaequalis* infection and salicylic acid treatment. *Plant Mol. Biol.* **40**: 945-957.

Korban S.S. and Chen H. 1992- Apple. *Biotechnology of perennial fruit crops.* Hammerschlag F. A. and Litz R. (eds). CAB International, Wellington, Oxon, UK, 203-207.

Krüger J. 1991- Scab incidence in progenies of crosses with the scab resistant apple cultivar Prima. *Zeitschrift für Pflanzenkrankenheiten und Pflanzenschutz* **98**: 73-76.

Kucheryava N., Fiss M., Auling G., Kroppenstedt R.-M. 1999- Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic in vitro to *Venturia inaequalis*, the causal agent of apple scab. *Systematic Appl. Microbiol.* **22**: 472-478.

Kuhlemeier C. 1992- Transcriptional and post-transcriptional regulation of gene expression in plants. *Plant Mol. Biol.* **19**: 1-14.

Kurosaki F., Tashiro N., Nishi A. 1988- Role of chitinase and chitin oligosaccharides in lignification response of cultured carrot cells treated with mycelial walls. *Plant Cell Physiol*. **29**: 527-531.

Kusters-van Someren M.A., Manders B.J.G., Visser J. 1992- Pectic degradation by *Botrytis cinerea*: a molecular genetic approach. In: *Recent advances in Botrytis research*. Proceedings of the 10th International *Botrytis* Symposium, Heraklion, Crete, Greece, 5-10 April 1992, Verhoeff K., Malathrakis N.E., Williamson B. (eds), 30-36.

Laimer M., da Câmara Machado A., Hanzer V., Weiss H., Mattanovich D., Himmler G., Katinger H. 1988- In-vitro Kultur zur Virusfreimachung alter Apfelsorten. *Mitt. Klosterneuburg* **38**: 247-249.

Laimer M., da Câmara Machado A., Hanzer V., Himmler G., Mattanovich D. and Katinger H. 1989- Regeneration of shoots from leaf discs of fruit trees as a tool for transformation. *Acta Hort*. **235**: 85-92.

Laimer da Câmara Machado M., da Câmara Machado A., Hanzer V., Weiß H., Regner F., Steinkellner H., Mattanovich D., Plail R., Knapp E., Kalthoff B., Katinger H. 1992- Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of Plum Pox Virus. *Plant Cell Rep.* **11**: 25-29.

Lamb R.C. and Hamilton J.M. 1969- Environmental and genetic factors influencing the expression of resistance to scab (*Venturia inaequalis* Cke. Wint.) in apple progenies. *J. Amer. Soc. Hort. Sci.* **94**: 554-557.

Lapopin L., Gianinazzi-Pearson V., Franken P. 1999- Comparative differential RNA display analysis of arbuscular mycorrhiza in *Pisum sativum* wild type and a mutant defective in late stage development. *Plant Mol. Biol.* **41**: 669-677.

Lawrence C.B., Joosten M.H.A.J., Tuzun S. 1996- Differential induction of pathogenesis-related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. *Physiol. Mol. Plant Pathol.* **48**: 361-377.

Lawton K., Ward E., Payne G., Moyer M., Ryals J. 1992- Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. *Plant Mol. Biol.* **19**: 735-743.

Lawton K.A., Beck J., Potter S., Ward E., Ryals J. 1994- Regulation of cucumber class III chitinase gene expression. *Mol. Plant Microbe Interact.* **7**: 48-57.

Leah R., Tommerup H., Svendsen I., Mundy J. 1991- Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**: 1564-1573.

Lespinasse Y. 2000- DARE Newsletter No. 3 European project FAIR5 CT97-3898.

Lespinasse Y., Durel C.-E., Laurens F., Chevalier M., Pinet C., Parisi L. 2000- A european project: D.A.R.E.- Durable apple resistance in europe (FAIR5 CT97-3898). Durable resistance of apple to scab and powdery-mildew: one step more towards an environmental friendly orchard. In: *Proceedings of the Eucarpia Symp. on Fruit Breeding and Genetics*. Geibel M., Fischer M., Fischer C. (eds). *Acta Hort.* **538**: 197-200.

Liang P. and Pardee A.B. 1992- Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**: 967-971.

Liang P., Bauer D., Averboukh L., Warthoe P., Rohrwild M., Muller H., Strauss M., Pardee A.B. 1995- Analysis of altered gene expression by differential display. *Methods in Enzymol.* **254**: 304-321.

Lin W., Anuratha C.S., Datta K., Potrykus I., Muthukrishnan S., Datta S.K. 1995- Genetic engineering of rice for resistance to sheath blight. *Bio/technology* **13**: 686-691.

Logemann J., Jach G., Logemann S., Leah R., Wolf G., Mundy J., Oppenheim A., Chet I., Schell J. 1993- Expression of a ribosome inhibiting protein (RIP) or a bacterial chitinase leads to fungal resistance in transgenic plants. In: *Mechanisms of plant defense responses*. Fritig B. and Legrand M. (eds). Kluwer Acad. Pub., Dordrecht, 446-448.

Lopes M.C. 1993- Algumas características culturais de *Spilocaea pomi*. *Revista de Ciências Agrárias* **XVI**: 355-360.

MacHardy W.E. 1996- Apple scab. Biology, epidemiology and management. APS Press.

Manganaris A.G., Alston F.H., Weeden N.F., Aldwinckle H.S., Gustafson H.L., Brown S.K. 1994- Isozyme locus Pgm-1 is tightly linked to a gene (V-f) for scab resistance in apple. *J. Amer. Soc. Hort. Sci.* **119**: 1286-1288.

Marrs K.A. 1996- The functions and regulation of glutathione-S-transferases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**: 127-158.

Martin J.T. 1964- Role of cuticle in the defense against plant disease. *Annu. Rev. Phytopathol.* **2**: 81-100.

Martin T., Wöhner R.-V., Hummel S., Willmitzer L., Frommer W. 1992- The GUS reporter system as a tool to study plant gene expression. In: *GUS protocols. Using the GUS gene as a reporter of gene expression.* Gallagher S.R. (ed.). Acad. Press, 23-43.

Martin-Laurent F., van Tuinen D., Dumas-Gaudot E., Gianinazzi-Pearson V., Gianinazzi S., Franken P. 1997- Differential display analysis of RNA accumulation in arbuscular mycorrhiza of pea and isolation of a novel symbiosis-regulated plant gene. *Mol. Gen. Genet.* **256**: 37-44.

Mathews H., Wagoner W., Kellogg J., Bestwick R. 1995- Genetic transformation of strawberry:stable integration of a gene to control biosynthesis of ethylene. *In vitro Cell. Dev. Biol.* **31P**: 36-43.

Mathias R.J. and Boyd L.A. 1986- Cefotaxime stimulates callus growth, embryogenesis and regeneration in hexaploid bread wheat *Triticum aestivum* L. Em. Thell. *Plant Sci*. **46**: 217-224.

Matias H. and Gonçalves L. 1993- Acção dos avisos agrícolas das regiões do Ribatejo e Dão no combate a inimigos das pomóideas. *Revista de Ciências Agrárias* **XVI**: 253-260.

Mauch F., Hadwiger L.A., Boller T. 1984- Ethylene: symptom, not signal for the induction of chitinase and ß-1,3-glucanase in pea pods by pathogen and elicitors. *Plant Physiol*. **76**: 607-611.

Mauch F., Mauch-Mani B., Boller T. 1988- Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and ß-1,3-glucanase. *Plant Physiol*. **88**: 936-942.

Mauch F. and Staehelin L.A. 1989- Functional implications of the subcellular localization of ethylene-induced chitinase and ß-1,3-glucanase in bean leaves. *Plant Cell* **1**: 447-457.

Mauch F., Mauch-Mani B., Gaille C., Kull B., Haas D., Reimmann C. 2001- Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *Plant J.* **25**: 67-77.

Maximova S.N., Dandekar A.M., Guiltinan M.J. 1998- Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. *Plant Mol. Biol.* **37**: 549-559.

Mazeyrat F., Mouzeyar S., Nicolas P., Tourvieille de Labrouhe D., Ledoigt G. 1998- Cloning, sequence and characterization of a sunflower (*Helianthus annuus* L.) pathogen-induced gene showing sequence homology with auxin-induced genes from plants. *Plant Mol. Biol.* **38**: 899-903.

McHughen A., Jordan M., Feist G. 1989- A preculture period prior to *Agrobacterium* inoculation increases production of transgenic plants. *J. Plant Physiol*. **135**: 245-248.

Mehlenbacher S. 1995- Classical and molecular approaches to breeding fruit and nut crops for disease resistance. *HortScience* **30**: 466-477.

Melchers L.S., Apotheker-de Groot M., van der Knaap J.A., Ponstein A.S., Sela-Buurlage M.B., Bol J.F., Cornelissen B.J.C., van der Elzen P.J.M., Linthorst H.J.M. 1994- A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *Plant J.* **5**: 469-480.

Merwin I.A., Brown S.K., Rosenberger D.A., Cooley D.R., Berkett L.P. 1994- Scab-resistant apples for the northeastern United States: new prospects and old problems. *Plant Dis.* **78**: 4-10.

Messiaen C.M. 1981- Les varietés résistantes. Institut National de la Recherche Agronomique.

Messner R., Schweigkofler W., Ibl M., Berg G., Prillinger H. 1996- Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18rRNA-gene. *J. Phytopathol*. **144**: 347-354.

Metraux J.P., Burkhart W., Moyer M., Dincher S., Middlesteadt W., Williams S., Payne G., Carnes M., Ryals J. 1989- Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proc. Natl. Acad. Sci. USA* **86**: 896-900.

Michelmore R.W., Paran I., Kesseli R.V. 1991- Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA* **88**: 9828-9832.

Milaire H.G. 1993- La protection phytosanitaire integrée des pommiers et des poiriers en France. *Revista de Ciências Agrárias* **XVI**: 211-245.

Millar A.A., Clemens S., Zachgo S., Giblin E.M., Taylor D.C., Kunst L. 1999- CUT1, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *Plant Cell* **11**: 825-838.

Mills W.D. 1944- Efficient use of sulfur dusts and sprays during rain to control apple scab. *Cornell Ext. Bull.* 630.

Moloney M.M., Walker J.J., Sharma K.K. 1989- High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep*. **8**: 238-242.

Moore-Landecker E. 1996- Fundamentals of the Fungi, 4th ed. Prentice Hall, New Jersey.

Moreno P.R.H., van deer Heijden R., Verpoorte R. 1994- Elicitor-mediated induction of isochorismate synthase and accumulation of 2,3-dihydroxybenzoic acid in *Catharanthus roseus* cell suspension and shoot cultures. *Plant Cell Rep*. **14**: 188-191.

Moser R. 1997- Evaluierung des Schutzes gegen Grapevine Fanleaf Virus in transgenen *Nicotiana benthamiana*. Diplomarbeit, Universität für Bodenkultur, Wien.

Müller M., Sierotzki H., Gessler C. 1994- Are phenolics and enzyme inhibitors involved in resistance of apple against *Venturia inaequalis*? *Norwegian J. Agric. Sci.* **Supplement 17**: 395-398.

Munoz C.I. and Bailey A. 1998- A cutinase-encoding gene from *Phytophthora capsici* isolated by differential-display RT-PCR. *Curr. Genet.* **33**: 225-230.

Murashige T. and Skoog F. 1962- A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15**: 473-497.

Naleway J.J. 1992- Histochemical, spectrophotometric, and fluorometric GUS substrates. In: *GUS protocols. Using the GUS gene as a reporter of gene expression*. Gallagher S.R. (ed). Acad. Press, 61-76.

NCBI Database http://www.ncbi.nlm.nih.gov/entrez/query.fcgi.

Nehra N.S., Chibar R.N., Kartha K.K., Datla R.S.S., Crosby W.L., Stushnoff C. 1990- Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disk regeneration system. *Plant Cell Rep*. **9**: 293-298.

Neuhaus J.M., Sticher L., Meins F. Jr, Boller T. 1991a- A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. *Proc. Natl. Acad. Sci. USA* **88**: 10362-10366.

Neuhaus J.M., Ahl-Goy P., Hinz U., Flores S., Meins F Jr. 1991b- High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris*. Susceptibility of transgenic plants to *Cercospora nicotianae* infection. *Plant Mol. Biol.* **16**: 141-151.

Neuhaus J.M., Fritig B., Linthorst H.J.M., Meins F. Jr., Mikkelsen J.D., Ryals J. 1996- A revised nomenclature for chitinase genes. *Plant Mol. Biol. Reporter* **14**: 102-104.

Nicetto N. 1998- Trends in the european apple production. http://www.idfta.org/cft/1998/vol31no4/interpomapapers.html.

Nicholson R.L., Kuc J., Williams E.B. 1972- Histochemical demonstration of transitory esterase activity in *Venturia inaequalis*. *Phytopathology* **62**: 1242-1247.

Nielsen K.K., Mikkelsen J.D., Kragh K.M., Bojsen K. 1993- An acidic class III chitinase in sugar beet: induction by *Cercospora beticola*, characterization, and expression in transgenic tobacco plants. *Mol. Plant Microbe Interact.* **6**: 495-506.

Nielsen H., Engelbrecht J., Brunak S., von Heijne G. 1997- Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**: 1-6.

Nishizawa Y., Kawakami A., Hibi T., He D.Y., Shibuya N., Minami E. 1999a- Regulation of the chitinase gene expression in suspension-cultured rice cells by N-acetylchitooligosaccharides: differences in the signal transduction pathways leading to the activation of elicitor-responsive genes. *Plant Mol. Biol.* **39**: 907-914.

Nishizawa Y., Nishio Z., Nakazono K., Soma M., Nakajima E., Ugaki M., Hibi T. 1999b- Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic rice by constitutive expression of rice chitinase. *Theor. Appl. Genet*. **99**: 383-390.

Norelli J.L. and Aldwinckle H.S. 1993- The role of aminoglycoside antibiotics in the regeneration and selection of neomycin phosphotransferase-transgenic apple tissue. *J. Amer. Soc. Hort. Sci.* **118**: 311-316.

Norelli J., Mills J.A., Aldwinckle H. 1996- Leaf wounding increases efficiency of *Agrobacterium*-mediated transformation of apple. *HortScience* **31**: 1026-1027.

Noveroske R.L., Williams E.B., Kuc J. 1964- ß-glycosidase and phenoloxidase in apple leaves and their possible relation to resistance to *Venturia inaequalis*. *Phytopathology* **54**: 98-103.

Nuss L., Mahé A., Clark A.J., Grisvard J., Dron M., Cervone F., De Lorenzo G. 1996- Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. *Physiol. Mol. Plant Pathol.* **48**: 83-89.

Ogawa J.M. and English H. 1991- Diseases of temperate zone tree fruit and nut crops. University of California, Division of Agriculture and Natural Resources.

Oldach K.H., Becker D., Lorz H. 2001- Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Mol. Plant Microbe Interact.* **14**: 832-838.

Palmiter D.H. 1934- Variability in monoconidial cultures of *Venturia inaequalis*. *Phytopathology* **24**: 22-47.

Parisi L., Lespinasse Y., Guillaumes J., Krüger J. 1993- A new race of *Venturia inaequalis* virulent to apples with resistance due to the Vf gene. *Phytopathology* **83**: 533-537.

Parisi L. and Lespinasse Y. 1999- Pathogenicity of a strain of *Venturia inaequalis* race 6 on apple clones (*Malus* spp.). In: *Proceedings of the Eucarpia Symp. on Fruit Breeding and Genetics*. Tobutt K.R. and Alston F.H. (eds). *Acta Hort.* **484**: 443-447.

Patino B., Posada M.L., Gonzalez-Jaén M.T., Martinez-Del-Pozo A., Vazquez C. 1997- Control of polygalacturonase synthesis in *Fusarium oxysporum* f.sp. *radicis lycopersici*. *Can. J. Microbiol.* **43**: 1084-1090.

Patocchi A., Gianfranceschi L., Gessler C. 1999a- Towards the map-based cloning of Vf: Fine and physical mapping of the Vf Region. *Theor. Appl. Genet.* **99**: 1012-1017.

Patocchi A., Vinatzer B.A., Gianfranceschi L., Tartarini S., Zhang H.B., Sansavini S., Gessler C. 1999b- Construction of a 550 kb BAC contig spanning the genomic region containing the apple scab resistance gene Vf. *Mol. Gen. Genet.* **262**: 884-891.

Pear J.R., Kawagoe Y., Schreckengost W.E., Delmer D.P., Stalker D.M. 1996- Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. *Proc. Natl. Acad. Sci. USA* **93**: 12637-12642.

Pellizzari E.D., Kuc J., Williams E.B. 1970- The hypersensitive reaction in *Malus* species: changes in the leakage of electrolytes from apple leaves after inoculation with *Venturia inaequalis*. *Phytopathology* **60**: 373-376.

Philion V., Carisse O., Paulitz T. 1997- *In vitro* evaluation of fungal isolates for their ability to influence leaf rheology, production of pseudothecia, and ascospores of *Venturia inaequalis*. *Eur. J. Plant Pathol.* **103**: 441-452.

Pinon L.K. 2001- Fruits à pépins. Tendances à la baisse. *L´arboriculture fruitière* **552**: 10.

Poulsen C. and Verpoorte R. 1991- Roles of chorismate mutase, isochorismate synthase and anthranilate synthase in plants. *Phytochemistry* **30**: 377-386.

Powell A.L., Stotz H.U., Labavitch J.M., Bennet A.B. 1994- Glycoprotein inhibitors of fungal polygalacturonases. In: *Advances in molecular genetics of plant-microbe interactions* vol 3. Daniel M.J., Downie J.A., Osbourn A.E. (eds). Kluwer Acad. Pub., Dordrecht, 399-402.

Powell A.L., van Kan J., ten Have A., Visser J., Greve L.C., Bennett A.B., Labavitch J.M. 2000- Transgenic expression of pear PGIP in tomato limits fungal colonization. *Mol. Plant Microbe Interact.* **13**: 942-950.

Predieri S., Malavasi F.F., Passey A.J., Ridout M.S., James D.J. 1989- Regeneration from *in vitro* leaves of ´Conference´and other pear cultivars (*Pyrus communis* L.). *J. Hort. Sci.* **64**: 553-559.

Preece T.F. 1959- A staining method for the study of apple scab infections. *Plant Pathol.* **8**: 127-129.

Pühringer H., Moll D., Hoffmann-Sommergruber K., Watillon B., Katinger H. and Laimer da Câmara Machado M. 2000- The promoter of an apple *Ypr10* gene, encoding the major apple allergen Mal d 1, is stress- and pathogen-inducible. *Plant Sci.* **152**: 35-50.

Puite K. and Schaart J. 1999- *Agrobacterium*-mediated transformation of the apple cultivars ´Gala´, ´Golden Delicious´and ´Elstar´, and the strawberry cultivars ´Gariguette´, ´Polka´ and ´Elsanta´. In: *Proceedings of the Eucarpia Symp. on Fruit Breeding and Genetics*. Tobutt K.R.and Alston F.H. (eds). *Acta Hort.* **484**: 457-553.

Raa J. 1968- Natural resistance of apple plants to *Venturia inaequalis*. Doctoral Thesis, University of Utrecht, Utrecht.

Rasmussen U., Giese H., Mikkelsen J.D. 1992- Induction and purification of chitinase in *Brassica napus* L. ssp. *oleifera* infected with *Phoma lingam*. *Planta* **187**: 328-334.

Raventós D., Anders B.J., Rask M.-B-, Casacuberta J.M., Mundy J., San Segundo B. 1995- A 20bp *cis*-acting element is both necessary and sufficient to mediate elicitor response of a maize PRms gene. *Plant J.* **7**: 147-155.

Register J.C. III, Peterson D.J., Bell P.J., Bullock W.P., Evans I.J., Frame B., Greenland A.J., Higgs N.S., Jepson I., Jiao S., Lewnau J.L., Sillick J.M., Wilson H.M. 1994- Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. *Plant Mol. Biol*. **25**: 951-961.

Ren Y.Y. and West C.A. 1992- Elicitation of diterpene biosynthesis in rice (*Oryza sativa* L.) by chitin. *Plant Physiol*. **99**: 1169-1178.

Richmond T.A. and Somerville C.R. 2000- The cellulose synthase superfamily. *Plant Physiol.* **124**: 495-498.

Robert N., Roche K., Lebeau Y., Breda C., Boulay M., Esnault R., Buffard D. 2002- Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. *Plant Sci.* **162**: 389-400.

Roberts A.L. and Crute I.R. 1994- Apple scab resistance from *Malus floribunda* 821 (Vf) is rendered ineffective by isolates of *Venturia inaequalis* from *Malus floribunda*. *Norwegian J. Agric. Sci.* **Supplement 17**: 403-406.

Rohini V.K. and Sankara R.K. 2001- Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. *Plant Sci.* **160**: 889-898.

Rosa M.T. 1993- Previsão do desenvolvimento dos pedrados das pomóideas. Aplicação de sistemas de simulação. *Revista de Ciências Agrárias* **XVI**: 107-114.

Rosenberger D.A. 2000- An update on scab-resistant cultivars and advanced selections for consideration in new plantings. http://orchard.uvm.edu/sap/srcupdate.html.

Rousselle G.L., Williams E.B., Hough L.F. 1974- Modification of the level of resistance to apple scab from the Vf gene. In: Proc. 19th Int. Hortic. Cong. Vol 3. Int. Soc. Hortic. Sci. Warsaw Antoszewski R., Harrison L., Nowosielski J. (eds), 19-26.

Rudloff C.F. and Schmidt M. 1934- *Venturia inaequalis* (Cooke) Aderh.II. Zur Zuchtung schorfwiderstandsfahiger Apfelsorten. *Zuchter* **6**: 288-294.

Rudloff C.F. and Schmidt M. 1935- Der Erreger des Apfelschorfes *Venturia inaequalis* (Cooke) Aderh. Grundlagen and Moglichkeiten für seine Bekämpfung auf Zuchterischen Wage II. *Zuchter* **7**: 65-74.

Ryals J., Neuenschwander U., Willits M., Molina A., Steiner H.-Y., Hunt M. 1996- Systemic acquired resistance. *Plant Cell* **8**: 1809-1819.

Samac D.A. and Shah D.M. 1994- Effect of chitinase antisense RNA expression on disease susceptibility of *Arabidopsis* plants. *Plant Mol. Biol.* **25**: 587-596.

Sambrook J., Fritsch E.F., Maniatis T. 1989- Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanger F., Nicklen S., Coulson A.R. 1977- DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Sangwan R.S., Bourgeois Y., Brown S., Vasseur G., Sangwan-Norrel B. 1992- Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta* **188**: 439-456.

Sansavini S. 1993- Il miglioramento genetico del melo per la resistenza alle avversità biotiche. *Rivista di Frutticultura* **5**: 61-73.

Sansavini S., Tartarini S., Barbieri M. 2000- Scab (*Venturia inaequalis*) resistance in apple: the Vf-gene in the breeding strategy and results at the University of Bologna, Dipartimento di Colture Arboree (DCA). In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Schaart J.G., Puite K.J., Kolova L., Pogrebnyak N. 1995- Some methodological aspects of apple transformation by *Agrobacterium*. *Euphytica* **85**: 131-134.

Schlumbaum A., Mauch F., Vögeli U., Boller T. 1986- Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**: 365-367.

Seehaus K. and Tenhaken R. 1998- Cloning of genes by mRNA differential display induced during the hypersensitive reaction of soybean after inoculation with *Pseudomonas syringae* pv. *glycinea*. *Plant Mol. Biol.* **38**: 1225-1234.

Seguin T. 2000- Topaz a séduit les bio. *L´arboriculture fruitière* **543**: 48.

Sela-Buurlage M.B., Ponstein A.S., Bres-Vloemans S.A., Melchers L.S., van den Elzen P.J.M., Cornelissen B.J.C. 1993- Only specific tobacco (*Nicotiana tabacum*) chitinases and ß-1,3-glucanases exhibit antifungal activity. *Plant Physiol*. **101**: 857-863.

Sharma K. and Anjaiah V. 2000- An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens*-mediated genetic transformation. *Plant Sci*. **159**: 7-19.

Shay J.R. and Hough L.F. 1952- Evaluation of apple scab resistance in selections of *Malus*. *Am. J. Bot.* **39**: 288-297.

Shay J.R. and Williams E.B. 1956- Identification of three physiologic races of *Venturia inaequalis*. *Phytopathology* **46**: 190-193.

Shay J.R., Dayton D.F., Hough L.F. 1953- Apple scab resistance from a number of *Malus* species. *Proc. Am. Soc. Hort. Sci*. **62**: 348-356.

Sierotzki H., Eggenschwiler M., Boillat O., McDermott J.M., Gessler C. 1994a- Detection of variation in virulence toward susceptible apple cultivars in natural populations of *Venturia inaequalis*. *Phytopathology* **84**: 1005-1009.

Sierotzki H., Eggenschwiler M., McDermott J., Gessler C. 1994b- Specific virulence of isolates of *Venturia inaequalis* on "susceptible" apple cultivars. *Norwegian J. Agric. Sci.* **Supplement 17**:83-93.

Silva J.M. 1993- A pêra e a maçã em Portugal. *Revista de Ciências Agrárias* **XVI**: 17-28.

Simantiras M. and Leistner E. 1989- Formation of O-succinylbenzoic acid from isochorismic acid in protein extracts from anthraquinone-producing plant cell suspension cultures. *Phytochemistry* **28**: 1381-1382.

Stanford A., Bevan M., Northcote D. 1989- Differential expression within a family of novel wound-induced genes in potato. *Mol. Gen. Genet.* **215**: 200-208.

Stanford A.C., Northcote D.H., Bevan M.W. 1990- Spatial and temporal patterns of transcription of a wound-induced gene in potato. *EMBO J.* **9**: 593-603.

Stotz H.U., Powell A.L., Damon S.E., Greve L.C., Bennett A.B., Labavitch J.M. 1993- Molecular characterization of a polygalacturonase inhibitor from *Pyrus communis* L. cv Bartlett. *Plant Physiol.* **102**: 133-138.

Stotz H.U., Contos J.J., Powell A.L., Bennett A.B., Labavitch J.M. 1994- Structure and expression of an inhibitor of fungal polygalacturonases from tomato. *Plant Mol. Biol.* **25**: 607-617.

Sturtevant J. 2000- Applications of differential-display reverse transcription-PCR to molecular pathogenesis and medical mycology. *Clinical Microbiol. Rev.* **13**: 408-427.

Subramaniam K., Ye Z., Buechley G., Shaner G., Solomos T., Ueng P.P. 1999- Isolation of a Zeta class wheat glutathione S-transferase gene. *Biochimica et Biophysica Acta* **1447**: 348-356.

Suslow T.V., Matsubara D., Jones J., Lee R., Dunsmuir P. 1988- Effect of expression of bacterial chitinases on tobacco susceptibility to leaf brown spot. *Phytopathology* **78**: 1556.

Tabaeizadeh Z., Agharbaoui Z., Harrak H., Poysa V. 1999- Transgenic tomato plants expressing a *Lycopersicon chilense* chitinase gene demonstrate improved resistance to *Verticillium dahliae* race 2. *Plant Cell Rep.* **19**: 197-202.

Tabei Y., Kitade S., Nishizawa Y., Kikuchi N., Kayano T., Hibi T., Akutsu K. 1998- Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). *Plant Cell Rep*. **17**: 159-164.

TAIR Database http://www.arabidopsis.org/Blast/index.html.

Tao R., Dandekar A.M., Uratsu S.L., Vail P.V., Tebbets J.S. 1997- Engineering genetic resistance against insects in japanese persimmon using the cryIA(c) gene of *Bacillus thurigiensis*. *J. Am. Soc. Hort. Sci.* **122**: 764-771.

Tartarini S. 1996- RAPD markers linked to the Vf gene for scab resistance in apple. *Theor. Appl. Genet.* **92**: 803-810.

Tartarini S., Sansavini S., Vinatzer B.A., Barbieri M., Patocchi A., Gessler C., Gianfranceschi L. 2000- Advances in marker assisted selection for scab resistance in apple and cloning of the Vf gene. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Tawe W.N., Eschbach M.L., Walter R.D., Henkle-Duhrsen K. 1998- Identification of stress-responsive genes in *Caenorhabditis elegans* using RT-PCR differential display. *Nucleic Acids Res.* **26**: 1621-1627.

Terakawa T., Takaya N., Horiuchi H., Koike M., Takagi M. 1997- A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal actvity to transgenic tobacco. *Plant Cell Rep.* **16**: 439-443.

Thakur V.S. and Sharma R.D. 1999- Effect of urea on microbial degradation of apple leaf litter and its relationship to the inhibition of pseudothecial development of *Venturia inaequalis*. *Indian J. Agri. Sci.* **69**: 147-151.

Theiler-Hedtrich C. and Theiler-Hedtrich R. 1990- Influence of TDZ and BA on adventitious shoot regeneration from apple leaves. *Acta Hort.* **280**: 195-199.

Töpfer R., Matzeit V., Gronenborn B., Schell J., Steinbiss H.H. 1987- A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res.* **15**: 5890.

Toubart P., Desiderio A., Salvi G., Cervone F., Daroda L., De Lorenzo G. 1992- Cloning and characterization of the gene encoding the endopolygalactorunase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L.. *Plant J.* **2**: 367-373.

Truesdell G.M. and Dickman M.B. 1997- Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. *Plant Mol. Biol.* **33**: 737-743.

Turner M.L., MacHardy W.E., Gadoury D.M. 1986- Germination and appressorium formation by *Venturia inaequalis* during infection of apple seedling leaves. *Plant Dis*. **70**: 658-661.

Uknes S., Winter A.M., Delaney T., Vernooij B., Morse A., Friedrich L., Nye G., Potter S., Ward E., Ryals J. 1993- Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.* **6**: 692-698.

Valsangiacomo C. and Gessler C. 1988- Role of cuticular membrane in ontogenic and Vf-resistance of apple leaves against *Venturia inaequalis*. *Phytopathology* **78**: 1066-1069.

Valsangiacomo C., Wagner K., Stadler B., Ruckstuhl M., Manini-Gessler P., Michel M., Gessler C. 1989- Aspects of host resistance and pathogenesis in the interaction between *Venturia inaequalis* and apple leaves. In: *Integrated control of pome fruit diseases*, Vol II, IOBC/WPRS Bull. XII/6, 191-204.

Valsangiacomo C., Ruckstuhl M., Gessler C. 1992- In vitro degradation of cell walls of apple leaves by pectinolytic enzymes of the scab fungus, *Venturia inaequalis*, and by commercial pectinolytic and cellulolytic enzyme preparations. *J. Phytopathol.* **135**: 20-27.

van den Bulcke M., Bauw G., Castresana C., Van Montagu M., Vandekerckhove J. 1989- Characterization of vacuolar and extracellular ß-1,3-glucanases of tobacco. Evidence for a stricly compartmentalized plant defence system. *Proc. Natl. Acad. Sci. USA* **86**: 2673-2677.

van der Fits L., Deakin E.A., Hoge J.H.C., Memelink J. 2000- The ternary transformation system: constitutive *vir*G on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* **43**: 495-502.

van Loon L.C., Pierpoint W.S., Boller T., Conejero V. 1994- Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Rep.* **12**: 245-264.

van Tegelen L.J., Moreno P.R., Croes A.F., Verpoorte R., Wullems G.J. 1999- Purification and cDNA cloning of isochorismate synthase from elicited cell cultures of *Catharanthus roseus*. *Plant Physiol.* **119**: 705-712.

Vancanneyt G., Schmidt R., O´Connor-Sanchez A., Willmitzer L., Rocha-Sosa M. 1990- Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol. Gen. Genet.* **220**: 245-250.

Vanderplank J.E. 1963- Plant diseases: epidemics and control. Acad. Press, New York

Venema J., Vos H.R., Faber A.W., van Venrooij W.J., Raue H.A. 2000- Yeast Rrp9p is an evolutionarily conserved U3 snoRNP protein essential for early pre-rRNA processing cleavages and requires box C for its association. *RNA* **6**: 1660-1671.

Verburg J.G. and Huynh Q.K. 1991- Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. *Plant Physiol*. **95**: 450-455.

Vercauteren I., Van Der Schueren E., Van Montagu M., Gheysen G. 2001- *Arabidopsis thaliana* genes expressed in the early compatible interaction with root-knot nematodes. *Mol. Plant Microbe Interact.* **14**: 288-299.

Vinatzer B.A., Zhang H.-B., Sansavini S. 1998- Construction and characterization of a bacterial artificial chromosome library of apple. *Theor. Appl. Genet.* **97**: 1183-1190.

Vinatzer B.A., Patocchi A., Gianfranceschi L., Tartarini S., Zhang H.B., Gessler C., Sansavini S. 2001- Apple contains receptor-like genes homologous to the Cladosporium fulvum resistance gene family of tomato with a cluster of genes cosegregating with Vf apple scab resistance. *Mol. Plant Microbe Interact.* **14**: 508-515.

Vögeli-Lange R., Bürckert N., Boller T., Wiemken A. 1996- Rapid selection and classification of positive clones generated by mRNA differential display. *Nucleic Acids Res.* **24**: 1385-1386.

Vogelsang R. and Barz W. 1993- Purification, characterization and differential hormonal regulation of a ß-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). *Planta* **189**: 60-69.

Wagner K., Hitz-Germann L., Seng J.M., Gessler C. 1988- Cellulolytic ability of the scab fungus, *Venturia inaequalis*. *J. Phytopathol.* **123**: 217-221.

Waniska R.D., Venkatesha R.T., Chandrashekar A., Krishnaveni S., Bejosano F.P., Jeoung J., Jayaraj J., Muthukrishnan S., Liang G.H. 2001- Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. *J. Agric. Food. Chem.* 49: 4732-4742.

Ward E.R., Uknes S., Williams S.C., Dincher S.S., Metraux J.P., Ryals J. 1991- Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* **3**: 1085-1094.

Watillon B., Kettmann R., Boxus P., Burny A. 1992- Cloning and characterization of an apple (*Malus domestica* [L.] Borkh) calmodulin gene. *Plant Sci.* **82**: 201-212.

Williams E.B. and Brown A.G. 1968- A new physiologic race of *Venturia inaequalis*, incitant of apple scab. *Plant Dis. Rep*. **52**: 799-801.

Williams E.B. and Kuc J. 1969- Resistance in *Malus* to *Venturia inaequalis*. *Ann. Rev. Phytopathol.* **7**: 223-246.

Wilson E.E. 1928- Studies of the ascigerous stage of *Venturia inaequalis* (Cke.) Wint. in relation to certain factors of the environment. *Phytopathology* **18**: 375-418.

Wiltshire S.P. 1915- Infection and immunity studies on the apple and pear scab fungi (*Venturia inaequalis* and *V. pirina*). *Ann. Appl. Biol.* **1**: 335-350.

Wong K.-W., Harman G.E., Norelli J.L., Gustafson H.L., Aldwinckle H.S. 1999- Chitinase-transgenic lines of ´Royal Gala´ apple showing enhanced resistance to apple scab. In: *Proceedings of the Eucarpia Symp. on Fruit Breeding and Genetics.* Tobutt K.R.and Alston F.H. (eds). *Acta Hort.* **484**: 595-599.

Wu H., Echt C.S., Popp M.P., Davis J.M. 1997- Molecular cloning, structure and expression of an elicitor-inducible chitinase gene from pine trees. *Plant Mol. Biol.* **33**: 979-87.

Xu X.M., Butt D.J., Van Santen G. 1995- A dynamic model simulating infection of apple leaves by *Venturia inaequalis*. *Plant Pathol.* **44**: 865-876.

Xu M., Song J., Jiang J., Korban S.S. 2000- Constructing a bacterial artificial chromosome (BAC) library of the apple cultivar Goldrush. In: *ISHS symposium on apple breeding for scab resistance*. 14-16 September 2000, Trento. *Acta Hort*. (in press).

Xu M., Huaracha E., Korban S.S. 2001- Development of sequence-characterized amplified regions (SCARs) from amplified fragment length polymorphism (AFLP) markers tightly linked to the Vf gene in apple. *Genome* **44**: 63-70.

Yamamoto T., Iketani H., Ieki H., Nishizawa Y., Notsuka K., Hibi T., Hayashi T., Matsuta N. 2000- Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Rep.* **19**: 639-646.

Yang H. and Krüger J. 1994- Identification of a RAPD marker linked to the Vf gene for scab resistance in apples. *Euphytica* **77**: 83-87.

Yang H. and Korban S.S. 1996- Screening apples for OPD20/600 using sequence-specific primers. *Theor. Appl. Genet.* **92**: 263-266.

Yang H.Y., Korban S.S., Krüger J., Schmidt H. 1997a- A randomly amplified polymorphic DNA (RAPD) marker tightly linked to the scab-resistance gene V-f in apple. *J. Amer. Soc. Hort. Sci.* **122**: 47-52.

Yang H.Y., Korban S.S., Krüger J., Schmidt H. 1997b- The use of a modified bulk segregant analysis to identify a molecular marker linked to a scab resistance gene in apple. *Euphytica* **94**: 175-182.

Yao C., Conway W.S., Ren R., Smith D., Ross G.S., Sams C.E. 1999- Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. *Plant Mol. Biol.* **39**: 1231-1241.

Yeboah N.A., Arahira M., Nong V.H., Zhang D., Kadokura K., Watanabe A., Fukazawa C. 1998- A class III acidic endochitinase is specifically expressed in the developing seeds of soybean (*Glycine max* [L.] Merr.). *Plant Mol. Biol.* **36**: 407-415.

Yepes L.M. and Aldwinckle H.S. 1993a- Pathogenesis of *Venturia inaequalis* on shoot-tip cultures and on greenhouse-grown apple cultivars. *Phytopathology* **83**: 1155-1162.

Yepes L.M., Aldwinckle H.S. 1993b- Selection of resistance to *Venturia inaequalis* using detached leaves from in vitro-grown apple shoots. *Plant Sci.* **93**: 211-216.

Yi S.Y. and Hwang B.K. 1998- Molecular cloning and characterization of a new basic peroxidase cDNA from soybean hypocotyls infected with *Phytophthora sojae* f.sp. *glycines*. *Mol. Cells* **8**: 556-564.

Young C.S. and Andrews J.H. 1990- Inhibition of pseudothecial development of *Venturia inaequalis* by the basidiomycete *Athelia bombacina* in apple leaf litter. *Phytopathology* **80**: 536-542.

Zhu Q., Doerner P.W., Lamb C.J. 1993- Stress induction and developmental regulation of a rice chitinase promoter in transgenic tobacco. *Plant J.* **3**: 203-212.

Zhu Q., Maher E.A., Masoud S., Dixon R.A., Lamb C.J. 1994- Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Bio/technology* **12**: 807-812.

Zhu L., Holefors A., Ahlman A., Xue Z., Welander M. 2001- Transformation of the apple rootstock M.9/29 with the rolB gene and its influence on rooting and growth. *Plant Sci* **160**: 433-439.

Appendix 1: Composition of culture media used

• Plant material:

Composition of MS (Murashige and Skoog, 1962) (1000ml): 1.65g NH_4NO_3, 1.90g KNO_3, 0.44g $CaCl_2.2H_2O$, 0.37g $MgSO_4.7H_2O$, 0.17g KH_2PO_4 (macroelements); 37.3mg $Na_2EDTA.2H_2O$, 27.85mg $FeSO_4.7H_2O$, 6.2mg H_3BO_3, 22.3mg $MnSO_4.4H_2O$, 8.6mg $ZnSO_4.4H_2O$, 0.83mg KI, 0.25mg $Na_2MoO_4.2H_2O$, 0.025mg $CuSO_4.5H_2O$, 0.025mg $CoCl_2.6H_2O$ (microelements); 0.5mg nicotinic acid, 0.5mg pyridoxine-HCl, 0.1mg thiamine-HCl, 2.0mg glycine, 100mg myo-inositol (vitamins)

• Culture media for bacteria and bacteriophages (Sambrook *et al.*, 1989):

LB liquid (1000ml): 10g tryptone (Difco), 5g yeast extract, 10g NaCl; pH 7.0

LB agar: LB liquid + 1.5% agar

SOC (1000ml): 20g tryptone (Difco), 5g yeast extract, 0.58g NaCl, 0.19g KCl, 2.03g $MgCl_2.6H_2O$, 3.96g $C_6H_{12}O_6.H_2O$, 2.46g $MgSO_4.7H_2O$; pH 7.0

NZY liquid (1000ml): NZY Broth (GibcoBRL) 21g

NZY solid: NZY liquid + 1.5% agar

TA7: NZY liquid + 0.7% agarose

• Salts/vitamins of liquid culture media for *Botrytis cinerea*:

Czapek-Dox (Moore-Landecker, 1996) (1000 ml): 2g $NaNO_3$, 1g KH_2PO_4, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 0.01g $FeSO_4$

Visser (Kusters-van Someren *et al.*, 1992) (1000 ml): 5g KNO_3, 2.5g KH_2PO_4, 0.25g $MgSO_4$, 0.4g $FeCl_3$

MSf (1000 ml): salts and vitamins identical to MS used for plant material.

MAMm* (1000ml): 11.0g $C_6H_{12}O_6.H_2O$, 2.0g tryptone (Difco), 2.8g $(NH_4)_2SO_4$, 4.0g KH_2PO_4, 12.53g $Na_2HPO_4.2H_2O$, 10ml microelements solution; pH5.0 with HCl
Microelements solution (100ml): 182mg $FeSO_4$, 100mg $ZnSO_4.7H_2O$, 50mg $CuSO_4.5H_20$

* adapted from Mandels-Andreotti-Medium (Messner *et al.*, 1996)

Appendix 2: Composition of solutions and buffers used

If not specified elsewhere, solutions and buffers were prepared according to Sambrook *et al*. (1989).

• Common buffers and solutions

TE pH8.0: 10mM Tris-HCl pH8.0, 1mM EDTA pH8.0

Denaturation Buffer: 1.5M NaCl, 0.5M NaOH

Neutralisation Buffer: 1M Tris pH7.4, 1.5M NaCl

20x SSC (1000ml): 175.3g NaCl, 88.2g Na-citrate; pH7.0 with 10N NaOH

50x Denhardt's Reagent (500ml): 5g Ficoll (Type 400, Pharmacia), 5g PVP, 5g BSA

HBI: 10x DH, 3x SSC, 0.1%SDS, 100μg/ml salmon sperm

Church buffer: 5x SSC, 7%SDS, 50%deionised formamide, 2%Blocking Reagent, 50mM sodium phosphate pH7.0, 0.1% sodium-lauryl-sarcosine

PAA Elution buffer: 0.5M NH_4CH_3COO, 10mM $Mg(CH_3COO)_2$, 1mM EDTA pH8.0, 0.1%SDS

SM buffer (1000ml): 5.8g NaCl, 2g $MgSO_4.7H_20$, 50ml 1M Tris-HCl pH7.5, 5ml 2% gelatin

• Nucleic acid extractions

2x CTAB buffer for plant DNA extraction (Doyle and Doyle, 1990): 2%CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, 1%PVP; pH 8.0

Extraction buffer for plant RNA extraction (Chang *et al.*, 1993): 2%CTAB, 2%PVP, 100mM Tris-HCl pH8.0, 25mM EDTA, 2.0M NaCl, 0.05% spermidine

SSTE (Chang *et al.*, 1993): 1.0M NaCl, 0.5%SDS, 10mM Tris-HCl pH8.0, 1mM EDTA pH8.0

CI: 24 chloroform:1 isoamyl alcohol

• DNA gels

50x TAE (1000ml): 242g Tris, 57.1ml glacial acid acetic, 100ml EDTA 0.5M pH8.0; pH7.8
1x TAE (electrophoresis buffer): 0.04M Tris-acetate, 0.001M EDTA

Loading buffer: 0.09%bromophenol blue, 0.09%xylene cyanol FF, 60%glycerol, 60mM EDTA

PAA gel solution (100ml): 45g urea, 10ml 5x TBE, 11.25ml Rotiphorese^{R}Gel 40 (Roth); 625µl 10%APS and 62.5µl TEMED added immediately before pouring the gel

5x TBE: 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA pH8.0
0.5x TBE (electrophoresis buffer): 0.045M Tris-borate, 0.001M EDTA

PAA Loading buffer: 98%deionised formamide, 0.098%bromophenol blue, 0.098%xylene cyanol FF, 10mM EDTA

- **Silver staining** (Bassam *et al.*, 1991)

silver nitrate solution (500ml): 0.5g $AgNO_3$, 750µl formaldehyde 37%

developer solution (500ml): 15g Na_2CO_3, 750µl formaldehyde 37%, 50µl stock solution $Na_2S_2O_3.5H_2O$ (20mg/ml)

- **RNA gels** (Fourney *et al.*, 1988)

10x MOPS: 0.2M MOPS, 50mM $NaCH_3COO$, 10mM EDTA; pH7.0

RNA gel solution (130ml): 1.82g agarose, 13ml 10x MOPS, 113ml $A.dest._{DEPC\ treated}$; 6.63ml formaldehyde 37% added after melting the agarose and let cool until ~50°C

Electrophoresis Sample Buffer: 0.75ml deionised formamide, 0.15ml 10x MOPS, 0.24ml formaldehyde, 0.1ml deionised DEPC-treated A.dest., 0.1ml glycerol, 0.08ml 10%(w/v) bromophenol blue

- **Hybridisation of membranes and DIG Detection** (Roche)

DNA dilution buffer: 10mM Tris-HCl pH8.0, 50µg/ml DNA herring sperm

RNA dilution buffer: 50%DEPC-treated A.dest., 30%20x SSC, 20%formaldehyde

10x Maleic Acid Buffer: 1M Maleic Acid, 1.5M NaCl; pH7.5

Blocking Reagent: 10%Blocking Reagent in 1x Maleic Acid Buffer

Wash buffer: 1x Maleic Acid Buffer, 0.3%Tween 20

Detection Buffer: 100mM Tris, 100mM NaCl; pH9.5

• GUS assays

GUS buffer (Naleway, 1992): 100mM Na_3PO_4, 10mM EDTA, 0.5mM $K_3[Fe(CN)_6]$, 0.5mM $K_4[Fe(CN)_6].3H_2O$, 0.1% Triton-X-100; pH7.0

X-Gluc: 10mg/ml in Dimethylformamide

GUS extraction buffer (Gallagher, 1992): 50mM Na_2HPO_4, 10mM Na_2EDTA, 0.1%sodium-lauryl-sarcosine, 0.1% Triton- X-100; pH7.0

Protein dilution buffer (1000ml): 6.8g KH_2PO_4, 8.76g NaCl; pH7.2

• 2-cyanoacetamide assay

citrate buffer (Titrisol^R): 0.056M $C_6H_8O_7$, 0.11M NaOH, 0.044M HCl; pH4.0
borate buffer (Titrisol^R): 0.05M H_3BO_3, 0.05M KCl, 0.022M NaOH; pH9.0

• Periodic acid-basic fuchsin staining (Preece, 1959)

periodic acid: 1% solution in A. dest.

decolorised basic fuchsin: 1g basic fuchsin dissolved in 200ml A.dest. by boiling; after cooling, 20ml N/1 HCl are added, followed by 1g $K_2S_2O_5$ crystals

sulphurous acid solution: 10ml 10% $K_2S_2O_5$ are added to 200ml A. dest., followed by 10ml N/1 HCl

Appendix 3: DNA sequences from isolated clones

DNA sequence from *pgip* clone **N17**

```
   1 ATCGATATTA TCCCGATATT TCCATCGATA TTTCCGTGTT TTCGAACTAC CGATATTTCC
  61 GATATTACCG ATATTTTCTT CCTTACTCCA GAGTCATTAC AAACAAAACC GAACCAACCA
 121 AATCGAACTT GAGTCAGTTT TGGTATGGTT GAATTGATAT AGCAATTTTC TTTTACAAAA
 181 TCAAATGGAA TTGAACCTAT CATTTATGCT TGATTTTTAG TTTCAAACAA AAACCGAACC
 241 CAGAGACCGA ATAGGACAAA CTTTTGTATT CACACTGATA AAACCTGAAT CAAATCGATT
 301 CCTTTTTTAA TAGTCAATTT AACAAAAAGC TTTGGTATTA TTAATTTTAA TAAAAAAAAT
 361 CATATTTTTA CATTAAAAAG TTAATTTTTA CTATTCATTT TATCATTTAT TTTGTCTTTA
 421 AAACTCAAAA TTTTCAAACA CTTTTTACTA GTTTTCCTTT TTTTAATATC TATACGTGCA
 481 ATTCTCTCTA CGTTAGAGGA TATTTTCATG TTATTTTGCC ATAATTTTTT ATAAAATATT
 541 AAATTAAAGA ATTTACCAAA AAAAAAAACC CTCTTTTTAT ATGTGGAGGC CAGATGACCT
 601 ACAAAAAAAA TAACTAAATA AGTAAAAGAT TTTTAGCTAA AATGGTCATG AAATTGGCAT
 661 AACTCCTTAC TTTAGTTTCT AAGATTTGAA ATCGATTAAG TAATTCTTGA GTTTGTCCAC
 721 CATCAATCAT TTTGATCATT CCATAAAAAT TTCTGTTAAA TAAGAATAAA AGGCCAAACA
 781 TACCTTCAAT TTAATAAACA ATGAGTTAAA ATAATTTGAC AAAAAATAAA GATATTTTTG
 841 TCATTTTATC TTTATGTAAT AGAGCAGCAG CAGGGCCTCT AATTATCTAA AATCTCCGTA
 901 TTTTAGTATG ACTAATGTTG TTGTATTAAA AAGAAGTCAC AAGGCAATTA ACAAGCAAAG
 961 CAAATAGTAA CATTTGACAG GCACAAGTTT ATTGGGTTTA ATTAAGTCTC AATTCCCCCC
1021 CATCAGTTGC CAATTGAGGA AGGGTGGAAG TCACTTGGTA AACACAATGT GGTGGAGGTT
1081 CATCTGCTTT TGCCAGATCA CAGCACGCAG ACCCCAAGGA TTCAGCAGAC CCCCTTCGTT
1141 TTTGACTTTT TTTTTTCTTT TTCAAGTTGT CATAACAGTA TAGAAGCGTG AAATTTTGGC
1201 AAATGTTGGA TTAGAGCATC TCTAACAACG CTAGCAAAAA CTTAGTATTA TATGATTTCA
1261 AATACCGTAA AATTATTTAT AACAAATTAC TCTTAACAAT TCTACTTCAC CGACAATCTA
1321 ATATGAATAA AGTTGGATCT TTTAAGAGTG GCCAACAACT GTCCGAAAAA AATAAAATAA
1381 CTTGCTACAT AGCTAGTTGG CTAGCACTTT GTTTATGAGG TTATGTTTGG ATGAGGAATT
1441 TGTGAATACC AATGAATTTT AATTAGTTAG AAATGCATTA TAAAACAAAG TAACGGATGA
1501 ATATGAAAAT GACTGCTTGA CATGAGTGAT ATGGAATTGT TTGTGTATTA TTAATAATCA
1561 TGTAGGGCAT TTCAATGCCT CTATCACATC TTTGTCAATT ATTTCTAATT ATTTTATCCT
1621 AAATCTGGTA GTACTTGCAA ATCGGCTCCT CCAAACATAG CTTATTTCAG TCAAAAGGAG
1681 CTGAGTAATC TTGAGAAACT CTTAGGTCCT CATCTCTATC AATCCAGCTT TGGCCGACCA
1741 TGGTTCCCAA ACCATGCTCC ATTTGTTCTC TTTAATTTTG TTTATTCCTA CGCTTATACT
1801 TCAGGTTTAT AACCGATACA TCTTGATTTT TGTATTTTTA ATTAAAAATC TGACCGTTCA
1861 AAGATTAGGA ATATCTTAAA CTTGAGGAGA TGTAAAAATT TCACAAAAGA CACTACATGT
1921 TTGAATGAAA TAAGGAGCAA AGCATGTTAC TTTTTGATGT CGATTAAATG CGCACAAGAT
1981 CTATGACCTG TCCACACTTC CTTCAACCAC AAATTCACTC TCTCAGGCTC CCAACCAAAA
2041 CCCAAAACA**A** **TG**GAGCTCAA GTTCTCCACC TTCCTCTCCC TAACCCTACT CTTCTCCTCC
2101 GTCCTAAAAC CCGCTCTCTC CGATCTCTGC AACCCCGACG ACAAAAAAGT CCTCCTACAA
2161 ATCAAGAAAG CCTTCGGCGA CCCCTACGTC TTGACCTCAT GGAAATCAGA CACTGACTGT
2221 TGTGATTGGT ACTGCGTCAC CTGTGACTCC ACCACAAACC GCATCAACTC CCTCACCATC
2281 TTCGCCGGCC AGGTATCCGG CCAAATCCCC GCCTTAGTCG GAGACTTGCC GTACCTTGAA
2341 ACCCTTGAAT TCCACAAGCA ACCCAATCTC ACTGGCCCAA TCCAACCCGC CATTGCCAAG
2401 CTCAAAGGAC TCAAGAGTCT CAGGCTCAGC TGGACCAACC TCTCAGGCTC TGTCCCTGAC
2461 TTCCTCAGCC AACTCAAGAA CCTCACATTC CTCGACCTCT CCTTCAACAA CCTCACCGGC
2521 GCCATCCCCA GCTCGCTTTC TCAGCTCCCA AACCTCAACG CTCTTCATCT AGACCGCAAT
2581 AAGCTCACAG GTCATATTCC GATATCGCTT GGGCAGTTCA TTGGCAACGT TCCAGACCTG
2641 TATCTCTCCC ACAACCAGCT CTCTGGCAAC ATTCCAACCT CATTCGCCCA GATGGACTTC
2701 GGCAGCATAG ACTTATCACG GAACAAGCTC GAAGGTGACG CATCCGTGAT ATTTGGGCTG
2761 AACAAGACAA CGCAGATTGT GGACCTGTCC AGGAACTTGC TAGAATTTAA TCTGTCAAAG
2821 GTGGAGTTTC CGACAAGCTT GACCTCGCTG GATATCAACC ACAATAAGAT CTACGGGAGT
2881 ATCCCAGTGG AGTTTACCCA ACTGAATTTC CAGTTCCTGA ACGTGAGCTA CAACAGGCTG
2941 TGTGGTCAGA TTCCGGTGGG TGGAAAGTTG CAGAGCTTCG ACGAGTATTC TTATTTCCAT
3001 AACCGATGCT TGTGCGGTGC TCCACTCCCA AGCTGCAAG**T** **AA**
```

ATG= translation start
TAA= translation stop

DNA sequence from *pgip* clone **Q8**

```
   1 TTGGAAAAAT GAGCCAAAAT TGAATATAAA ATTTTTGTTC GACAAAGTAT AGACTAAATA
  61 GTCAATGACG AACACCAAAA TCTAGGGATA GTGCAAACTC ACATATGTCA TGTTAAATTT
 121 CAGAAAGATG GGTTAGCGGC CCAATAATAG CAACATTGAC ATGGTTCCCA ACTTAACCAC
 181 CTATAAAGCC CAGTAAGTGT GAGATTTTAT CACAAAAAAC CTCGATGATA TTAGTAGTGG
 241 AACTGTTCAT TATATCTTGT ATTTTATTTA GTCAAGTTTC CGATGTGGGA CTTATATTCT
 301 TTAACATGCT CAAGTTCATT GAAAGGTCAA GAGCTCTATT TTTCTGATAC TTGACAAAAC
 361 TGAACAGCGG CTTTAGAATC ACATTCTACC TCAAGGTTAG TGTTCCTGAA CAATTGTTTT
 421 TGTTCTTCTT TAATTTGAAA GTCTGAAAAA GAATTTTAAA ATTGTTAGAA GGATGTTCAT
 481 TAAAGAAGAA GATGATGGAC TCATTGAATA AATGCATGAT TTGTTAGAAA AATTAAGTAA
 541 GAGAAAAGCA TACATGGATT AGACTAGTTG ACCAGGATTA AAAAATTATT AATTTTTACA
 601 ACATGATATC ATATAAAATG ACCCTATTGT TATGATGGTG TTGTACAGAT TGAGTAGAAT
 661 TGAACATGGT TATATATGAA TAAACTCGAC GAAGGAAACC CGCCGCATAA GGAGATCGAC
 721 CTATCCCGAT GAAGAAGCAT AATGTCCTCT TGAGTTTGTG AGACCAGTAG ATCCTTGGGC
 781 TGAAAGCAGA AGCAAAGGAT CCCCCGACAT GATAATGCTT ACTCATCATC ATAGTCAATA
 841 CTACAACCTA AACAAGCAAA CAAACACCAC TGACTTTCCA CACGTTTATT CCCGCCATCA
 901 ATTCAGCAGT TGAGGAAGAC AACTTGGCAA TTGAATTAGC TTTCAAAGTC ACCTCCATTT
 961 GCCAGATCTC AGCACACAAT AAATAAAGAA AAAGAGAGCC TTCATTTTTG ACTTGTTGAA
1021 GTTCTCAGCA CAACAAAGTT ATGCAGCTAG CAAACCTAAA GTTTTCTAGA TTTGGCTCCT
1081 CACAGTCGCA ACTATCAGTC TTGTTTAGAT ATAGGTCCTC AGCTTTGGCA CTCCACCTCC
1141 CCCTTTGGCC ATCCAAACCA TGTTCCCATG GATTCCACAT TCTTTACCCT TCTTCCTCTC
1201 CTCGTCTCAA TTTTACTTTT CCTAAATAAT CCAGATTATG TTATACAAAG AATTTATTTT
1261 CTTAACCGTA ATGATATATA AGTGAATTAA CAACATCATG TGATAATATT ACGCTCACGT
1321 CGAGAATTTA CTGCAGATTA TGAAAGCAGC CTTTAGCAAC TGAGAGAGAC TGGCTATTGA
1381 ATTGCCAAAT ATATATCATG TTTGAATGGA GTACATGGAG CAAAGCATGT TACTTTTGTC
1441 ATATATATTA ATTAAATACC TCCAATAAGA CCTTCACCAC CTGTGCAAAC TCCAAAATCT
1501 TTCGTCATTA TATCCATCAT CCAAAATCAC AAACACTTTG ACTTACATGC GCATGCAATA
1561 CCTATATGTA TATCCAACTC TTCGTTCAAT GGCAATCACA TTTCTTATCC AAAACCCACA
1621 AAATGGACGT CAAGTTCCCC ACCCTCCTCT GCTTGACCCT ACTCTTCTCC ACCATCCTAA
1681 ACCCAGCGCT CTCTGAGCTC TGCAACCCGG AAGACAAGAA AGTTCTCCTA CAAATCAAGA
1741 AAGCCTTCAA CGACCCCTAC GTCTTGACCT CATGGAAGCC AGAGACAGAC TGCTGTGACT
1801 GGTACTGTGT CACCTGTGAC TCCACCACAA ACCGCATCAA CTCCCTCACC ATCTTCGCCG
1861 GCCAAGTCTC CGGTCAAATT CCGACCCAAG TCGGTGACTT GCCATATCTT GAAACACTTG
1921 AGTTTCACAA GCAACCCAAT CTTACCGGAC CAATCCAACC CTCCATTGCC AAGCTTAAGC
1981 TCCTCAAGGA GCTGCGCCTC AGCTGGACTA ACATCTCAGG CTCTGTACCT GACTTCCTCA
2041 GCCAACTCAA GAACCTCACC TTTCTTGACC TCTCATTCAG TAACCTCACA GGCTCCATCC
2101 CCAGCTCGCT TTCTCAGCTT CCCAACCTCA ACGCTCTTCG TCTAGACCGT AACAAGCTCA
2161 CAGGTCTGTT CTTCCTTGAT ATATCTCTTT CTACCAAGTG CCCAGAAAGA AAGATACAAC
2221 TTTGTTCAAA ATTTTCATAT AATTTATCTT GATACCGATA GTTAATATTT TCTGTTAACT
2281 TCCATAGTTA ATACCCTTGC TTGCTTACAG GTCATATTCC GAAGTCATTT GGAGAATTCC
2341 ATGGCAGTGT TCCAGATCTC TATCTCTCTC ACAACCAGCT CTCAGGCACC ATACCAACCT
2401 CATTAGCCAA ACTGAACTTC AGCACCATAG ACTTCTCCCG GAACAAGCTC GAAGGCGATG
2461 CATCCATGAT CTTTGGATTG AACAAGACAA CCCAGATTGT GGATCTGTCG AGGAACTTGC
2521 TGGAATTTAA TCTGTCAAAT GTGGAGTTTT CCAAGAGCTT GACTTCGTTG GATCTTAACC
2581 ATAACAAGAT CACAGGCGGT ATTCCGGTGG GGCTGACCCA ACTGGATTTG CAGTTCCTGA
2641 ACGTGAGCTA CAACAGGTTG TGTGGTCAGA TTCCAGTGGG CGGGAAGTTG CAGAGCTTCG
2701 ACTCCTCGAC TTATTTCCAT AACCGCTGCT TGTGTGGTGC TCCACTCCCA AGCTGCAAAT
2761 AA
```

ATG= translation start
TAA= translation stop

DNA sequence from clone **M2**

```
  1 TACAACGAGG CCACCGCCGC CGCCCTTCTT ATTCCTCAAC CCAGACTCCC GCCGTTTTCG
 61 ACTCCCTCAA AAGTCTTTTT TTTTTCTCCA CTCTCATTTA TTTCCACTTA AATTATGTCA
121 CTTTTATTTT CTTTTTCGTA TTATTTTTGT TTTTAAGGCC GTTGGAGRAG TAAGATTCGC
181 CCGGAGATCG AAGTCGGCGA GGTGTCGGAA ACGGAGGACG GTGACTTGGT GATCGAGACC
241 GGCGTCACGT GGACGCTGAC TCCGGCGTTG ACTCTTCCTC AAGGACTTGA AAAGATCAGA
301 GAGGCCGTTG AGGAGTTGAA GCTCAATCCT CCTTCCACTT GTACCGGAAT TCATAGGTTT
361 CAAGTAATTA AAAATCTCCG GTTTCATACT GGtTTATCAC ACAATGTGCG TTGAATTGTG
421 GATTTTGATG GCGAATTTCA CAGATGGCTG TGCCGCTGAG TGCCAAAGCC TGAACTGGTT
481 TTGCATGCAG TTATCCTCTG TTTTTCATGG GCAAGGATGT CGAAAACCCG AGTTATAAAT
541 CGCTGTACGT GAATGAAACC CGAGGAGTTT TCGGAATTGG TGCCGCGGTT CACTTTGCAC
601 CTTCATCATC TTCTTCTTCG TTCAAAGGGT TAGTTCTTGT CTTTCTTTTT GCCCTCGTTG
661 TA
```

DNA sequence from clone **M3**

```
  1 TTTTGGCTCC AAGGTAATGG AAAATTCTTG GTGGGTGGCC TCAAACCATC CATAGCAGAT
 61 CTCAGCATGG TTTGTGAAAT CATGCAACTT GAGGTAAATA GATGCGACTC ATGCTTATAA
121 ATGTTGCTTT ATGGGTCAAT TTCATTTAAA TAATTGGAAT GGGTTTGTGG GTTCGGAAGG
181 ATTTTCTTCA ACTTGTCATG TATAATGAAC CAAATTCTAC TGATGAAGTA AATAGGTAAA
241 AAGTAGTACA CGATGAGAGA TGACGTATTA AAATTTATAG TTGATTGAAG CAATGGTTTC
301 TTGGTGTGTT AATTATTTAT GAAAAGTGGT AAAAAACGCG TCATCCTTCC ACCTTTGACG
361 TGTTCTTAGT CTGCCAAAAA AAAAAAA
```

DNA sequence from clone **M4**

```
  1 AACTCAAACG CCAGAAGAGA ATTAAGATGT ACAAATACAT TGGCATTTTC ATCGTCTTTC
 61 AAATCGTTGT CATCAGCATT TTTGGTTTGA CTGTGATGAA AGTTAAGTCC CCAAAAATCA
121 GGTTAGGCAA CATCTATGTC CTAAGTCTAA ACTCCGTCCC AGCAGCACCT TCATTCGACA
181 TGAGCTTCGT AACCCAAATC AGAGTCAGGA ACTTAAACTG GGGTACCTTT AAGTTTGATG
241 CCGGCATGGC CACGTTTATG TACCAAGGTG TGCCTGTTGG GCAAGTTGCT ATTCCGAATA
301 GCAAAGTCCG AATGCGATCC ACAAAAAAAA TTGATGTTGT GGTGAGCGTG AATTCTGCAG
361 CATTGCCGAG CAACTCCGCT CTTGGAAGTG AGTTGAGCAA TGGGCTGCTG ATGTTGAGCA
421 GTCAAGCCAA GTTGAGTGGA AAGGTTAAGT TGATGATGAT CATGAAGAAA AACAAGTCTG
481 CTGAAATGAG TTGCTCTATG GTGTTTAATT TGGCAGCAAA GTCTGTCCAA AATTTGGACT
541 GTAACTGATA GAATATGGNT ATACCTTGAG CCAATTTTAT GNT
```

DNA sequence from clone **M5**

```
  1 TCGATACAGG GATGGACAGG AGGTGGAATT ATAACTCTTG GAGGAAGACA AATTCCCACT
 61 TGGTTCAATC ATGTCAACGA GGGTACCCAA GTCTCTTTTG AAGTGCCTAA GGAAATTGGT
121 TCTAATGCAA AAGCATTGGC TGTGTGCCTG GCTTTTGTTC CTCATGATTA CGTCAGCTCG
181 TGTTATATTT ATGTTATTAA TCACACCAAG GGTACTAGTT TTTATGTCGA CATAGTAGAT
241 ATTTTTCCCC TTGAAGAGAT CATTTGGATG GGAAATCTGT CATTGTCGGA AACCGAGTTC
301 AATT
```

DNA sequence from clone **M6**

```
  1 TCGATACAGG GATGGACAGG AGGTGGAACC ATGTGTTTTG TAGGAAGACA AATTCCCACT
 61 TGGTTCAATC ATGTGAACGA GGGTACCCAA GTCTCTTTTG AAGTGCCTAA GGAAATTGGT
121 TGTAATGCAA AAGCATTGGT TGTGTGCCTG GCTTTTGTTC CTCGTGATTA CGTCAGCTCG
181 TGTTATATTT ATGTTATTAA TCACACCAAG GGTACTAGTT TTTATGTCGA CATAGTAGAT
241 ATTTTTCCCC TTGAAGAGAT CATTTGGATG GGAAATCTGT CATTGTCGGA AACCGAGTTC
301 AATTTGGAAG AAAGCGATTT GGTCCATGTT ATTGCACATT TTCCGGTGAA GAAAATAGGG
361 TACGTTTAGT ATGTGACAAA CTTATGACTT TCGAAGGTTC GTTATTAGAT TACCATTATA
421 TCCCTTACGA ACGGGCCTTA GAAGAAATTT CTACCGAAGT TGATGATTTT GATGAGGATT
481 ATGAAGATGA TGATTTTGAT GAGGATGATG ACAACACGAT GAGGATTTTG ATGGGGATGA
541 CGAGGAGGAT TTTGATAAGG AGGNGGGGGG N
```

DNA sequence from clone **M9**

```
  1 TGCCTGGCCC TGTCTTTGGT AGGCTGTTCT TTGCCATTTG GGTCATCGTC CATCTTTACC
 61 CGTTCCTAAA AGGTTTGGTG GGAAGACAAG AGAGACTGCC GACCATCATT GTGGTGTGGT
121 CAATTCTTCT GGCATCCATA TTCTCCCTGC TGTGGGTGCG AATCAATCCA TTTGTGAATA
181 AGGGTGGCAT TGTACTAGAA GTTTGCGGGC TGGATTGTAA CTGAGATACG CTATAGAGAA
241 GAAGGCTTAG ACAGTGTGAG TTCTGTAGAA AAGCTGTTTG GACAGAAAAC AAAGAAAGGA
301 TCCAAGTTCC AAGTGCGCGA CCGAAGGAAG GTAAAGTTGT TTCAAACAGT TTGTATTCGT
361 AAAAGGATAA ATCTTCGCGG TTAGAGCAAA TCGTTGCCAT AGGTGACTGT AGATACAAAG
421 GGACTGGATC TGACACATGA ATTTGTTTTG CAAAGTGTTC ATTAATTCTT TGATACATGG
481 ATATTGGAAG GGGGATTGTA TTTTGAGGGC TGNCTGTAAT CCAATGAGAA GATAAACGAG
541 TTTAGTATTG ATTTCATAAA TTTATGTCCC TTTCCGAATA AGGGG
```

DNA sequence from clone **M10**

```
  1 CGGGAGGGCG GTGATCGACG AGCTGCAGAA GAATTTACAG CTGTCGGTCG AGCACTGCGA
 61 GGCGTCGAGG ATGACACTGC ACAGGTTCGG CAATACGTCG TCGTCGTCGC TGTGGTACGA
121 GTTGGGGTAC AACGAGGCGA AGGGTAGGAT GAAAAGAGGT GATAGGGTTT GGCAGATTGC
181 GTTCGGGAGC GGGTTTAAGT GCAACAGCGC GGTCTGGAAG TGCAATCGGA CCATCACAAC
241 AACGTCAGAT GGGCCCTGGG CTGATTGCAT CGATAATTAC CCGGTTCATA TCCCTGAGGT
301 GGTCAAGCTC TAAAGTCAAA GCAAACAACA AACAAACAAA ATTCATAATG AAATGGTGAA
361 TGGGATTGGT GGTTGCTTTT TTTTTTTTTT TTTGTAAAAC CATATTACAT ATATTTGAAT
421 TGAACAAATA TTTAATTAGT TATAGTTTTA TAACTGGATC AAAGACAGGG CCAGGCA
```

DNA sequence from clone **M11**

```
  1 TACAACGAGG TCACATATAG ATGATGAGTA TGCAAACGCA ACTCAGAGAG ATCCGAAAAT
 61 TTTGCTGACT ACTTCCCGGG ATCCGAGTGC TCCTCTTATA CAATTTACTA AGGAACTGAA
121 ACTCGTTTTT CCCAATGCGC AACAAATGAA TCGTGGTGGT CAGGTTATTT CTGAAATTAT
181 TGAAACATGC CGTTCACACG AATTTACGGA TGTTGTGCTG GTTCATGAAC ATCGTGGTGT
241 ACCAGATGGT TTGATTATAA GCCACCTTCC TTTTGGTCCA ACAGCTTATT TTCAACTACT
301 CAATGTGGTC ACAAGACATG ACATCAAAGA CAGGGCCAGG CA
```

DNA sequence from clone **M13**

```
  1 TTTTGGCTCC CACTTCGAAG GCGAAAACGA CGGTGGCATG CATCAAGAAG AAGAAGATGA
 61 TGATGGTGGA ACAAATTATG GTGGTTATCA TAAGGATGAA GAGAGTGATG ATTCAATGGT
121 CTCTGATGCA TCCTCCGGTC CTAGTCATCA TCACGGCCGT CCATGTGGAA AAGAGAGGGT
181 GGCAGCTGAG AAAAAGGGCA AGAAGAAACC AGAGAAGGAG AAGCAAACCA GAGGTGGAAG
241 GAGAAAGCAG GAGGAGAAGA AAGACAAGGC ACTGTTGATT CATGGCAAGA AACGATGAGA
301 GGTCAACTGG AAAGGGGTAT TTTGAAAAAA AAAAAAAAAA AAAAAAAAA
```

DNA sequence from clone **M14**

```
  1 AAACTCCGTC CCAGCAACAC CTTCATTCAA CACTAAATTC ACAGCACAAA TCAGAGTCAG
 61 AAACACAAAT TTTGGTCCAT ACAAGTATGA TGCAGGCATT GTCACGTTTT TGTACCAAGG
121 TGCGACTGTC GGGCAAGTTT CTATTCCCAA GAGCAAGGCT GGAATGCTTT CCACCAAAAA
181 AATCAATGTC GAGGTGAGCT TGAGTTCAAG TGCATTGAGC GGTACAAACC TTGGCAGTGA
241 AATGAGCAAC GGGGTGTTGA CTCTCAACAG CGCGGCTAAG TTGACGGG
```

DNA sequence from *chitinase* clone **C1**

```
   1 ATGGCTCCAA AGTCCACAAT GTCCCTAGCA TTGCTCTCTT TAGTTACACT AGTTCTGGCT
  61 TTGGGGGCTA ATGCCGGTGG AATCGCTATA TATTGGGGTC AAAATGGGAA TGAAGGCACG
 121 TTAGCGGAAA CTTGTGCTAC AGGGAATTAC CAATTTGTAA ACGTAGCTTT TCTCACCACC
 181 TTCGGCAATG GCCGCACCCC CGCCATCAAC CTAGCCGGTC ACTGTGACCC AACGACCGAT
 241 GAATGCACCA AGTTGAGCCC AGAGATCAGG TCATGCCAAG CCAAGGGGAT TAAGGTCATA
 301 CTTTCCATTG GAGGAGCTTC TGGGAGCTAC TCTCTAACTT CAGCAGCTGA TGCAAGGCAA
 361 GTTGCAACTT ACTTGTGGAA CAACTTCTTG GGAGGGCATT CATCATCGAG GCCATTGGGA
 421 GCTGCAGTAT TGGATGGAAT TGACTTTGAT ATTGAGGGAG GGACTGACCA ATATTGGGAT
 481 GACCTTGCTA GGTACCTATC TGGATATAGC AAGAGAGGCA AGAAAGTTTA CTTGACTGCT
 541 GCCCCACAAT GTCCCTTTCC TGATGCTTGG ATTGGAAATG CACTTAAGAC AGGCCTCTTT
 601 GACAATGTTT GGGTTCAGTT CTACAACAAC CCTCCCTGTC AGTACACTTC CGGCGACGTG
 661 GCCAATCTAG AGGACGCTTG GAAGCAGTGG ACTTCAGCCA TCCCTGCACA TAAGATTTTC
 721 TTGGGGTTGC CTGCTGCTCC TCAGGCTGCC GGTAGCGGTT TTATTCCTGC TTCTGATCTC
 781 AACTCACAAG TCCTTCCGGC TATTAAAAAT TCGGGTAAGT ATGGAGGTGT CATGCTTTGG
 841 TCCAAATATT ATGATGATCG AGCT
```

DNA sequence from *chitinase* clone **C5**(Acc. no. AF309514)

```
   1 ATGGCTTCAA AGTCCACAGC AACGTTCCTA GCATTGCTCT CTTTAGTGAC ACTAGTTCTG
  61 GCTCTCGGGG CTAATGCCGG TGGAATCGCA ATATATTGGG GTCAGAATGG TAATGAAGGC
 121 ACATTAGCAG AAACATGCGC TTCAGGGAAT TACCAATTTG TAAACGTAGC TTTTCTCACC
 181 ACCTTCGGCA ACGGCCAAAC GCCTGCAATC AACCTAGCCG GTCACTGTGA CCCAACGACA
 241 GAAGAATGCA CCAAATTGAG CCCAGAAATC AAGTCATGCC AAGCCAAGGG GATTAAAGTC
 301 ATACTCTCCA TAGGAGGAGC TTCTGGGAGC TACTCTCTAA CTTCAGCTGA TGATGCAAGG
 361 CAAGTTGCAA CTTACCTGTG GAATAATTTC TTGGGAGGGC AATCGTCGTC GAGGCCATTG
 421 GGAGCTGCCG TTTTGGATGG AATTGATTTT GACATCGAGG GAGGGACTGA CCAACATTGG
 481 GATGACCTAG CAAGGTACCT CTCTGGATAT AGCAAGAGAG GCAAGAAAGT TTACTTGACT
 541 GCTGCCCCAC AATGTCCCTT TCCTGATGCT TATGTTGGAA ATGCACTTAA GACAGGCCTC
 601 TTTGACAATG TTTGGGTTCA GTTCTACAAC AACCCTCCCT GCCAGTACGC TTCTGGGGAT
 661 GTGACCAACC TTGAAGACGC CTGGAAGCAG TGGACTTCAG CCATCCCTGC AGATAAGATT
 721 TTCTTGGGAT TGCCTGCTGC ACCTCAGGCT GCTGGTAGCG GGTTTATTCC TGCTACCGAT
 781 CTTAGCTCAC AAGTTCTTCC GGCTATTAAA AGTTCGGCTA AGTATGGAGG CGTCATGCTT
 841 TGGTCCAAGT ATTATGATGA TCCTGATGGA TACAGCTCCT CCATCAAGAA TGATGTCTAG
```

DNA sequence from *chitinase* clone **C20**

```
   1 ATGGCTTCAA AGTCCACAAT GTCCATAGCA TTGCTCTCTT TAGTGACACT AGTGCTGGCT
  61 CTCGGGGCTA ATGCCGGTGG AATCGCTGTA TATATTGGGG TCAGGATGGT AACGAAGGCA
 121 CATTAGCTGA AACATGTGCT TCAGGGAATT ACCAATTTGT AAACCTAGCT TTTCTCACCA
 181 CATTCGGCAT CGGCCAAACC CCCGCCATCA ACCTAGCCGG CCGGTCACTG TGACCCAACG
 241 ACCGATGAAT GCACCAAATT GCCCAGAAAT CAAGTCATGC CAAGCCAAGG GGATTAAAGT
 301 CATACTCTCC AGAGGAGGAG CTTCTGGGAG CTACTCTCTA GCTTCAGCTG AGGATGCAAG
 361 GCAAGTTGCA ACTGACTTGT GGAACAACTT CTTGGGAGGG CATTCCTCGT CAAGGCCGTT
 421 GGGAGCTGCA GTATTGGATG GAATTGACTT TGATATTGAA GGAGGGACTG ACCAACATTG
 481 GGATGACCTT GCTAAGTACC TATCTGGATA TAGCAAGAGA GGCAAGAAAG TCTACTTGAC
 541 TGCTGCACCA CACTGTCCCT TTCCTGATGC TTGGGTTGGA AATGCACTTA AGACCGGCCT
 601 CTTTGACAAT GTTTGGGTTC AGTTCTACAA CAATCCTCCC TGTGAGTACA CTCCCGGCAA
 661 TGTGGATAAT CTCGAAATTG CTTGGAAGCA GTGGACTTCA GCCATCCCTG CACATAAGAT
 721 TTTCTTGGGG TTGCGTGCTG CACCTCAGGC TGCTGGTAGT GGTTTTATTC CTGCTTCTGA
 781 TCTCAACTCA CAAGTCCTTC CGCATATGGA GGTGTCATGC TTTGGTCCAA GTATTATGAT
 841 GATCTTGATG GATGAGTTTA TGCAATTCAA TACTATTAAT TTCCTAGGTT TTTATGA
```

ATG= translation start
TAG= translation stop