Genetic Variation in Resistance to Fungal Storage Diseases in Apple

Inoculation-Based Screening, Transcriptomics and Biochemistry

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Cover: Infected fruits with *Penicillium expansum*, chemical analysis, gene expression and functional analysis.
   (Figure: Masoud Ahmadi Afzadi)

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Abstract

Apple is one of the economically and culturally most important fruit crops and has many health-related benefits. Apple production is, however, sensitive to several fungal diseases including blue mold, caused by *Penicillium expansum*. Problems are more pronounced in organic production or in countries where postharvest application of fungicides is prohibited. To limit or overcome this problem, many studies have been focused on investigations of the mechanism of resistance/tolerance. No major gene(s) have as yet been identified, but quantitatively inherited traits, some of which are related to fruit texture and content of chemical compounds, have been shown to affect the ability of cultivars to withstand storage diseases. In the present thesis, inter-cultivar variation in terms of resistance to fungal storage diseases was investigated at two locations, i.e. Balsgård in Sweden and Njøs in Norway. The association of harvest date, fruit firmness and softening with lesion decay was investigated on large sets of cultivars. The contribution of four fruit texture-related genes (*Md-ACO1*, *Md-ACS1*, *Md-Exp7* and *Md-PG1*) in explaining the fruit texture characteristics was examined. Fruit content of chemical compounds with a potential impact on disease resistance was also investigated, and finally the regulation of apple genes upon fungal infection was studied in order to identify candidate genes responsible for disease resistance.

Inoculation-based screening indicated large variation across the investigated cultivars in terms of blue mold and bitter rot susceptibility. Harvest date and softening rate of fruits during storage had a large impact on resistance to fungal diseases, thus cultivars with moderate to firm fruits that soften comparatively little during storage could withstand the fungal infection comparatively well. Softening rate is, in its turn, closely associated with harvest date whereas four fruit texture-related genes had lower predictive power than expected. Quantifying the chemical compounds in the fruit samples revealed that some of these compounds, especially flavonols and procyanidin B2, could contribute to resistance against blue mold, whereas contents of malic acids or total titratable acidity had considerably less impact. Differential expression of *FLS, LDOX,* and *CHS* genes involved in biosynthesis of flavonoids and *PGIP*, *TT10*, *WAK1* and *CTL1* genes related to cell wall structure indicate the importance of fruit characteristics and biochemical compounds in the resistance mechanism.

Keywords: cell wall, chemical contents, defense mechanism, fruit firmness, gene expression, microarray, polyphenols, qRT-PCR, quercetin.

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Dedication

To my parents who have always been praying for my success, and to my wife who has been a great source of strength and motivation

My Lord! Grant me the serenity to accept the things I cannot change, the courage to change the things that I can, and the wisdom to know the difference.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Kellerhals M., Szalatnay D., Hunziker K., Duffy B., Nybom H., **Ahmadi-Afzadi M.**, Höfer M., Richter K. and Lateur M. (2012). European pome fruit genetic resources evaluated for disease resistance. *Trees*. 26: 179-189.

II Nybom H., **Ahmadi-Afzadi M.**, Sehic J. and Hertog M. (2013). DNA marker-assisted evaluation of fruit firmness at harvest and post-harvest fruit softening in a diverse apple germplasm. *Tree Genet. Genomes*. 9: 279-290.

III **Ahmadi-Afzadi M.**, Tahir I. and Nybom H. (2013). Impact of harvesting time and fruit firmness on the tolerance to fungal storage diseases in an apple germplasm collection. *Postharvest Biol. Tec*. 82: 51-58.

IV Tahir I., Nybom H., **Ahmadi-Afzadi M.**, Røen K., Sehic J. and Røen D. Susceptibility to blue mold caused by *Penicillium expansum* in apple cultivars adapted to a cool climate. *Eur. J. Hortic. Sci.*, in press.

V **Ahmadi-Afzadi M.**, Nybom H., Ekholm A., Tahir I. and Rumpunen K. Biochemical contents of apple peel and flesh affect level of partial resistance to blue mold. (Manuscript)

VI **Ahmadi-Afzadi M.**, Nybom H., Orsel M., Pelletier S., Bruneau M., Proux-Wéra E. and Renou J.P. Gene expression analysis of resistant and susceptible apple cultivars challenged with *Penicillium expansum* using an oligonucleotide microarray. (Manuscript)

Papers I-III reproduced with the permission of the publishers.

The contribution of Masoud Ahmadi-Afzadi to the papers included in this thesis was as follows:

I Performed the storage disease experiment in Sweden, analysed the data and participated in writing the corresponding part of the paper.

II Performed the firmness evaluations, and participated in analyses and writing of the manuscript.

III Performed most of the experimental work, and all data evaluation and statistical analysis, and wrote the manuscript.

IV Participated in the planning and performance of the experimental work, and in writing the manuscript.

V Planned the experiment together with co-authors, performed all the practical work and data evaluation, and wrote the manuscript.

VI Planned the experiment together with co-authors, performed all the practical work and data evaluation, and wrote the manuscript.

Related works by Masoud Ahmadi-Afzadi but not included in this thesis:

I Nybom H., **Ahmadi-Afzadi M.**, Garkava-Gustavsson L., Sehic J. and Tahir I. (2012). Selection for improved fruit texture and storability in apple. *Acta Hortic*. 934: 849-854.

II Dey E.S., **Ahmadi-Afzadi M.**, Nybom H. and Tahir I. (2013). Alkylresorcinols isolated from rye bran by supercritical fluid of carbon dioxide and suspended in a food-grade emulsion show activity against *Penicillium expansum* on apples. *Arch. Phytopathol. Plant Protect*. 46: 105-119.

III Tahir I., **Ahmadi-Afzadi M.**, Nybom H. and Dey E.S. (2014). Rye bran alkylresorcinols inhibit growth of *Penicillium expansum* and *Neofabraea perennans* in vitro and in vivo on different apple cultivars. *Eur. J. Hortic. Sci*. 79: 218-225.

IV **Ahmadi-Afzadi M.**, Rumpunen K., Ekholm A., Renou J.P., Orsel M., Pelletier S., Bruneau M., Tahir I. and Nybom H. Genetics of resistance to blue mould in apple; inoculation-based screening, transcriptomics and biochemistry. (Submitted manuscript)

V Nybom H., Røen D., Karhu S., Garkava-Gustavsson L., Tahir I., Haikonen T., Røen K., **Ahmadi-Afzadi M.**, Ghasemkhani M. and Hjeltnes S.H. Prebreeding for future challenges in Nordic apples; susceptibility to fruit tree canker and storage diseases. (Submitted manuscript)

Abbreviations

ACO1	1-Aminocyclopropane-1-Carboxylate Oxidase
ACS1	1-Aminocyclopropane-1-Carboxylate Synthase
ANOVA	Analysis of Variance
BHT	2,6-di-*tert*-butyl-4-methylphenol
CAT	Catechin
cDNA	Complementary DNA
CHL	Chlorogenic Acid
EPI	Epicatechin
GLM	General Linear Model
HCA	Hierarchical Cluster Analysis
hpi	hours post inoculation
HR	Hypersensitive Reaction
LG	Linkage Group
MAL	Malic Acid
MeJA	Methyl Jasmonate
NBS-LRR	Nucleotide-Binding Site Leucine-Rich Repeat
NEO	Neo-Chlorogenic Acid
PAL	Phenylalanine-Ammonia Lyase
PB2	Procyanidin B2
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGIP	Polygalacturonase Inhibiting Protein
PHL	Phloridzin
PLS-DA	Partial Least Square Discriminant Analysis
PR	Pathogenesis-Related
QAR	Quercetin 3-*O*-Arabinoside
QGA	Quercetin 3-*O*-Galactoside

QGL	Quercetin 3-*O*-Glucoside
QRH	Quercetin 3-*O*-Rhamnoside
QRU	Quercetin 3-*O*-Rutinoside
QTL	Quantitative Trait Loci
QXY	Quercetin 3-*O*-Xyloside
RT-qPCR	Reverse Transcription - quantitative PCR
SA	Salicylic Acid
TPH	Total Phenols
TTA	Total Titratable Acidity
wpi	weeks post inoculation

1 Introduction

Apple is one of the economically and culturally most important fruit crops and contributes significantly to human daily consumption due to its high accessibility and comparatively low price. Apple production is, however, sensitive to several diseases, and damage occurring during and after the harvesting is the major cause of postharvest fruit loss. The damage is caused mainly by fungal diseases, e.g. blue mold (*Penicillium expansum*), bitter rot (*Colletotrichum gloeosporioides*) and bull's eye rot (*Neofabraea* spp.), especially in countries where fungicide application after harvest is prohibited. This problem is especially pronounced in organic production since the use of fungicides prior to harvest is also prohibited, making the stored fruit even more vulnerable (Jönssson *et al*., 2010). In Europe, fungal diseases have increased significantly in the last decades, most likely with global warming being one of the major causes (Weber, 2009). In addition, many apple cultivars are inherently sensitive to blue mold, thus more than 10% of the harvested fruit was lost during storage for several cultivars grown in commercial orchards in Sweden (Tahir, 2014).

Many studies have focused on the control of postharvest diseases of apple by chemical compounds, biological treatments or by controlling the environmental and physical conditions (Conway *et al*., 2007; Tahir, 2006; Janisiewicz & Korsten, 2002). Neither of these alternative control methods is as efficient as fungicide application. Moreover, application of any kind of substance will involve more work and higher costs for the grower and ultimately also the consumer. A better solution would be to develop new apple cultivars that are as resistant or tolerant as possible to the major fungal diseases.

Screenings in the field with natural infection and in trials with artificially inoculated fruits have demonstrated large inter-cultivar variation between apple cultivars in terms of resistance to blue mold (Jurick *et al*., 2011; Tahir &

Nybom, 2008; Tahir & Jönsson, 2005). Investigation of the genetic mechanisms behind the resistance/tolerance and utilization of the resistant/tolerant cultivars in plant breeding programs could improve postharvest quality and grower economy considerably. No major gene(s) have as yet been identified for resistance against blue mold, but quantitatively inherited traits, some of which are related to fruit texture, content of chemical compounds and ripening behavior, apparently affect the ability of different cultivars to withstand fungal attacks (Nybom *et al*., 2008; Blazek *et al*., 2007; Prusky *et al*., 2004).

In the present thesis, inter-cultivar variation in terms of resistance to fungal storage diseases was first investigated, and the association of harvest date, fruit firmness and softening with lesion development in several cultivars was determined. The contribution of four different genes in explaining the fruit texture characteristics was also examined. Fruit content of chemical compounds with a potential impact on disease resistance was also investigated, and finally the regulation of apple genes upon fungal infection was studied.

2 Background

2.1 Apple (*Malus* × *domestica*)

Apple (*Malus* × *domestica* Borkh.) is one of the major horticultural crops in many countries in the world. Apples and apple-based products contain several important phytochemicals with health-promoting effects. Recent studies suggest that consumption of apple products can be associated with a positive impact on the risk of cancer, cardiovascular disease, asthma, and Alzheimer's disease (Hyson, 2011). Apple fruit has multiple uses; either being consumed fresh or after storage or being processed into, e.g., juice, sauce, slices, vinegar and cider.

2.1.1 History and origin

The history of apple seems to be interwoven with the history of humankind. Apple has been culturally important in many countries and religions where it has been referred to as a symbol of forbidden fruit, fruit of healthiness from heaven or a symbol of love in Greek mythology where an epigram states:

> *"I throw the apple at you, and if you are willing to love me, take it and share your girlhood with me; but if your thoughts are what I pray they are not, even then take it, and consider how short-lived is beauty."*
>   Plato, Epigram VII

Historical studies have shown that apple has probably been distributed by human or animals from its center of origin (the region where the species originated) to other parts of the world. The greatest diversity of *Malus* species has been observed in Central Asia, Asia Minor, the Caucasus, Himalayan India and Pakistan and Western China, where at least 25 species are found (Brown, 2012). Vavilov therefore suggested that wild apples originating from Central

Asia and their close relatives are the progenitors of the domesticated apple (Harris *et al*., 2002).

The wild species *M. sieversii* which grows in Kazakhstan and Kyrgyzstan, is thought to be the major progenitor of domesticated apple (Pereira-Lorenzo *et al*., 2009). This species may have hybridized with *M. prunifolia*, *M. baccata* and *M. sieboldii* to the East and with *M. turkmenorum* and *M. sylvestris* to the West. Selected and well-established cultivars were then introduced into Europe and the Mediterranean regions by the Romans (Juniper *et al*., 1999). However, analyses of genetic variation at the cytoplasmic level, i.e. chloroplast diversity, have lately re-opened the question about the origin of the domesticated apple. Coart *et al*. (2006) have thus reported a closer relationship than presently accepted, between *M. sylvestris*, i.e. European wild species (Figure 1) and domesticated apple based on the detection of eight shared chloroplast haplotypes, thereby re-opening the discussion about whether *M. sieversii* really is the main progenitor of domesticated apple. In the apple genome project, sequencing of the genome indicated more similarity between domesticated apple and *M. sieversii* than with *M. sylvestris* (Velasco *et al*., 2010), whereas another study indicates that domesticated apple is more genetically similar to *M. sylvestris* than to *M. sieversii* due to a bidirectional gene flow (Cornille *et al*., 2014; Cornille *et al*., 2012).

2.1.2 Taxonomy and biology

Apple belongs to the Rosaceae family and subfamily Maloideae which includes many of the commercially most valuable fruits like apples and pears, some ornamentals and also invasive plants. Different studies including cytology and morphology, flavone analysis and isozyme analysis, have suggested that Maloideae originates from hybridization between a Spiraeoideae ancestor and a Prunoideae ancestor, followed by fusion of unreduced gametes to form a fertile organism (Currie, 2000). Maloideae consists of approximately 1000 species in 30 genera (including *Malus*) characterized by the distinctive fruit, the pome (Brown, 2012; Kellerhals, 2009).

The genus *Malus* consists of five sections (*Malus*, *Sorbomalus*, *Chloromeles*, *Eriolobus* and *Docyniopsis*) based on morphological traits and flavonoid similarities. Section *Malus* consists of series *Malus*, including many European and Asian species and series *Baccatae*. Section *Sorbomalus* includes series *Sieboldianae* (native to Japan), *Florentinae* (from South-East Europe), *Kansuenses* and *Yunnanenses*. Section *Chromeles* consists exclusively of North American species. Section *Eriolobus* consists of only one species from the eastern Mediterranean, and section *Docyniopsis* includes some species originally from Japan, Taiwan and South-East Asia. The total number of

species in the genus *Malus* varies between studies, with as many as 78 (Robinson *et al*., 2001), 55 (Harris *et al*., 2002) or 30–35 species (Zhi-Qin, 1999).

Malus sylvestris	*Malus sieversii*
Fruit diameter: 1–3 cm	**Fruit diameter: up to 8 cm**

Figure 1. The morphology and fruit diameter of two wild species, i.e. *M*. *sylvestris* and *M*. *sieversii* that are proposed as progenitors of domesticated apple according to different studies. Figure modified from Cornille *et al*. 2014.

The majority of apple cultivars are diploid with 2n = 34 whereas some cultivars are triploid with 2n = 3x = 51. Such triploids (like 'Mutsu' and 'Jonagold') are pollen sterile, but they instead have larger fruits and leaves than their diploid relatives (Brown, 2012; Pereira-Lorenzo *et al*., 2009). As the haploid chromosome number is x = 17, the Maloideae may have resulted from an ancient auto-polyploidization of a 9 chromosome progenitor to 18 chromosomes. This was followed by a chromosome loss resulting in current 17–chromosome apple cultivars whereas other subfamilies in Rosaceae family have x = 7, 8 or 9 (Giovannoni, 2010; Kellerhals, 2009).

Blooming and ripening of apple vary considerably among different cultivars, and these are often categorized as early, middle and late flowering or ripening. Flowers have a gametophytic type of self-incompatibility (S locus); therefore planting at least two different cultivars (or one cultivar and a pollinator accession of a wild *Malus* species) is necessary to ensure a high level of cross-pollination. Flowering in apple, like in many other fruit crops, is a result of several physiological changes, and winter chilling is necessary to break bud dormancy. Inadequate winter chilling can delay both flower buds and vegetative buds. In addition to environmental effects, inherent differences between cultivars can also influence the physiological processes during the development from bud formation to fruit ripening (Janssen *et al*., 2008).

2.1.3 Growing conditions and production

Apple can be cultivated in a wide range of environments across the world, but a temperate climate where the chilling requirement is met to break bud dormancy is optimal. However, there is also an increasing interest in growing apples in subtropical and tropical countries like e.g. India, Mexico, Brazil, Zimbabwe and Kenya at high altitudes (Ashebir *et al*., 2010; Wamocho & Ombwara, 2001).

Total apple production is approximately 76.4 million tons in the world, of which more than 60% is produced in Asia. China presently produces approximately half of the apple production in the world (37 million tons), followed by USA, Turkey, Poland, India, Italy, Iran, Chile, Russia and France (FAO, 2013) (Figure 2).
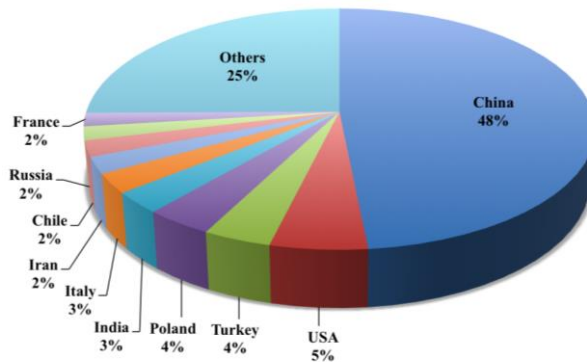

Figure 2. Contribution (%) of top ten countries in apple production (tons) in the world (FAO, 2013). China is responsible for approximately half of the world production.

2.1.4 Cultivars

Domesticated apple includes over 7500 different cultivars that are grown in many different countries. Selection and cultivation of different cultivars by small-scale apple growers was probably the earliest strategy in apple breeding. Desirable cultivars were thus selected based on traits that were important for the growers' own use and for the local markets (Ashebir *et al*., 2010). Later, understanding the biology and reproduction mechanisms in apple (e.g. pollination and grafting) opened a new window to breeding attempts. Many cultivars with desirable characteristics have now been bred but only a few dozens of these are grown commercially on a worldwide scale.

In general, breeding for a new cultivar is a money- and time-consuming process since several good characteristics must be gathered into one cultivar. Breeding attempts are, nowadays, more focused on traits that are demanded by consumers, e.g. fruit size and shape, color, taste and flavor. In addition, other factors like high and consistent yields, postharvest storability and shipping quality, and resistance to disease, pests and disorders also have to be considered. Unlike the old cultivars that were simply selected in or around established orchards as chance seedlings, modern (new) cultivars generally derive from long-term breeding programs (Brown & Maloney, 2005; Janick *et al*., 1996).

Apple fruits are available throughout the year while the production period is a maximum of three months, i.e. beginning of August to middle of October in Northern and Central Europe. Therefore, much fruit is stored for a long period before being marketed. During this period, apples are subjected to a variety of losses that can be categorized into three groups: mechanical damages, physiological disorders and biological damages (Wu, 2010).

2.1.5 Mechanical and physiological damage

Mechanical and physiological damage cause much fruit loss during picking and handling of fruits after harvest, and can amount to 5–25% of the total production in developed countries and 20–50% in developing countries (Kader, 2005). Damage due to mechanical handling systems or impact forces during packing or transportation, produces soft and brown bruise marks thereby reducing consumer acceptance and market value. In addition, mechanical damage also promotes the incidence of several fungal diseases during the storage and shelf life.

Physiological disorders are changes in metabolic processes in fruits due to unsuitable conditions (both environmental and nutritional). Similar to mechanical damages, physiological disorders produce significant changes in fruit shape, color or attractiveness, which leads to unmarketable fruits. Some of these disorders are bitter pit (related to calcium transportation and deficiency), soft and superficial scald (due to low temperature during storage), watercore (dysfunction in carbohydrate physiology) and internal browning (due to CO_2 injury) (Martins *et al*., 2013; Ferguson *et al*., 1999).

2.1.6 Biological damage

The biological damages can be caused by pests or pathogens. Apple fruit is host to over 70 infectious diseases most of which are caused by pathogenic fungi. However, bacteria (like fire blight caused by *Erwinia amylovora*), viruses and phytoplasma can also produce significant diseases on apple. The

symptoms are root rots, leaf spots, leaf blights, blossom blights, fruit spots, canker and postharvest decay. Apple diseases caused by fungi like apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*), brown rot (*Monilinia fructicola*), bitter rot (*C. gloeosporioides*), bull's eye rot (*Neofabraea* spp.) and blue mold (*P. expansum*) can cause severe losses in apple production (Martins *et al.*, 2013; Jijakli & Lepoivre, 2004).

Among all these fungal diseases, postharvest diseases caused by prevalent pathogens like *Penicillium* or *Neofabraea* can destroy a large part of the production during storage, especially on inherently susceptible cultivars. The damage is more pronounced under warm and humid conditions, and can decrease the yield by 50–80% in orchards without effective plant protectant spraying (Jurick *et al.*, 2011; Jones & Aldwinckle, 1990).

2.2 Postharvest diseases; Blue mold

2.2.1 Importance

Blue mold, caused by the necrotrophic fungus *P. expansum*, is one of the most common postharvest diseases of apple (Pianzzola *et al.*, 2004). In addition, this fungus produces patulin, a carcinogenic mycotoxin that has attracted public concern due to its impact on human health (Barreira *et al.*, 2010; Beretta *et al.*, 2000). In Europe, blue mold and other storage rots have increased significantly during the last decades, probably due to a combination of global warming, increased organic production and prohibition of several fungicides (Weber, 2009; Tahir & Jönsson, 2005).

Typical symptoms of blue mold are soft, circular and tan-colored rots with sharp margins between the watery soft rot and healthy fruit flesh (Figure 3). Symptoms usually increase very rapidly after a few days at room temperature. A dense and powdery mass of blue-green spores on the surface of decay can occur during development of disease. Airborne conidia present in decayed fruits in the orchards or in packinghouses and storage, and can be transmitted to newly harvested fruits and thus spread the disease (Jijakli & Lepoivre, 2004).

2.2.2 Blue mold control by chemical treatment

Until now, pre- and postharvest treatment of the fruit with protective compounds has been the main strategy to control diseases. Several applications of fungicides like benzimidazole, phenylcarbamate, phtalimide, thiabendazole or sulfamide prior to harvest can decrease the incidence of postharvest diseases considerably (Jijakli & Lepoivre, 2004). In Sweden, commercial orchards usually receive 5–6 yearly administrations of standard fungicides in spring and

summer, with the last one at least three weeks prior to harvest. No postharvest applications are, however, permitted.

A well-integrated approach with application of fungicides, pesticides and bactericides (the latter usually not allowed in Europe), selection of resistant or tolerant rootstocks and scion varieties, biological disease control and selection of a suitable site for the orchard can decrease damage considerably (Dewasish & Amal, 2010; Jönsson, 2007). However, new legislation has recently limited the number of permitted fungicides in several countries (Jijakli & Lepoivre, 2004).

Figure 3. Blue mold symptoms in apple fruits inoculated with spores of *Penicillium expansum*, after six weeks of storage. Photo by M. Ahmadi-Afzadi.

2.2.3 Breeding for resistant cultivars

Disease control is a major annual expense for growers in most apple-producing areas. The grower needs to control early-season diseases like apple scab as well as summer diseases and also some storage diseases. Although control of postharvest diseases can be achieved by the application of chemical fungicides, environmental concerns and increasing public concern about the impact of chemicals on human health requires the development of new approaches. Therefore, research has presently focused on identifying the genetic background of resistance in different cultivars. Identification and breeding of such cultivars will increase the level of disease tolerance in the field. A main step in breeding is to gain better knowledge about genetic resources that could be suitable in breeding programs, and this will also help to conserve genetic diversity.

Traditionally, crosses using controlled pollination and selection of desirable recombination products are the first steps in breeding of improved cultivars. Thus breeders mate parents with suitable traits like disease resistance and superior fruit quality. This is the most effective way to increase the frequency of the desirable alleles due to the relatively high additive variance in most of the traits (Brown & Maloney, 2005; Janick *et al*., 1996).

2.3 Resistance to fungal infection

2.3.1 Mechanism of resistance

Understanding the pathogenicity mechanism and the interaction between plant defense system and pathogen are crucial steps in defining the resistance and producing tools for marker-assisted plant breeding and cultivar improvement. In general, research on the interaction between plant and pathogen shows that response to a pathogen attack depends mainly on the type of disease resistance mechanism in the host plant. Two main categories have been defined, i.e. qualitative and quantitative resistance (Figure 4). Other terms have also been used to refer to this concept, including vertical versus horizontal, complete versus incomplete and major-gene versus minor-gene (Zhang *et al*., 2013; Poland *et al*., 2009).

In a qualitative gene-for-gene interaction, plants usually react through activation of an R gene(s) in a hypersensitive reaction (HR). By contrast, plants may respond to the pathogen in a more complex manner when multiple genes are involved in a quantitative resistance reaction. The qualitative defense reaction is mediated by different nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, while quantitative resistance is achieved by a wide range of reactions including the promotion of basal defense, activation of signaling pathways, encoding of detoxifying enzymes, cell wall modification and lignification, and changes in developmental and biosynthesis processes (Zhang *et al*., 2013).

As shown in Figure 4, defense response towards necrotrophic fungi is generally assumed to be controlled by quantitative resistance whereas plant response to biotrophic pathogens is largely due to gene-for-gene interaction (Glazebrook, 2005). Activation of hormone-dependent pathways has also been reported upon infection by necrotrophic fungi, resulting in expression of certain pathogenesis-related (PR) proteins. Jasmonic acid- and ethylene-mediated defense responses have thus been proposed to play an important role in the resistance to necrotrophic pathogens (Zhang *et al*., 2013).

The genetic mechanism of resistance/tolerance in apple to blue mold infection has, as yet, not been fully understood, but a contribution of inter-

cultivar genetic variation to the amount of blue mold damage has been indicated in previous studies (Jurick *et al*., 2011; Janisiewicz *et al*., 2008).
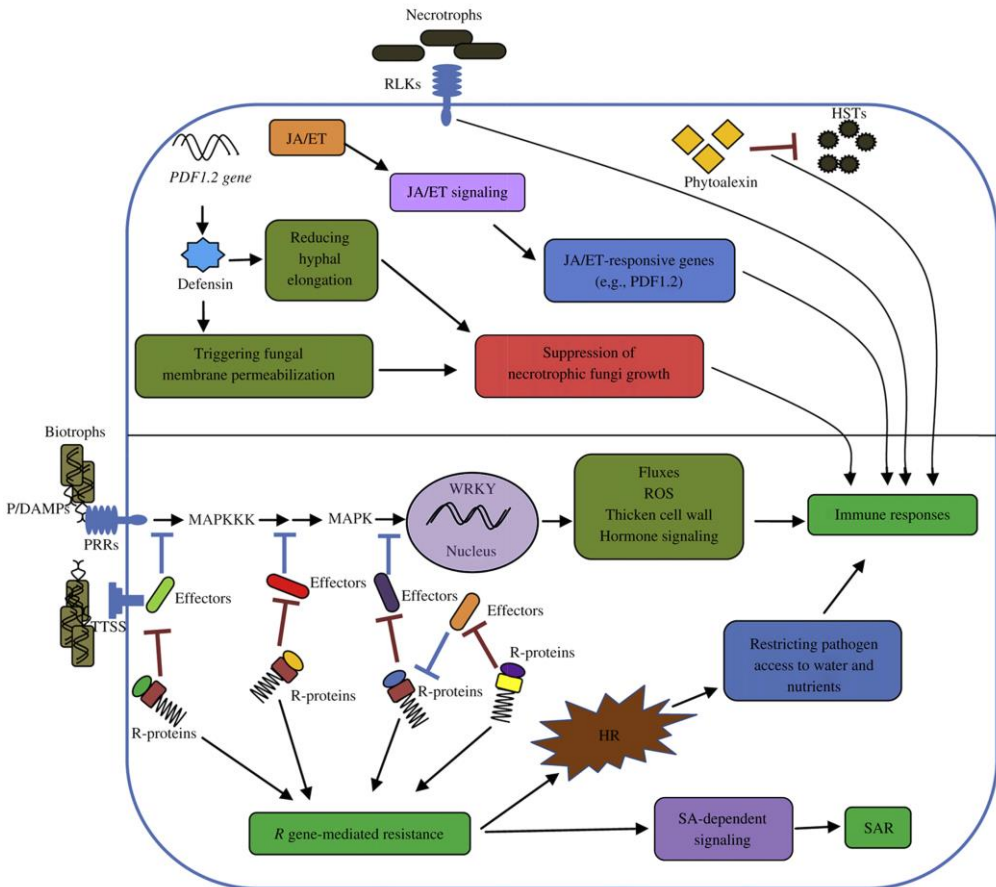

Figure 4. The mechanism of disease resistance in necrotrophic and biotrophic fungi. Figure reproduced from Zhang *et al*., 2013, *Journal of Genetics and Genomics*, reproduced by permission.

2.3.2 Resistance and fruit characteristics

Fruit ripening and maturation are complex processes in the development of apple fruit, resulting from several biochemical and physiological changes. These changes include internal enzymatic activities like polysaccharide hydrolysis which produces rearrangements and modifications of cell wall structure (Giovannoni, 2001). Several enzymes related to the cell wall, e.g. polygalacturonases, pectin-methyl esterases, pectate lyases, glycosidases

and/or expansins have thus been proposed to play an important role in fruit development (Payasi *et al*., 2009; Brummell & Harpster, 2001).

Initial firmness (i.e. firmness at harvest), firmness after storage and softening rate (i.e. the difference between initial firmness and firmness after storage) are quantitatively inherited traits associated to fruit characteristics, and they have been hypothesized to have an influence on resistance to fungal decay. For instance, it is well known that apples with high storability and low ethylene production will stay more firm (less softening) during storage (Costa *et al*., 2010b; Sansavini *et al*., 2004) and show less decay (Conway *et al*., 1991).

Apparently, increased firmness and reduced softening due to calcium spraying can decrease fungal decay (Conway *et al*., 2002; Sams *et al*., 1993; Conway *et al*., 1991). In addition, several quantitative loci on linkage groups LG01, LG08, LG10, LG15 and LG16 have been associated with fruit firmness and flesh texture (Costa *et al*., 2008; Seymour *et al*., 2002; King *et al*., 2001). Fruit maturity has also been suggested to influence the development of rots during ripening and storage of apples (Torres *et al*., 2003). Lignification upon fungal infection was less in over-mature fruits indicating that maturity stage of fruit plays an important role in fungal resistance to *Penicillium* infection (Vilanova *et al*., 2012).
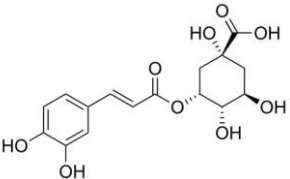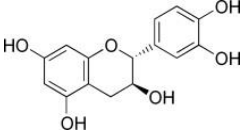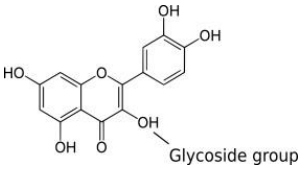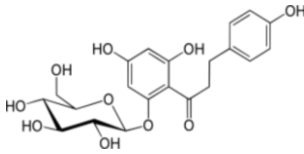
2.3.3 Resistance and fruit chemical compounds

Several compounds with health-related benefits such as organic acids, sugar alcohols, soluble fibers (like pectin), phenolic compounds and other phytochemicals have been found in apple fruit (Roen *et al*., 2009; Boyer & Liu, 2004). These chemical compounds of the fruit flesh and peel may have an important role in resistance to storage diseases. Many plants can respond to pathogens either by accumulation of pre-formed compounds (phytoanticipins, i.e., chemicals that are already present in different concentrations and forms) or by production of new compounds (phytoalexins) due to induction of genes involved in the defense system.

Phenolic compounds constitute a comprehensive class of phytoanticipin and phytoalexin molecules, many of which are involved in the natural defense reactions of plants, and can be toxic to invading organisms (Lattanzio *et al*., 2006; Grayer & Kokubun, 2001). Major groups of polyphenolic compounds in apple are hydroxycinnamic acids (chlorogenic acid and neo-chlorogenic acid), flavanols (catechin, epicatechin and procyanidins), flavonols (quercetin glycosides) and dihydrochalcones (phloridzin) (Table 1). Sixteen individual polyphenolic compounds have been found in both apple peel and flesh (Tsao *et al*., 2003). Hydroxycinnamic acids, phloridzin, and flavanols were found in

both flesh and peel while flavonols were found only in the peel. However, the predominant groups of polyphenols, in the apple peel and flesh, are procyanidins followed by quercetin glycosides in the peel and chlorogenic acids in the flesh (Roen *et al.*, 2009; Tsao *et al.*, 2003).

A contribution of polyphenolic compounds to the plant defense system against apple scab disease has been reported in several studies (Slatnar *et al.*, 2012; Mikulic-Petkovsek *et al.*, 2009; Mikulic-Petkovsek *et al.*, 2008; Mayr *et al.*, 1997). The incidence of apple scab has been reported to be lower in apple cultivars with a high level of flavanols (Mayr *et al.*, 1997). Phloridzin has also been proposed to be involved in the defense system of apple leaves against scab disease (Lattanzio *et al.*, 2006).

Table 1. *Different groups of important polyphenolic compounds present in apple, and their skeletal formula.*

Phenolic group	Compound	Skeletal formula
Hydroxycinnamic acids	Chlorogenic acid Neo-chlorogenic acid	
Flavanols (Flavan-3-ols)	Procyanidins Catechin Epicatechin	
Flavonols	Quercetin 3-*O*-rutinoside Quercetin 3-*O*-galactoside Quercetin 3-*O*-glucoside Quercetin 3-*O*-rhamnoside Quercetin 3-*O*-arabinoside Quercetin 3-*O*-xyloside	
Dihydrochalcones	Phloridzin	

Production of polyphenols in apple may be initiated by activation of phenylalanine-ammonia lyase (*PAL*) due to fungal attack (Mikulic-Petkovsek *et al.*, 2011; Lattanzio *et al.*, 2006). Schovankova and Opatova (2011) have shown that the total phenol content increased in apple fruits when inoculated with *P. expansum* followed by a decline after one week. Experiments with exogenous application of different phenolic compounds have shown that quercetin is an effective compound in controlling blue mold (Sanzani *et al.*, 2009a). Subsequent studies have demonstrated that this control is achieved through an increased transcription of genes involved in the quercetin biosynthetic pathway (Sanzani *et al.*, 2010; Sanzani *et al.*, 2009b).

2.4 Molecular breeding for resistance

2.4.1 Molecular markers and breeding

Different molecular markers, especially DNA-based markers, have been used in studying the genetic diversity of numerous plant species. These molecular markers can be applied for different purposes; some are just used for generating genotype-specific fingerprinting profiles while others are used specifically to tag genes and thus help to, e.g., select desirable genotypes (Nybom *et al.*, 2014). Many DNA markers linked to quantitatively inherited traits, including markers for fruit characteristics, ethylene production, scab resistance, and biochemical compounds, have the potential to enhance the efficiency of apple breeding programs.

Several studies have been directed to investigation of DNA markers linked to genes controlling important traits like resistance to disease, e.g. *Vf*, *Vb*, *Vbj*, *Vm*, *Vr* and *Va* scab resistance genes (Patocchi *et al.*, 2004; Cheng *et al.*, 1998; Hemmat *et al.*, 1998; Koller *et al.*, 1994), *Fb_MR5* gene for fire blight resistance (Fahrentrapp *et al.*, 2013; Peil *et al.*, 2008) and *Pl* gene for powdery mildew (James *et al.*, 2004; Markussen *et al.*, 1995). Moreover, complete or partial genetic maps based on linkage analysis have also been developed for the apple genome. QTLs associated to fruit softening during storage are co-located with *Md-PG1* on linkage group (LG) 10 (Longhi *et al.*, 2012; Costa *et al.*, 2010b) and with *Md-Exp7* on LG1 (Costa *et al.*, 2008). Two other well-studied genes affecting fruit firmness are *Md-ACS1* (1-aminocyclopropane-1-carboxylate synthase) on LG15 and *Md-ACO1* (1-aminocyclopropane-1-carboxylate oxidase) on LG10, both of which independently affect the ethylene production of apple cultivars (Costa *et al.*, 2005; Oraguzie *et al.*, 2004).

Quantitative trait loci associated to fruit maturity and co-located with *MdMYB10* (a candidate gene for red flesh) have been identified on LG9 (Morimoto *et al.*, 2013). QTLs for fruit chemical contents and acidity

(Vilanova *et al*., 2014a; Prusky *et al*., 2004), and a QTL controlling phenolic compounds co-located with *LAR1* on LG16 (Chagne *et al*., 2012) have already been identified. Moreover, a QTL associated to blue mold resistance has recently been reported on LG 4 and 10 in a mapping population of 'Royal Gala' × *Malus sieversii* PI613981 (Norelli *et al*., 2013).

Molecular marker information can be efficiently used in different ways in breeding programs of apple, e.g. for early selection of traits (traits can be screened during the juvenile phase), for evaluation of large sets of cultivars for favorable traits linked to the marker, for identification of the transformed plant after transformation (marker traits) and for selection of traits which are too expensive or difficult to measure directly (Currie, 2000). Moreover, by incorporation of QTLs data to the data produced by two international projects, RosBreed (www.rosbreed.org) and FruitBreedomics (www.fruitbreedomics .com) based on the genotyping of numerous pedigree families, a high density SNP array has been developed and will be used to improve apple research and breeding.

2.4.2 Gene expression and identification of candidate genes

Although many QTL markers have been developed in different populations, several questions remain unanswered regarding the genetic mechanism of resistance to postharvest diseases, including the number of loci that underlie variation in resistance, magnitude of their effects, their molecular mechanisms of action and interaction, and their dependence on environmental variables. To understand the regulatory mechanism of resistance and gene function, knowing when, where and to what extent a gene is expressed is a very central part. Global gene expression analysis can reveal the answers to these questions as well as provide useful information on the network of genes acting together to regulate a function, i.e. resistance to postharvest diseases.

There are several ways to measure the abundance of mRNA and the changes in gene expression, including northern blots, reverse transcription of polymerase chain reaction (RT-PCR), cDNA sequencing, differential display, subtractive hybridization and microarray technique (Lockhart & Winzeler, 2000). Depending on the method, for instance RT-PCR versus microarray, respectively, limited numbers of genes or the whole 'transcriptome' can be studied (Figure 5), and specific candidate genes can be identified. Moreover, these methods provide very useful information about the biological reasons behind the studied function. For instance, genes with similar expression behavior, e.g. increasing and decreasing together upon fungal infection, are likely to be related functionally (Lockhart & Winzeler, 2000).

Gene expression analysis has recently become a routine tool for studying genes under specific conditions or during a particular developmental stage, e.g. genes controlling skin color in apple (Vimolmangkang *et al*., 2014; Telias *et al*., 2011), fruit and cuticle development (Albert *et al*., 2013; Soria-Guerra *et al*., 2011; Janssen *et al*., 2008; Lee *et al*., 2007), polyphenol and anthocyanin biosynthesis during development (Bizjak *et al*., 2013; Henry-Kirk *et al*., 2012; Soglio *et al*., 2009; Kondo *et al*., 2002), response to disease (Vilanova *et al*., 2014b; Musetti *et al*., 2013; Jensen *et al*., 2012; Sarowar *et al*., 2011; Baldo *et al*., 2010; Sanzani *et al*., 2010; Yao *et al*., 1999) and ethylene biosynthesis and ripening (Yang *et al*., 2013; Zhu *et al*., 2012; Costa *et al*., 2010a).
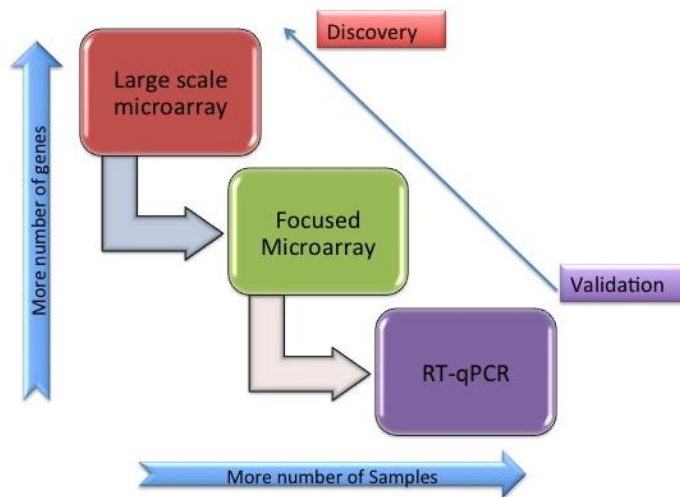

Figure 5. Different methods to study the gene expression, i.e. microarray analysis which investigates the whole genome by evaluating a large number of genes while qPCR is used for a lower number of genes. Figure modified from VanGuilder *et al*., 2008.

2.5   Gene expression analysis

The first reports about gene expression analysis were published after the introduction of Northern blotting for RNA analysis in 1977. With the advent of the polymerase chain reaction (PCR), small amounts of RNA could be converted to complementary DNA (cDNA), and a new technique, i.e. reverse-transcription quantitative PCR (RT-qPCR), with more accuracy in detection was developed (VanGuilder *et al*., 2008). More recently, nano-technology has

led to the development of microchip arrays thus enabling large-scale transcriptome analysis.

2.5.1 RT-qPCR

Gene expression analysis by RT-qPCR is a very sensitive technique, and it is used mostly when only a few numbers of genes are studied. This technique was initially developed to amplify distinct nucleic acid sequences to allow detection of target RNAs. Compared to end-point PCR (which provides a quantification only at the end of cycles), real quantification of amplified DNA is instead made possible during the cycles in RT-qPCR (VanGuilder *et al*., 2008).

The RT-qPCR analysis can be conducted either at a relative (target gene relative to reference gene) or an absolute level (numbers of copies of a target gene). For analyses of gene expression upon a physiological change, relative quantification is used in most cases (Pfaffl, 2001). Non-differentially regulated genes (reference genes) with stable and consistent expression in various tissues or conditions are then used for normalization of the target gene. There are several ways to calculate and quantify gene expression, but the method developed by Pfaffl is one of the most common:

$$ratio_{control/sample} = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

E: RT-qPCR efficiency

ΔCP: CP* deviation of control – sample

∗ Crossing point: the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

2.5.2 Microarray technique

Large-scale studies of gene expression by microarray technique have recently received a great deal of attention. The microarray experiment is in principle very similar to northern blot analysis, in which labeled mRNA from a sample is hybridized in parallel to a large number of DNA sequences. However, tens of thousands of transcripts can be immobilized on a microarray chip, and they can be detected and quantified simultaneously with the microarray analysis (Schulze & Downward, 2001).

Microarray systems can be divided into two main groups according to the probes used on the array; oligonucleotide microarray and PCR-based (or cDNA) microarrays. In oligonucleotide (nt) arrays, oligomers are synthesized, *in situ*, onto array slides whereas PCR products generated from cDNA libraries or by gene-specific primers are printed onto cDNA microarray slides. The length of the probes varies depending on the manufacturer and type of probe: typically from 20–25 nt to 50–100 nt for oligonucleotide probes, while PCR-based probes are generally more longer; 200–2000 bp (He *et al.*, 2005). Development of a PCR product-based microarray is a time-consuming procedure that requires PCR primer design, amplification, size verification, product purification, and quantification. Therefore, oligonucleotide arrays with longer probes (usually longer than 40 nt) have recently been developed and are used frequently in many studies.

In practice, RNA templates of control and target (e.g. infected tissue in case of disease) samples are obtained and reverse transcribed in an RT-PCR reaction (Figure 6). These templates are then labeled and deposited into the array and hybridized with oligoprobes. The hybridized array is scanned, and signal intensity data are extracted and subjected to quality control, normalization and differential expression identification analyses with statistical packages. Further gene classification, functional or pathway analyses are performed with gene lists that are differentially expressed between samples (Sanches & Ruiz de Villa, 2008).

Several microarray chips have recently been developed for apple and applied in order to elucidate the molecular mechanisms of fruit development (Soria-Guerra *et al.*, 2011; Janssen *et al.*, 2008; Lee *et al.*, 2007), aroma production and red color pigmentation (Vimolmangkang *et al.*, 2014; Schaffer *et al.*, 2007), genetic response to fire blight (Jensen *et al.*, 2012; Sarowar *et al.*, 2011) and host/non-host mechanism of resistance to blue mold (Vilanova *et al.*, 2014b). The obtained results can provide a better understanding of the genetic components that confer specific characteristics to apple cultivars, and thus be very valuable for breeding programs.

Microarray experiment and data analysis workflow

Infected   Intact

RT-PCR and labeling

Load on the array

Hybridization

Array Scaning

Bioconductor
OPEN SOURCE SOFTWARE FOR BIOINFORMATICS
Differential expression analysis with statistical packages

Quality control and normalization

Image analysis and signal intensity extraction

Venn diagram

Cluster analysis

Gene functional categories
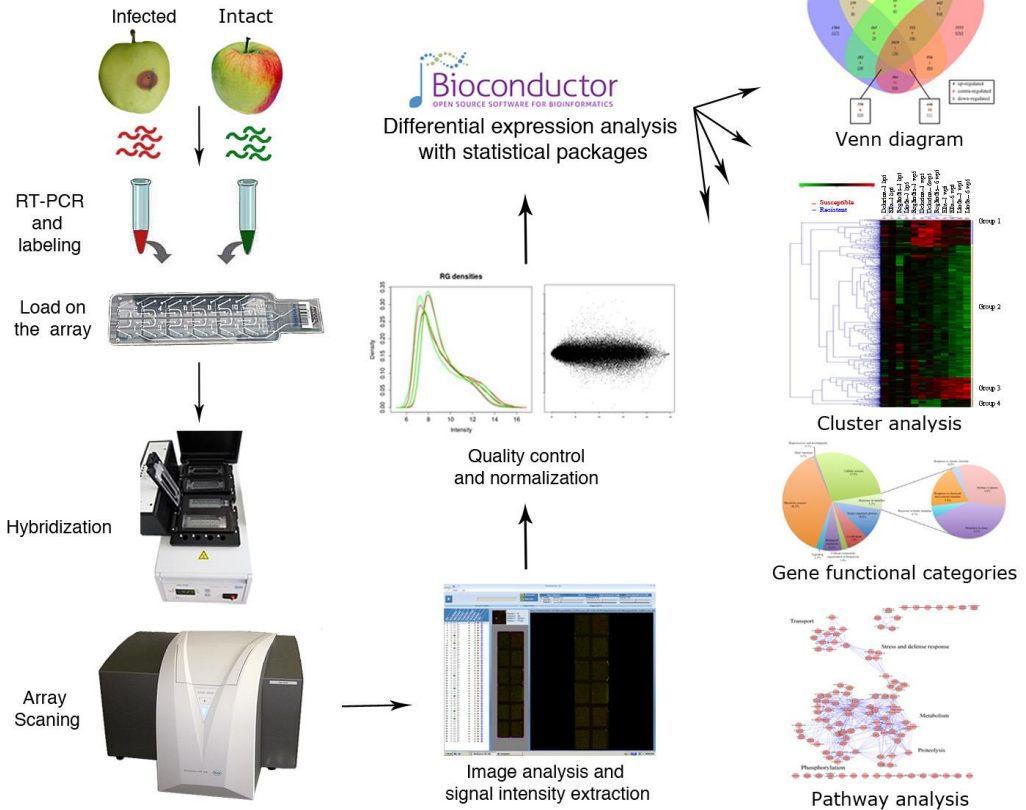
Pathway analysis

Figure 6. Schematic picture showing the microarray gene expression technique and data analyses. Figure by M. Ahmadi-Afzadi.

3 Aim and objectives

Although many studies have investigated different ways to control or inhibit blue mold development on apple fruits, there is little information on the genetic mechanisms of resistance/tolerance to this fungal storage disease. We have therefore quantified disease susceptibility in different apple cultivars, and investigated the association between disease resistance and fruit characteristics (both physical and biochemical). In addition, we have carried out a microarray transcriptome analysis using the whole apple transcriptome of two susceptible and two resistant cultivars. The specific objectives of this study were to:

➢ Quantify resistance to blue mold and bitter rot in an apple germplasm collection, and investigate associations between the level of resistance and harvest date, firmness and softening rate during storage (Papers I, III, IV).

➢ Investigate the contribution of harvest date, initial firmness and allelic configuration of four genes responsible for fruit texture in apple (i.e. *Md-ACS1*, *Md-ACO1*, *Md-Exp7* and *Md-PG1*) in explaining the variation in fruit softening in a large germplasm collection (Paper II).

➢ Quantify selected chemical compounds in peel and flesh of several apple cultivars in infected and uninfected (control) fruits, and investigate associations between these chemical compounds and the level of resistance to blue mold (Paper V).

➢ Utilize the microarray gene expression technique to identify candidate genes that are differentially regulated and could be responsible for inter-cultivar variation in resistance to blue mold (Paper VI).

4 Material and methods

This thesis contains four sections, in which different types of analysis have been used, i.e. 1) inoculation-based screening of disease resistance and fruit texture characterization, 2) DNA marker analysis and fruit texture characterization, 3) analysis of chemical components in fruit flesh and peel, and 4) gene expression and candidate gene identification.

4.1 Plant materials

Plant materials (apple fruits) were harvested at Balsgård, SLU, in southern Sweden (N 56° 06′ 23″, E 14° 09′ 55″), at a former SLU research station in Kivik (N 55° 40′ 57″, E 14° 13′ 18″) about 60 km southwest of Balsgård, and at a research station in Njøs in Southwestern Norway (N 61° 10′ 47″, E 06° 51′ 44″). The orchards were fertilized, irrigated, pruned and sprayed as a commercial orchard, including 5–6 yearly administrations of standard fungicides in spring and summer. All fruits were harvested at a harvest date suitable for long-term storage, i.e. a pre-climacteric stage according to the iodine starch test (starch conversion value 4–5 on a 9-point scale) (Smith *et al*., 1979).

4.2 Inoculation-based screening

In this part of the thesis, the level of storage disease resistance was investigated in 92 cultivars in 2010, in 45 cultivars in 2012, and in 43 cultivars in 2013, at Balsgård, Sweden (Papers I, III and IV). A corresponding analysis was performed with 45 cultivars at Njøs, Norway for two years; 2012 and 2013 (Paper IV).

4.2.1 Fruit inoculations

In these experiments, fruits were harvested and washed with distilled water in order to remove pesticide residues and naturally occurring fungi, wiped dry and then inoculated on opposite sides at a depth of 3 mm with 20 µL of a solution containing conidiospores (1×10^5 conidia mL^{-1}) of either *P. expansum* or *C. gloeosporioides*. Naturally infected apples showing typical disease symptoms were used for production of conidia (Figure 7). Mycelium was maintained on Petri dishes with potato dextrose agar (PDA). Pathogen virulence was confirmed by periodic inoculation of apples over a time. Conidia were removed from the surface of 10-day-old cultures and suspended in 5 mL sterile distilled water containing 0.05% (v/v) Tween 80. The suspension was filtered through four layers of sterile cheesecloth to remove any adhering mycelia, and spore concentration was adjusted to above-mentioned concentration using hemacytometer (Tahir *et al.*, 2009).

Inoculation of fruits   **Inoculated fruits after storage**

Fruit firmness test

Figure 7. Inoculation of fruits with *Penicillium expansum* spores (top left), symptoms of blue mold decay after storage (top right), and measurement of fruit firmness by a penetrometer (below). Figure by M. Ahmadi-Afzadi.

Inoculated fruits were stored in open-faced plastic boxes at 2–4 °C and 90% RH for either 6 weeks for early-ripening cultivars (summer cultivars) or 12 weeks for late-ripening cultivars (fall and winter cultivars). Lesion decay was measured as lesion diameter on the surface of infected apples at both sites at the end of the storage period, and expressed as mm lesion per week of storage.

Fruit firmness was also measured on opposite, peeled sides of each fruit with a penetrometer (model FT-327, Effigy, Alfonsine, Italy, plunger diameter 11.1 mm, depth 7.9 mm), and expressed as Newtons (Figure 7). Twenty fruit per cultivar were picked on the same day as harvesting for the inoculation tests. Firmness of 10 fruit was measured at harvest and the rest were stored in regular air (2–4 °C and 90% RH) for 6 and 12 weeks of storage for early- and late-ripening cultivars, respectively. Fruit were removed after storage and left in room temperature for 4 h before measuring the firmness. Average fruit firmness at harvest and after storage, and fruit softening rate (difference between firmness at harvest and firmness after storage) were calculated for each cultivar. Moreover, harvest date was also calculated by denoting the first harvested cultivar as '1', and then adding number of days to harvest for each cultivar.

In addition, fruit genetic resources were phenotypically and genotypically evaluated for resistance to several diseases like apple scab (*V. inaequalis*), powdery mildew (*Podosphaera leucotricha*), fire blight (*Erwinia amylovora*) and pear rust (*Gymnosporangium sabinae*) in projects throughout Europe (Paper I), but only data for blue mold and bitter rot in apple will be presented and discussed in the present PhD thesis.

4.2.2 Data evaluation and statistical analysis

Inter-cultivar variation was investigated by subjecting the lesion decay data to one-way analysis of variance with the general linear method (GLM). A series of regression analyses were performed with lesion decay data as response variable and harvest date, firmness and softening rate as explanatory variables. These statistical analyses were performed using Minitab ver. 16 (Minitab Inc., State College, PA, USA). In addition, partial least square discriminant analysis (PLS-DA) was applied to quantify the contribution of harvest date, firmness, and softening rate, respectively, in predicting lesion decay in 2010 and 2012 (Paper I). PLS-DA was applied using The Unscrambler® X.1 (CAMO Software, 2006).

4.3 Fruit texture and DNA analysis

Fruits of 127 apple cultivars were used to investigate the impact of *Md-ACS1*, *Md-ACO1*, *Md-Exp7* and *Md-PG1* genes, fruit firmness, and harvest date in explaining the variation in fruit softening (list of cultivars is available in paper II).

4.3.1 DNA analysis

The whole material was screened for the above-mentioned genes with specific primers. For *Md-ACS1*, cultivars were screened with allele-specific primers according to Nybom *et al*. (2008). A SCAR marker amplifying two alleles (allele 1 of 525 bp and allele 2 of 578 bp) was used for detecting alleles of the *Md-ACO1* locus (Costa *et al*., 2005). For *Md-Exp7*, a SSR marker amplifying 'CT' repeat motif was used according to Costa *et al*. (2008). The *Md-PG1* locus was amplified using a CAPS marker which detected two different alleles, i.e. one large fragment allele and two smaller fragments allele. Details of PCR reactions are available in Paper II.

4.3.2 Firmness measurements and data analysis

For all cultivars, fruit firmness at harvest, amount of fruit softening per week of storage and harvest date (maturity) were calculated as described in 4.2.1. PLS-DA was used to investigate the ability of the different loci of four tested genes, firmness and harvest date (as explanatory variables) to explain the variation observed in softening rate (as response variable).

4.4 Chemical analysis

In this section of the thesis, selected chemical compounds of apple fruits with a potential impact on resistance to blue mold were investigated in two years, 2012 and 2013.

4.4.1 Sample preparation

Fruits of 20 cultivars were harvested at the pre-climacteric stage in 2012, inoculated and stored as described earlier in 4.2.1. Non-inoculated fruits were stored under the same conditions and used as control. All procedures were repeated for 13 cultivars in 2013 (nine cultivars were included in both years). Lesion decay was measured at each inoculation point, and the average value was calculated for each cultivar and divided by number of weeks in storage. For chemical analyses, peel and flesh were sampled separately from one pool of 10 inoculated fruits and from another pool of 10 control fruits. The pooled

samples were extracted by 80% methanol and 1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) and filtered (Figure 8) and stored at -20 °C until analysis.

4.4.2 Chemical compounds analyses

For total titratable acidity (TTA), flesh and peel samples were, respectively, titrated by 0.1 M and 0.01 M NaOH to pH 8.4 by an automated titrator and a SAC-80 sample changer controlled by a TIM-90 titration controller (Radiometer Analytical Inc., Copenhagen, Denmark), and the results were expressed as mL of 0.1 M NaOH per mL of extract. Malic acid (MAL) was quantified as the major organic acid according to an HPLC method with UV-Vis detector (Rumpunen *et al*., 2002). Content of total phenols (TPH) was quantified according to the Folin-Ciocalteu method (Singleton *et al*., 1999), and individual phenolic compounds were measured with HPLC using a diode array detector (DAD) according to (Gao *et al*., 2000) with minor modifications (Roen *et al*., 2009). All samples were analyzed in triplicates. The details of each procedure are described in Paper V.

Figure 8. Preparation of samples for chemical analyses. Fruit flesh samples were ground in an extraction solution (left) and then centrifuged and filtered through 0.45 μm membranes (right) prior to injection into the HPLC systems. Photos by M. Ahmadi-Afzadi.

4.4.3 Data analysis

ANOVA was used to investigate variation among cultivars for each chemical compound. A series of Pearson correlation analyses were used to investigate associations between the content of different chemical compounds on the one

hand, and lesion diameter (indicating the level of susceptibility to blue mold) on the other hand. All statistical analyses were performed using R version 3.0.2 (R Core Team, 2013). In addition, multivariate analyses including Partial Least Squares (PLS) analysis, Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were conducted to study the impact of explanatory variables (i.e. all measured chemical compounds) on variation in the response variable (lesion diameter) using Unscrambler® X10.3.

4.5 Gene expression analysis

To study the regulation of genes upon infection by blue mold, an AryANE chip covering 60K apple transcripts was used with samples of two resistant ('Delorina' and 'Reglindis') and two susceptible cultivars ('Ella' and 'Linda') collected at three time points, i.e. one hour post inoculation (1 hpi), one week (1wpi) and six weeks (6 wpi) post inoculation.

4.5.1 RNA extraction, cDNA synthesis and microarray hybridization

Total RNA was extracted from frozen samples as described by (Gasic *et al*., 2004) with some modifications. All RNA samples were checked for quality and quantity, and then reverse transcribed using MessageAmp™ II aRNA amplification kit (Ambion™) and then purified and labeled with Cyanine-3 (Cy3) or Cyanine-5 (Cy5). Labeled samples were loaded to an AryANE 12x135K containing 135,000 60-mer oligoprobes synthesized by NimbleGen (Madison, WI, USA) (Celton *et al*., 2014), and hybridized for 16 h at 42 °C. The results of the microarray study were validated by qRT-PCR. For details of the procedures see Paper VI.

4.5.2 Inoculation and hormone treatment

The regulation of identified candidate genes was explored across a wide variety of biological experiments in Arabidopsis by the Genevestigator plant biology tool (https://www.genevestigator.com). Based on the differential regulation of genes, two hormones (methyl jasmonate and salicylic acid) were used to study the inhibitory impact of these hormones on lesion decay development in 10 cultivars in 2014. Fruits were sprayed with either MeJA, SA or control (Figure 9) and stored for 24 hours and then inoculated with spores of *P*. *expansum* as described in 4.2.1. Lesion diameter was evaluated after six weeks of storage.

4.5.3 Statistical analysis

The inoculation and hormone-treatment data (expressed as average lesion diameter) were subjected to ANOVA with general linear model (GLM), and

subjected to post-hoc comparison analysis with Tukey's HSD tests ($\alpha = 0.05$). For microarray analysis, signal intensity data were then extracted from image files and checked for quality control and normalized by Lowess normalization method, and LIMMA package (Smyth, 2005) was used for differential expression analysis and data treatment according to Celton *et al*. (2014) using R version 3.0.2 (R Core Team, 2013). For each cultivar, genes were regarded as significantly differentially expressed when $P < 0.01$ and FDR < 0.05 and absolute fold change ≥ 2. Moreover, candidate differentially expressed genes were identified by comparing regulation of genes between susceptible and resistant cultivars.

Figure 9. Fruits of resistant and susceptible cultivars for microarray analysis (left) and spraying the fruits with hormones MeJA and SA for validation of identified genes in microarray experiment (right). Photos by M. Ahmadi-Afzadi.

4.5.4 HCA and gene function analyses

Hierarchical Cluster Analysis (HCA) was performed with normalized $\log_2$ ratios using Genesis ver. 1.7.6 (Sturn *et al*., 2002), and functional analysis using the gene ontology (GO) terms from Genome Database for Rosaceae (GDR) (Velasco *et al*., 2010) was performed. Gene enrichment analysis using AgriGO (Du *et al*., 2010), gene network analysis using ReviGO (Supek *et al*., 2011) and visualization of gene network using Cytoscape ver. 2.8.3 (Shannon *et al*., 2003) were also performed.

5 Results and discussion

5.1 Inter-cultivar genetic variation and impact of harvest date and fruit traits on disease resistance

Genetic variation is an essential part of all plant breeding programs. Identification and utilization of disease resistant material has thus proven valuable in the improvement of many different crops. This approach could also be very useful in the breeding of apple cultivars with improved resistance against fungal storage diseases. In order to identify useful sources of resistance, reliable evaluation data are needed. In Paper I, the phenotypic and genotypic variation of apple cultivars in response to several diseases was investigated and discussed. Results of inoculation-based trials in Germany, Switzerland, Belgium and Sweden thus revealed a wide range of tolerance and susceptibility to fire blight, scab, blue mold and powdery mildew (Paper I). However, in this thesis only the results of the blue mold investigation in Sweden will be focused.

Inoculation-based studies of a large number of apple cultivars during three years in Sweden (Balsgård) revealed very variable levels of susceptibility to blue mold (Figures 1 and 3 in Paper III and Figure 2 in Paper IV). Corresponding experiments in Norway (Njøs) produced a similar result (Figure 1 in Paper IV). In addition, inoculation of a large number of cultivars with *C. gloeosporioides* also revealed significant variation among cultivars (Figure 2 in Paper III). Data for year 2010 and year 2012 at Balsgård were significantly correlated (r = 0.42, P = 0.02, df = 8) and between 2012 and 2013 (r = 0.50, P < 0.001, df = 41). A similar result was obtained when data for 2012 and 2013 at Njøs were compared (r = 0.48, P value < 0.001, df = 43), indicating good reproducibility of data.

Tolerance to blue mold was negatively associated with the harvest date in both locations and years, especially when the lesion decay data of late-ripening

cultivars were associated to the harvest date, indicating that the tolerance to *P. expansum* increases with later harvest date (Table 1 and 2 in Paper III and Table 4 in Paper IV).

The impact of fruit characteristics, e.g. firmness at harvest and softening rate, i.e. loss of firmness during the storage period, on the susceptibility to blue mold and bitter rot was investigated. Fruit firmness had, on the whole, a negative impact on decay development whereas softening rate was positively associated to the decay development.

Moreover, a negative relationship was found between harvest time ('maturation' in Paper II) and softening rate when the data of 127 cultivars were analyzed, suggesting that later-ripening cultivars have a tendency to keep their firmness better than early-ripening cultivars (Papers II). This finding together with the results of Paper III suggests that cultivars with a higher firmness at harvest that tend to keep their firmness during storage (lower softening rate) are more tolerant to blue mold and bitter rot.

DNA analyses showed that only eight of 127 investigated cultivars are homozygous for the desirable allele 2 (low ethylene) in the *Md-ACS1* locus. As expected, cultivars that are homozygous for allele 1 (normal ethylene) had a higher softening rate than heterozygous and those that are homozygous for allele 2 (Paper II). For the *Md-ACO1* locus, the majority of cultivars were homozygous for the undesirable allele 2 (high ethylene), and no association was found between softening rate and allele configuration. For the *Md-Exp7* locus, two allele configurations, 202:202 and 198:202 (allele 198: firm fruit, allele 202: intermediate), were the most common. Unexpectedly, both firmness at harvest and softening rate were significantly lower in 202:202 genotypes compared to in 198:202 genotypes. For the *Md-PG1* locus, the majority of the cultivars were homozygous for the undesirable allele (regular softening), and only 11 cultivars were heterozygous and none was homozygous for desirable allele (lower softening). No association was found between softening rate and allele configuration in the *Md-PG1* locus in early-ripening cultivars whereas a significantly lower softening rate was found in heterozygous cultivars compared to homozygous cultivars in late-ripening cultivars. In summary, the four analysed loci explained only a minor part of the inter-cultivar variation in initial firmness and softening rate, while the three postharvest factors (harvest time, storage time and initial firmness) explained a larger part of the variation in softening rate.

5.2 Impact of contents of chemical compounds in flesh and peel of fruits on blue mold resistance

The chemical composition of fruit is expected to contribute to disease tolerance. Changes in fruit biochemical composition, e.g. organic acid degradation and acidification (Vilanova *et al*., 2014a; Prusky *et al*., 2004) or accumulation of pre-formed compounds like polyphenolic compounds (Mikulic-Petkovsek *et al*., 2011; Schovankova & Opatova, 2011; Mikulic-Petkovsek *et al*., 2008) have already been reported. Therefore, fruit chemical compounds, e.g. TTA, MAL, TPH and several individual phenolic compounds were investigated in the flesh and peel of several apple cultivars upon inoculation by blue mold. Moreover, to investigate possible associations between content of these compounds and the level of blue mold susceptibility (estimated as lesion diameter), a series of correlation analyses were performed across all cultivars.

Contents of all studied compounds varied significantly among different cultivars indicating large genetic variation in apple germplasm. In terms of acids content, the flesh samples had higher level of both TTA and MAL in comparison with the peel samples (Table 1 in Paper V). A strong influence of these two chemicals on the level of susceptibility to blue mold was, however, not indicated (Table 2). Still, the positive but non-significant (close to significance threshold of 0.05) correlation found between the acidity level and susceptibility to blue mold may indicate that cultivars with comparatively high MA and TTA, i.e. lower pH, could provide a better substrate for the fungus. A general acidification may occur in fruits after infection by *P*. *expansum* (Vilanova *et al*., 2014a), which has been related to accumulation of gluconic acid produced by this fungus in order to acidify the fruit environment (Prusky *et al*., 2004) and facilitate the activation of fungal enzymes to degrade the fruit cell wall, e.g. polygalacturonase (Barad *et al*., 2012; Hadas *et al*., 2007). In addition, it has been reported that development of *P*. *expansum* could be reduced by local alkalinization with $NaHCO_3$ (Prusky *et al*., 2004).

Content of total phenolic compounds was considerably higher in the peel samples than in samples of fruit flesh (Table 1 in Paper V). Several other studies have also reported this finding (Schovankova & Opatova, 2011; Roen *et al*., 2009; Khanizadeh *et al*., 2008; Escarpa & Gonzalez, 1998). Although correlation study did not produce significant association between level of susceptibility and total phenols in the control samples of flesh and peel, consistently negative correlations were obtained (Table 2). Correlations were, however, higher when samples from inoculated fruits were analysed instead (Table 2).

In terms of individual polyphenolic compounds, ten major compounds (listed in Table 2) were quantified in the fruit samples. Chlorogenic acid was the most abundant compound in the flesh samples whereas different quercetin glycosides (flavonols) were the most abundant compounds in samples of fruit peel (Table 3 in Paper V). Among all individual polyphenolic compounds, flavonols and procyanidin B2 produced the most consistent and highly negative association with the level of susceptibility (Table 2). Polyphenolic compounds have been suggested to contribute in the plant defense mechanism against apple scab disease in several studies (Slatnar *et al.*, 2012; Mikulic-Petkovsek *et al.*, 2011; Mikulic-Petkovsek *et al.*, 2008; Mayr *et al.*, 1997). Phenylalanine-ammonia lyase (PAL) is an important enzyme in the flavonoid biosynthesis pathway and could be activated due to fungal attack (Mikulic-Petkovsek *et al.*, 2011; Schovankova & Opatova, 2011; Lattanzio *et al.*, 2006), thereby influencing the biochemical composition of different fruit parts.

Partial least square analysis also confirmed that total phenols and procyanidin B2 in the flesh samples have the largest contribution in explaining the variation in level of susceptibility to blue mold, whereas flavonols followed by total phenols and procyanidin B2 had the largest impact on disease susceptibility in samples of fruit peel (Figure 10).
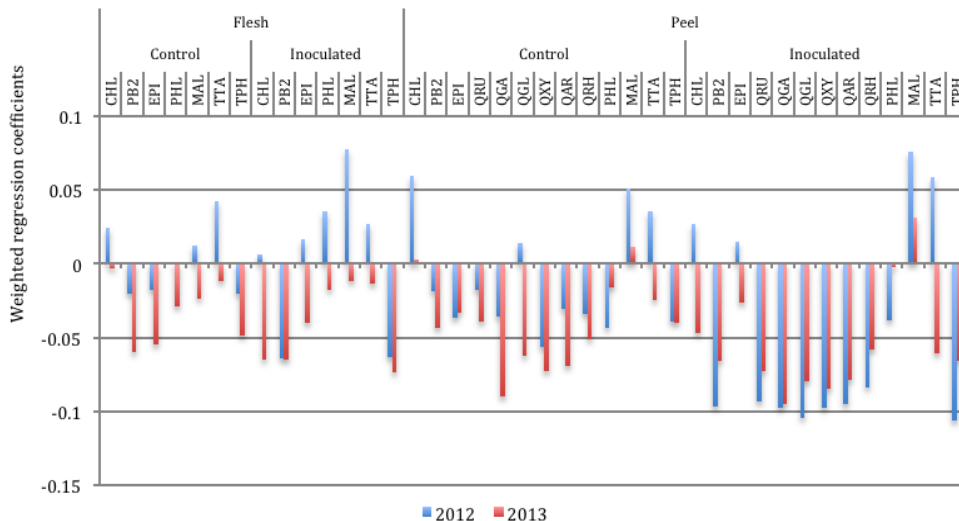

Figure 10. Partial least square (PLS) regression analysis showing the impact of chemical compounds in flesh and peel of control and inoculated fruits on level of susceptibility (lesion diameter data), in 2012 and 2013. PLS data in 2012 and 2013 are highly correlated (r = 0.74, P < 0.001).

Table 2. *Pearson correlation between level of susceptibility to blue mold and contents of different chemicals in flesh and peel in 2012 and 2013.*

Compound	Abbreviation	2012				2013			
		Flesh		Peel		Flesh		Peel	
		Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
Total titratable acidity	TTA	0.28 ns	0.17 ns	0.23 ns	0.38 ns	-0.09 ns	-0.1 ns	0.05 ns	0.36 ns
Malic acid	MAL	0.08 ns	0.50 *	0.32 ns	0.48 *	-0.18 ns	-0.09 ns	0.09 ns	0.24 ns
Total phenols	TPH	-0.13 ns	-0.41 ns	-0.26 ns	-0.70 **	-0.38 ns	-0.57 *	-0.31 ns	-0.51 ns
Hydroxycinnamic acids	CHL	0.16 ns	0.04 ns	0.28 ns	0.02 ns	-0.02 ns	-0.50 ns	0.02 ns	-0.37 ns
Flavanols	PB2	-0.13 ns	-0.40 ns	-0.09 ns	-0.60 **	-0.46 ns	-0.70 **	-0.34 ns	-0.51 ns
	EPI	-0.11 ns	0.11 ns	-0.23 ns	0.03 ns	-0.42 ns	-0.30 ns	-0.26 ns	-0.21 ns
	QRU	–	–	-0.20 ns	-0.62 **	–	–	-0.31 ns	-0.56 *
	QGA	–	–	-0.26 ns	-0.61 **	–	–	-0.70 **	-0.74 **
	QGL	–	–	-0.07 ns	-0.69 **	–	–	-0.48 ns	-0.61 *
Flavonols	QXY	–	–	-0.38 ns	-0.62 **	–	–	-0.56 *	-0.66 **
	QAR	–	–	-0.23 ns	-0.60 **	–	–	-0.53 ns	-0.61 *
	QRH	–	–	-0.27 ns	-0.55 *	–	–	-0.40 ns	-0.45 ns
Dihydrochalcones	PHL	-0.002 ns	0.23 ns	-0.33 ns	-0.28 ns	-0.23ns	-0.14 ns	-0.13 ns	-0.12 ns

ns, not significant; * P<0.05; ** P<0.01; – below level of detection.

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Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed using all chemical contents in the peel of inoculated fruit. HCA could separate three comparatively resistant cultivars, 'Olga', 'Tönnes' and 'Gloster', from the remaining cultivars. Highly and intermediately susceptible cultivars were also grouped into different clusters (Figure 2A and B in Paper V). According to the PCA analysis, up to 72% of the variation in lesion diameter could be explained by the two first PCs, with flavonols, procyanidin B2 and total phenols being the main contributors to PC1 (Figure 3A–D in Paper V).

5.3 Gene regulation in fruits of susceptible and resistant cultivars in response to blue mold infection

Gene expression of two resistant and two susceptible cultivars was investigated in order to identify candidate genes and possible pathways involved in resistance to blue mold. The highest number of differentially expressed genes was obtained in samples at six and one wpi, whereas very few genes were differentially regulated at one hpi (Figure 1 and Table 1 in Paper VI). Vilanova *et al*. (2014b) reported that the expression of four investigated genes (*PAL1*, *PAL2*, *POX2* and *COMT2*) in apple fruits inoculated with *P*. *expansum* and *P*. *digitatum* did not differ until 8 h after infection, and the differences in gene expression became significant at 24 hpi, while the greatest differences were obtained at 48 hpi.

Comparison of gene expression between resistant and susceptible cultivars resulted in 1,382 differentially expressed genes associated with important functional pathways, e.g. defense and stimulus response, transport, proteolysis, phosphorylation and cell wall-related processes (Figures 2 and 3 in Paper VI). Gene network analyses using enriched gene ontology (GO) terms revealed that metabolic processes are connected to the stress and defense response processes through a signal transduction process and then to localization and transport processes (Figure 11). In addition, several genes associated with TIR-NBS-LRR proteins were differentially expressed between resistant and susceptible cultivars. These TIR-NBS-LRR proteins belong to the PR proteins and are commonly produced by plants in response to pathogen infection (Sarowar *et al*., 2011; McHale *et al*., 2006; Venisse *et al*., 2002).
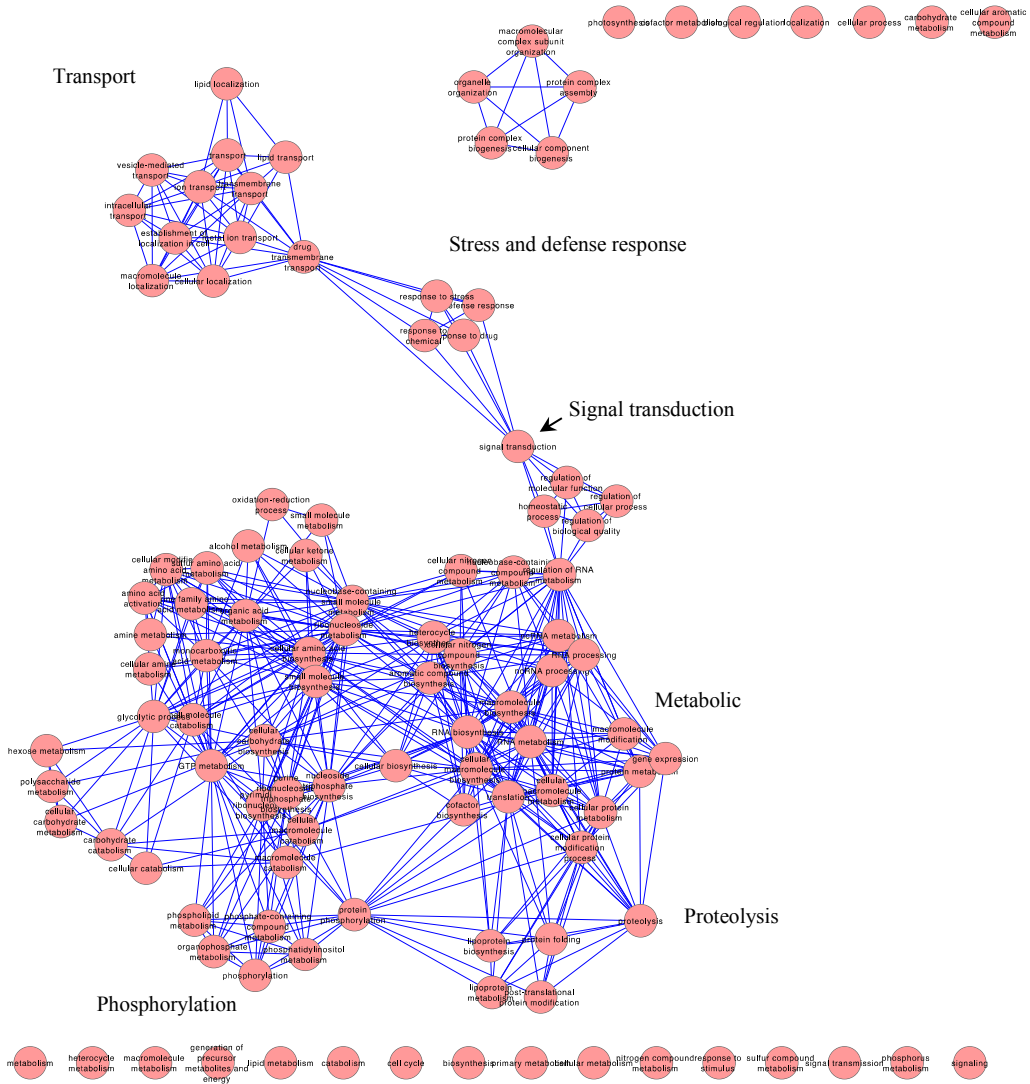
Figure 11. Gene network analysis using enriched gene ontology (GO) terms based on semantic similarities. Metabolic processes are the major cluster of genes and connected to defense response genes via a signal transduction process.

Several genes involved in the flavonoid biosynthesis pathway (Figure 12) were differentially regulated and induced in resistant cultivars, e.g. flavonol synthase (*FLS*), two chalcone synthases (*CHS*) and four leucoanthocyanidin dioxygenases (*LDOX*). The *FLS* gene regulates the synthesis of flavonols like kaempferol and quercetin, *CHS* catalyzes the production of chalcones and naringenin chalcones, and *LDOX* catalyzes the cyanidins (Winkel-Shirley, 2001; Pelletier *et al*., 1999).

Expression of *PAL* genes was induced in all cultivars. The *PAL* gene contributes to the first step of flavonoid biosynthesis in the phenylpropanoid pathway converting phenylalanine into cinnamic acid (Figure 12). Therefore, we hypothesize that although the flavonoid biosynthesis is induced through the *PAL* gene upon fungal attack in all cultivars, induction of other genes like *CHS*, *FLS* and *LDOX* synthesizing down-stream molecules (naringenin chalcone, quercetin and anthocyanidins) in the flavonoid pathway have a stronger impact on the differences between resistant and susceptible cultivars. The chemical analyses also showed that content of polyphenols in apple skin, in particular different quercetins, is positively associated with the level of resistance to blue mold infection (see section 5.2).

Several cell wall-associated genes, e.g. *PGIP*, *TT10*, *WAK1* and *CTL1* (*POM1*) were differentially regulated between resistant and susceptible cultivars. Changes in the plant cell wall are major factors in ripening and postharvest fruit softening (Mann *et al*., 2008; Johnston *et al*., 2002), and they can contribute to the level of susceptibility to fungal diseases. Thus, fruits with a firmer texture can be expected to show higher tolerance to fungal infection (see section 5.1). In addition, any changes in the cell wall that can improve its structure, e.g. substrate generation, glycosyl transferase activities, cell wall disassembly or lignification may enhance disease resistance to fungal infection (Zhu *et al*., 2012; Bhuiyan *et al*., 2009). Lignification of tissue surrounding the wound site has been reported to play an important role in the resistance mechanism (Vilanova *et al*., 2012).

Microarray results showed that several genes associated with phosphorylation (like serine/threonine kinases and receptor-like protein kinases), proteolysis activity (like serine carboxypeptidase and cysteine proteinase) and transportation (like lipid transfer protein and zinc transporter) are also differentially regulated between resistant and susceptible cultivars. Detailed lists of differentially expressed genes are present in additional files in Paper VI.
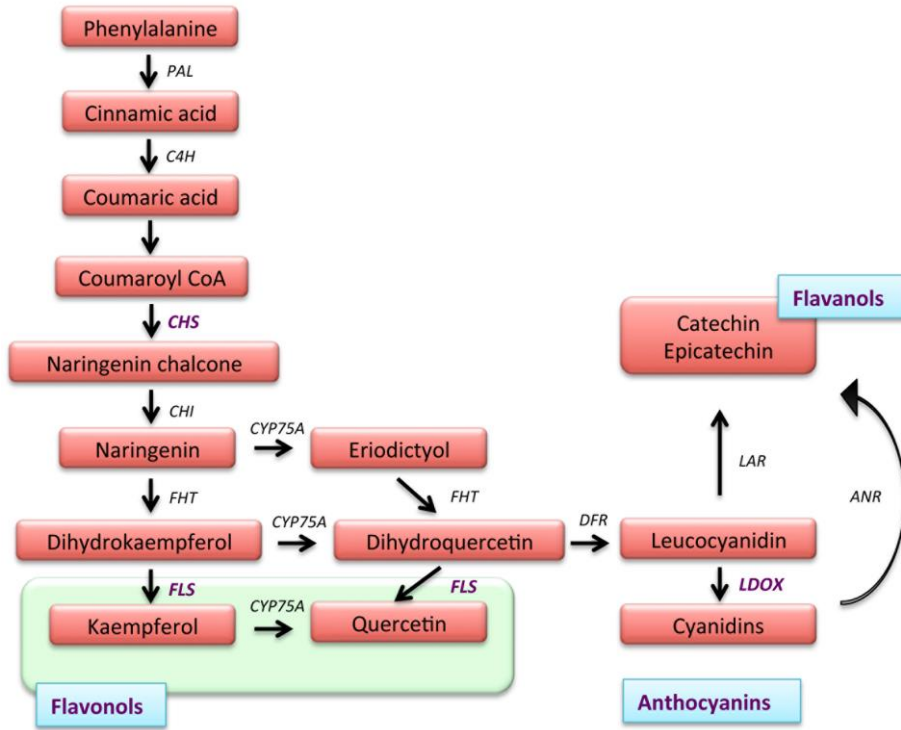
Phenylalanine

↓ PAL

Cinnamic acid

↓ C4H

Coumaric acid

↓

Coumaroyl CoA

↓ CHS

Naringenin chalcone

↓ CHI

Naringenin → CYP75A → Eriodictyol

↓ FHT ↓ FHT

Dihydrokaempferol → CYP75A → Dihydroquercetin → DFR → Leucocyanidin

↓ FLS ↓ FLS ↓ LDOX

Kaempferol → CYP75A → Quercetin   Cyanidins

Flavonols

Catechin Epicatechin — Flavanols

↑ LAR   ANR

Anthocyanins

Figure 12. Simplified and schematic representation of flavonoid biosynthesis in plants. Figure is drown according to KEGG pathway (Kanehisa & Goto, 2000).

Genevestigator analysis using genes identified in a wide range of Arabidopsis experiments showed that MeJA could limit fungal development. In keeping with this result, exogenous application of MeJA significantly reduced lesion diameter on fruits inoculated with *P*. *expansum* in comparison with inoculated and water-DMSO treated fruits (Figure 13). The maximum inhibitory impact was obtained on fruits of 'Prima' (60%) and 'Ella' (51%). By contrast, impact of SA appeared to be cultivar-dependent, since lesion development was limited in only four of the investigated cultivars (Figure 14). A positive impact of MeJA on enhancing disease resistance to *Monilia fructicola* and *P*. *expansum* infection on sweet cherry and peach fruit has been reported in previous studies (Yao & Tian, 2005b; Yao & Tian, 2005a). Based on our results, we believe that exogenous application of MeJA hormone could trigger defense-related mechanisms in fruits that enhance the disease resistance

to fungus. However, the specific regulation of our candidate genes in response to MeJA needs to be further studied.
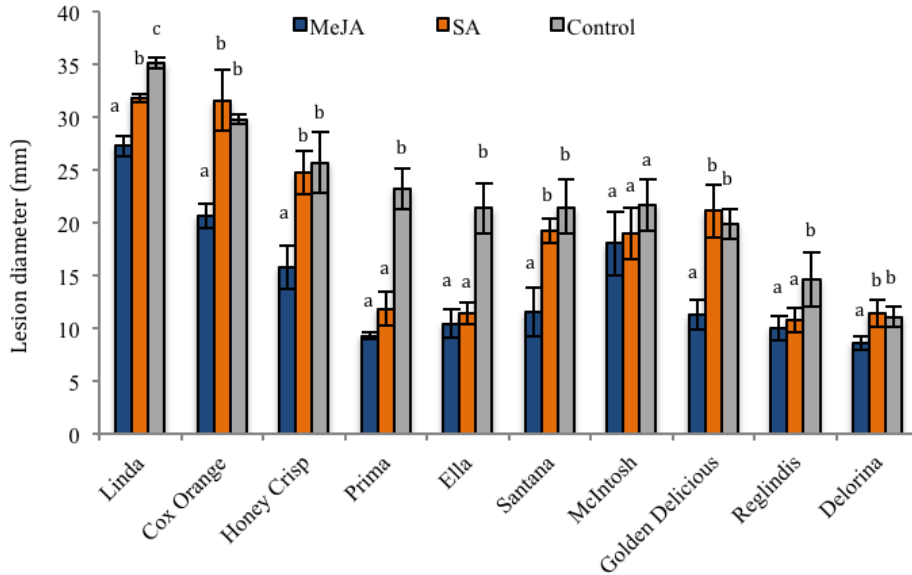

Figure 13. Exogenous application of MeJA and SA hormones on fruits inoculated with *Penicillium expansum*. Bars represent mean ± SD of lesion diameter (n = 30). For each cultivar, treatments with the same letters are not significantly different (Tukey method, α = 0.05).

6   Conclusions and future perspectives

In the present thesis, different strategies and techniques, i.e. inoculation-based screening of disease resistance, DNA-based analysis of fruit texture genes, chemical components investigation and finally studies of gene regulation were implemented in order to understand the mechanism of disease resistance to fungal infection by *P*. *expansum*. Our findings, on the whole, can conclude that:

- Apple cultivars differ considerably in terms of level of resistance to blue mold, suggesting that carefully selected material could be a valuable source for breeding programs.

- Level of resistance to blue mold is associated with several fruit texture characteristics, thus cultivars with moderate to firm fruits that soften comparatively little during storage could withstand the fungal infection comparatively well. In addition, harvest date is positively associated with firmness at harvest and negatively associated with fruit softening; very early-ripening cultivars are, overall, less resistant to infection.

- Four fruit texture-related alleles (*Md-ACO1*, *Md-ACS1*, *Md-Exp7* and *Md-PG1*) are associated with higher firmness at harvest and less softening during storage, but their predictive power was low for the observed variation for these variables. However, further research is needed on genes that affect maturation time since this parameter plays a very important role in explaining the variability in both firmness at harvest and softening rate.

- Content of the investigated chemical compounds varied considerably among different cultivars. Some of these chemical compounds, especially flavonols and PB2, also appear to contribute to resistance against blue mold. The importance of these compounds is especially noticeable in the apple peel where polyphenol contents are higher than in the fruit flesh.

- By contrast, contents of malic acids or total titratable acidity had much less impact on the resistance of fruits to blue mold.

- Numerous genes are induced after *Penicillium* infection, in fruits of all investigated cultivars. Several of these genes are differentially expressed between resistant and susceptible cultivars, and some of them are likely to contribute to functional biosynthetic pathways with an important role in the defense mechanism.

- Genes involved in processes related to cell wall structure, lignification and phosphorylation, transportation, biosynthesis of flavonoids as well as several known defense genes are likely to be responsible for differences between resistant and susceptible cultivars.

- Identification of *FLS*, *LDOX* and *CHS* genes, that synthesize polyphenolic compounds like quercetins and cyanidins, may reveal the role of these compounds in contributing to disease resistance.

- In addition, identification of several cell-wall related genes (*PGIP*, *TT10*, *WAK1* and *CTL1*) indicate that the cell wall is an important component in the resistance to blue mold. This finding is linked to the above-mentioned association between fruit firmness and softening rate with level of resistance.

- Exogenous application of methyl jasmonate hormone could inhibit the development of blue mold by enhancing the defense mechanism in apple fruit.

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