Candidate genes for resistance and susceptibility to the bird cherry-oat aphid (Rhopalosiphum padi L.) in barley (Hordeum vulgare L.)

Sara Mehrabi
Candidate genes for resistance and susceptibility to the bird cherry-oat aphid (*Rhopalosiphum padi* L.) in barley (*Hordeum vulgare* L.)

Sara Mehrabi
“Dedicated to my beloved parents”
Sammanfattning


cDNA för protashämmaren överfördes till backtrav med kontroll av två olika promotorer, varav den ena ger genuttryck i all växtväv och den andra särskilt i floemväv. Havrebladlus kan inte överleva på backtrav, och testen utfördes därför med persikbladlus (*Myzus persicae* Sulzer). Persikbladlus
kan till skillnad från havrebladlus livnära sig på många olika arter, både tvåhjärtbladiga (som backtrav) och enhjärtbladiga (som korn). Resultaten visade att persikbladlusen föredrog att äta från och producerade större antal avkomma på kontrollplantor jämfört med flera av linjerna som uttryckte genen för proteashämmaren.

Sammanfattningsvis ger denna avhandling stöd för idéer om att ett tioxin och en proteashämmare kan vara involverade i resistens mot havrebladlus och att ett lipoxygenas och två glukanaser kan påverka mottagligheten för havrebladlus positivt. Studien visar vidare att cDNA från korn som valts ut på grund av att det induceras i en enhjärtbladig växt av en tämligen specialiserad bladlus kan påverka resistensen mot en helt annan bladlusart i en tvåhjärtbladig växt.
List of papers

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


IV. Mehrabi S, Beste L, Stephens J, Jonsson LMV. Aphid performance on Arabidopsis and barley overexpressing an open reading frame, up-regulated in Rhopalosiphum padi resistant barley lines during aphid infestation. (Manuscript).

Paper I and II are reproduced with the permission of their respective publisher.
My contributions to the papers:

Paper I: Participated in the experimental design, performed the experiments and the analysis of the data. Contributed to writing the paper.

Paper II: Was main responsible for the experimental design, performed the experiments and the analysis of the data. Contributed to writing the paper.

Paper III: Participated in the experimental design and in performing the experiments. Commented on the manuscript.

Paper IV: Was main responsible for the experimental design, performed the experiments and the analysis of the data, except for cloning and transformation. Wrote the paper in cooperation with co-authors.
Contents

General introduction.................................................................................................................. 12
  Barley – an ancient agricultural important crop ................................................................. 12
  Aphids as pests ..................................................................................................................... 13
    General background .......................................................................................................... 13
    Aphid damage to plants .................................................................................................... 14
    Bird cherry-oat aphid (R. padi) ....................................................................................... 15
  Pest management ................................................................................................................. 16
  Resistance breeding ............................................................................................................ 17
    Constitutive and induced plant defense ........................................................................... 17
    Morphological and surface barriers ................................................................................. 18
    Secondary metabolites ...................................................................................................... 19
    Induced defense mechanisms and different types of resistance ....................................... 20
  Breeding methods ............................................................................................................... 22
  Plant material used in the study .......................................................................................... 23
  Main approaches in this thesis ............................................................................................ 25
    Constitutive gene expression in a large number of barley genotypes with known resistance levels to R. padi (Paper I) ............................................................................. 25
    Aphid-induced gene expression of putative susceptibility factors in a large number of barley genotypes with known resistance levels to R. padi (Paper II) ................. 25
    Aphid behavior and performance on transgenic Arabidopsis with a putative aphid resistance sequence from barley (Paper III) .............................................................. 27
    Aphid behavior and performance on transgenic Arabidopsis and barley with a putative aphid resistance sequence from barley (Paper IV) ............................................ 29
  Main results and discussion ............................................................................................... 31
  General discussion about methods .................................................................................... 33
  Conclusions ......................................................................................................................... 37
  Suggestions for further studies ......................................................................................... 38
  Acknowledgements ............................................................................................................ 39
  References .......................................................................................................................... 40
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>Back-cross</td>
</tr>
<tr>
<td>BCI</td>
<td>Barley Chemically Induced</td>
</tr>
<tr>
<td>BYDV</td>
<td>Barley yellow dwarf virus</td>
</tr>
<tr>
<td>Col-0</td>
<td>Columbia</td>
</tr>
<tr>
<td>F1</td>
<td>First generation of offspring after crossing</td>
</tr>
<tr>
<td>HAMPs</td>
<td>Herbivore-associated molecular patterns</td>
</tr>
<tr>
<td>Hsp5</td>
<td><em>Hordeum vulgare subspecies spontaneum</em> accession 5</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>MAMPs</td>
<td>Microbe-associated molecular patterns</td>
</tr>
<tr>
<td>NBS-LRR</td>
<td>Nucleotide-binding site leucine-rich repeat</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PI</td>
<td>Proteinase inhibitor (paper I) = BCI-7 (paper III)</td>
</tr>
<tr>
<td>PI (s)</td>
<td>Proteinase inhibitor (s)</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Resistant</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA-mediated interference</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>S</td>
<td>Susceptible</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>STK</td>
<td>Putative serine/threonine kinase sequence (paper I) = (contig16360_at, paper IV)</td>
</tr>
<tr>
<td>THIO</td>
<td>Thionin</td>
</tr>
<tr>
<td>VIGS</td>
<td>Virus induced gene silencing</td>
</tr>
<tr>
<td>βGLU-GIII</td>
<td>beta-1,3-glucanase III (paper I) = (contig1636_at, paper II)</td>
</tr>
</tbody>
</table>
General introduction

The overall objective of this thesis was to identify genes related to resistance or susceptibility towards the bird cherry-oat aphid (*Rhopalosiphum padi* L.) in barley (*Hordeum vulgare* L.).

Aphids are a major threat to crop production reducing the yield if not controlled. *R. padi* attacks all small grain cereals including e.g. wheat, triticale and barley (Blackman and Eastop 2007). According to the Food and Agriculture Organization of the United Nations statistics in 2013, barley had the fourth rank in the world cereal production after maize, rice and wheat (FAOSTAT 2013). Consequently, sustaining high yields of barley under conditions of abiotic and biotic stresses is needed (Maheswari et al. 2012).

Aphids are the most important insect pests on barley and damage the plants both directly by feeding and indirectly by transmitting viral diseases (Mornhinweg 2010). Chemical control is the most efficient and widely used management tool to control aphids but using insecticides has negative environmental consequences and causes insecticide resistance in the aphids. As an example, resistance against the major insecticides has developed in the green peach aphid (*Myzus persicae* Sulzer) worldwide (Radcliffe et al. 2007).

An alternative to the chemical control of aphids is to breed for aphid host plant resistance. The work in this thesis aims to identify resistance or susceptibility factors in barley. This is done by the study of constitutive and induced gene expression in barley genotypes with different degree of resistance against *R. padi*, and by transformation of candidate resistance sequences and evaluating their effects on aphids in barley and Arabidopsis.

Barley – an ancient agricultural important crop

Barley belongs to the tribe Triticeae and the family Poaceae. *Hordeum vulgare* ssp. *spontaneum* (wild barley) is considered as the ancestor of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) (von Bothmer and Komatsuda 2010). There are two- and six-rowed barley varieties as well as winter and spring types. Archaeological evidence shows that the domestication of barley started about 10,000 years ago in what is called “the fertile crescent”, that is the region in the Middle East, with a curved shape from the Persian Gulf to the Mediterranean Sea. Barley is a diploid (2n=14), self-pollinated
plant with a simpler genome compared to the hexaploid wheat and it has been argued that it is a very good model organism in molecular research for bread wheat (Schulte et al. 2009). The barley genome was shotgun sequenced recently and its genome size was reported to be about 5.1 gigabases and contain 85% repetitive elements (International Barley Genome Sequencing Consortium 2012). This advancement is a significant help to develop new cultivars of barley, which are able to withstand pests and diseases.

Barley is used as animal feed, in beverage production and as human food. Barley is valuable in the production of malt that is mainly used in the beer and beer-like beverage industry and to a lesser extent in baking, distilled beverage production, fuel (ethanol) and confectionary industry. As a whole grain food, various studies have put forward positive health effects, i.e. that the consumption could reduce blood cholesterol (Åman 2006), and blood glucose (Beta et al. 2012).

Barley is attacked by more than 100 arthropod pests and many of them cause economic damage in outbreak years, such as Russian wheat aphid (*Diuraphis noxia* Mordvilko), bird cherry-oat aphid (*R. padi*) and greenbug (*Schizaphis graminum* Rondani). Other serious pests on barley are the army cutworms (*Euxoa auxiliaris* Grote), grasshoppers (*Melanoplus sanguinipes* Stål) and wireworms (*Agriotes species*) (Mornhinweg 2010).

**Aphids as pests**

**General background**

Aphids are important and common pests in temperate regions. They are members of the superfamily Aphidoidea, suborder Sternorrhyncha, order Hemiptera. There are ca. 4700 aphid species, but only about 100 of them are considered as economically important on crop plants (Blackman and Eastop 2007). Aphids have soft bodies, vary in size between 1 to 10 mm and have different colors (yellow, green, red or black). They have slender piercing-sucking mouthparts and cornicles on the abdomen. The cornicles are tube-like structures, which release alarm pheromones after attack by natural enemies of aphids (Gullan and Martin 2009).

Aphids feed on plant phloem sap. They penetrate the epidermis with their stylet and push it through the parenchymatic tissue extracellularly until they reach the vascular bundles of the plant (Fig. 1). When the aphid stylet reaches the phloem, the aphid can feed on phloem sap for several hours and even days (Tjallingii 1995). As soon as an aphid stylet enters between the borders of two epidermal cells, saliva is secreted that jellifies and facilitates the stylet’s penetration of plant tissue. Watery saliva is released as the stylet
reaches the sieve element. This saliva has been shown to suppress protein clogging which would otherwise inhibit phloem transport and aphid feeding (Tjallingii 2006; Will et al. 2009). Aphid feeding causes the nutrients to flow into the aphid-infested tissue. As a consequence, the flow of nutrients into other sink areas such as the primary growth zone will be reduced (Mittler and Sylvester 1961). The phloem sap is very rich in sucrose and poor in amino acids, which are the main essential nutrients for aphids. Aphids cannot synthesize all their essential amino acids and are dependent on endosymbiotic bacteria in order to obtain those (Shigenobu et al. 2000). Occasionally aphids also ingest xylem sap.

Fig. 1 Aphid feeding on phloem sap. st: stylet, scl: sclerenchyma, p: phloem, x: xylem. Scale bar = 1 mm. The picture is adapted from Plants in Action, published by the Australian Society of Plant Scientists (http://plantsinaction.science.uq.edu.au/edition1).

Aphid damage to plants

Most of the crops in the world are attacked by at least one aphid species. Aphids cause asymptomatic (not visible) or symptomatic (visible) injury. Some species, including R. padi on cereals, pea aphid (Acyrthosiphon pisum Harris) on legumes and soybean aphid (Aphis glycines Matsumura) on soybean, do not cause visible damage but they can reduce plant growth. In contrast to the previous group, some species cause visible damage on plants including the Western wheat aphid (Diuraphis tritici Gillette) that causes
stunting, *D. noxia* that causes chlorosis, leaf rolling and stunting and *S. graminum* that causes necrosis in cereals (Quisenberry and Ni 2007).

Aphids are well-known as plant virus vectors and over 190 aphid species have been described as viral vectors (Katis et al. 2007). The barley yellow dwarf virus (BYDV) is a worldwide virus, infecting important cereals causing dwarfing and leaf discoloration. In years with heavy aphid infestation, BYDV leads to severe yield losses in cereals. Worldwide, most barley cultivars are susceptible to BYDV and the virus may reduce the plant yield by up to 46% (Schliephake et al. 2013). In addition to the above negative consequences, the sugary honeydew accumulating on leaves infested by aphids promotes fungal growth. Thereby, growth of saprophytic fungi can result in blocking of the stomata, reduction of the photosynthesis and earlier shedding of leaves (Morkunas et al. 2011).

**Bird cherry-oat aphid (R. padi)**

*R. padi* is one of the most serious pests on cereal crops and one of the five most important barley pests worldwide (Mornhinweg 2010). Its color is varying from green-olive to yellow-green, dark olive or greenish-black with a red-orange pigmentation around the bases of the posterior part of the abdomen in the apterous (wingless) individuals. *R. padi* can have an holocyclic life cycle with cyclic parthenogenetic reproduction on both a primary (winter) and a secondary (summer) host; or an anholocyclic life cycle with obligate parthenogenetic reproduction all year round on the secondary host in areas where the primary host is rare or during mild winters (Simon et al. 1996).

*R. padi* with holocyclic life cycle (Fig. 2) passes the winter in the form of eggs on bird cherry (*Prunus padus* L.). The eggs hatch in spring and after some apterous generations on the primary host, winged aphids develop that migrate to grasses (secondary hosts). Several apterous generations are born on the summer host. As the density of aphids increases or the nutrient source is depleted, winged aphids develop that migrate to new summer hosts. With the approach of autumn, winged females and males are born. The winged females (gynoparae) give birth to apterous egg-laying forms (oviparae) that mate with winged males and lay eggs on the winter hosts (Dixon 1971). As mentioned above, this aphid does not cause visible damage, but at high infestation rates, it can reduce the growth and yield by removal of plant nutrients (Dixon 1971; Mornhinweg 2010). It is also an efficient vector of most small grain viruses (e.g. BYDV) worldwide (Blackman and Eastop 2007, Mornhinweg 2010).
Fig. 2 *R. padi* holocyclic life cycle. Fundatrix is an apterous female that parthenogenetically (non-sexually) produces live offspring. Gynopara is a female winged aphid that migrates to the primary host and produces oviparae. Ovipara is an apterous sexual female that mates with a winged male aphid and lays eggs (Figure inspired by Dixon 1971).

**Pest management**

Insecticides are used extensively to control aphid infestation. However, there are disadvantages with this method, such as negative effects on the ecosystem, notably on non-target beneficial insects (predators, parasitoids and pollinators) and the risk of aphids developing resistance to the insecticides. There is a long list of pesticides active against aphids and new aphicides are being developed worldwide. In the 1990s, mostly organophosphates, carbamates and pyrethroid insecticides were used to control aphids and some of these insecticides have high toxicity to non-target insects (Dewar 2007). In the European Union, the regulations for the application of pesticides are becoming increasingly restrictive and e.g. in Sweden at
present there are few products available for farmers (Gustafsson 2015; Bekämpningsmedelsregistret 2016).

**Resistance breeding**

In view of the above situation, there is an urgent need to develop alternative ways to fight aphids, such as breeding for host plant resistance. Growing cultivars that are insect-resistant is an environmentally friendly, efficient and easy to use method for the farmer. The resistant traits can be classified as antixenosis, antibiosis and tolerance traits. Antixenosis or non-preference is when the herbivore avoids feeding or reproducing on the plant. Antibiosis is the negative effect of plant biology on pest physiology. It can result in the reduction of insect life span and reproduction as well as increasing the insect mortality. Tolerance is a mechanism that enables plants to withstand the herbivore, and recover from its damage and thus maintain biomass and yield production. The difference between antixenosis or antibiosis and tolerance is that the first two types of resistance measure the response of the insect to the plant, whereas tolerance measures the response of the plant to the insect (Smith 2005).

**Constitutive and induced plant defense**

When searching for resistance factors to be used in breeding, it is important to know the inherent defense mechanisms in plants. Although plants have a sedentary lifestyle, they have a dynamic interaction with insects (Mello and Silva-Filho 2002; Mescher and De Moraes 2015). Plants have both constitutive and inducible defense strategies against insect herbivores (Fig. 3). Constitutive defenses are including physical and chemical barriers that exist before insect attack, whereas induced defenses become active by insect attack. Induced plant defenses can be either direct or indirect. Direct defenses are the plant traits that affect herbivore biology directly, e.g. delaying their development or even killing them. Indirect defenses are when e.g. plants volatiles are induced which attract other organisms that may aid to suppress the growth of the herbivores (War et al. 2012; Fürstenberg-Hägg et al. 2013).
Fig. 3 Generalized scheme of plant defense reactions to insects, with aphids and ladybirds as examples of herbivores and insect natural enemies (Figure inspired by Mello and Silva-Filho 2002).

**Morphological and surface barriers**

Plant leaves and stems may carry trichomes, which can constitute a physical barrier against insects. Certain trichomes contain essential oils and other secondary metabolites that act as chemical defense (Wagner et al. 2004). Trichomes have been much studied in the context of insect defense and among them are some studies on their relevance in aphid defense. The results suggest that the effects of trichomes vary depending on the plant and aphid species. There are reports of negative effects of trichomes on aphid performance in wild species of *Lycopersicon* against *M. persicae* (Simmons et al. 2005) and against melon aphid on melon (Sarria et al. 2010), but there are also reports of no effects of trichome density in soybean upon the abundance of *A. glycines* (Dai et al. 2010). In barley, *R. padi* survival and nymphal growth was studied in a number of breeding lines of which some were densely hairy and others not at all hairy. There was no difference in the aphid parameters between these categories of lines, indicating that hairs are not important in barley against *R. padi* (Åhman et al. 2000).

The wax and cutin layers of the leaf are also considered as structural layers of defense. These components have been shown to reduce aphid population growth in some cases. As an example, English grain aphid (*Sitobion avenae* Fabricius) had longer non-probing periods on waxy compared to
waxless triticale plants and surface waxes from waxy plants had aphicidal activity against S. avenae (Wójcicka 2015).

**Secondary metabolites**

Plant secondary metabolites may be part of the defense against insect herbivores. Such metabolites accumulate constitutively or are induced after pathogen or herbivore attack. They may have different activities including toxicity, deterrence and repellence (Bennett and Wallsgrove 1994). Among the wide variety of secondary metabolites found in the plant world, there are some that have been found to play a role in the interaction between plants and aphids.

A basic idea concerning the role of secondary metabolites and plant defense is that they are toxic to generalist insects but that specialized insects have found ways to overcome their toxicity and use them to their own favor. Such insects may even need them as feeding or oviposition stimuli. This is nicely illustrated in the case of aphids on lupines (Lupinus angustifolius L.). Lupines contain quinolizidine alkaloids, but the contents vary between the cultivars. Generalist aphids, including M. persicae, feed on sweet lupines, which are almost alkaloid-free varieties, but avoid alkaloid-rich varieties. In contrast, the specialized lupine aphid (Macrosiphum albifrons Essig) is well adapted to lupines with high alkaloid content. This aphid stores the alkaloids and uses them for its defense against predators (Philippi et al. 2015). Similar relations have been shown in the case of the glucosinolates in the Brassicaceae and their associated aphids.

Glucosinolates are secondary metabolites restricted mainly to the Brassicaceae family. Upon tissue damage, glucosinolates are hydrolyzed by myrosinases into products, including isothiocyanates, which have toxic and deterrent effects on some herbivores (Halkier and Gershenzon 2006). There is much evidence that these compounds are deterrents and repellents towards generalist insects but may act as attractants or feeding and oviposition stimuli to specialist insects, including aphids (Bruce 2014). The specialist cabbage aphid (Brevicoryne brassicae L.) was shown to sequester glucosinolates from its host and to compartmentalize myrosinase into microbodies in the muscles, which in combination might help this aphid to defend itself against predators (Bridges et al. 2002).

Benzoazinoid hydroxamic acids constitute another group of secondary metabolites and are present in cereals including maize, wheat, rye and wild Hordeum species but not found in cultivated barley (Niemeyer 2009). There are reports of correlations between the performance of cereal aphids and the content of benzoazinoid hydroxamic acids in different wheat cultivars (Argandoña et al. 1980). Clear negative effects of the benzoazinoid 2, 4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one (DIMBOA) in maize have been reported on R. padi (Ahmad et al. 2011) and corn leaf aphid
(R. maidis Fitch) (Meihls et al. 2013), using genetically well-defined maize lines.

With regard to barley there are some studies on the indole alkaloid gramine indicating that it is a resistance factor against cereal aphids (Zúñiga et al. 1985; Zúñiga and Corcuera 1986). However later investigations raised doubt on the role of gramine in barley resistance to R. padi by showing no relationship between gramine content and settling preference (Forslund et al. 1998) or aphid growth (Åhman et al. 2000) in two different large selections of barley genotypes.

**Induced defense mechanisms and different types of resistance**

Induced defense mechanisms have the advantage that they are only expressed when the plant is attacked. Thus, there will be no yield penalty under the conditions that there is no aphid infestation.

Current models of plant responses to pathogens and other attackers suggest that plants have two types of defense responses, basal defense and resistance (R) gene mediated defense (also called effector-triggered immunity) (Chisholm et al. 2006; Jones and Dangl 2006; Bent and Mackey et al. 2007).

In the basal defense, the plant recognizes pathogen associated molecular patterns (PAMPs), microbe associated molecular patterns (MAMPs) or herbivore associated molecular patterns (HAMPs) by plant pattern recognition receptors that result in the activation of PAMP/MAMP/HAMP-triggered immunity.

PAMPs/MAMPs/HAMPs activate basal early defense responses that take seconds to minutes (e.g. ion flux and oxidative burst), intermediate defense responses that take minutes to hours (e.g. kinase cascade activation) and late responses that take hours to days, e.g. salicylic acid (SA) accumulation and callose deposition (Zipfel and Robatzek 2010). There are similarities in signaling pathways in plant-pathogen and plant-herbivore interactions including ion fluxes, kinase cascade activations, phytohormone generation, activation of defense-related genes and synthesis of secondary compounds and volatiles that suggest similar recognition processes in plant-herbivore and plant-pathogen interactions (Mithöfer and Boland 2008).

Aphid feeding on plants activates both the SA- and the jasmonic acid (JA) -mediated signal pathways (Morkunas et al. 2011; Louis et al. 2012). It has been suggested that efficient plant defence against phloem-feeding insects is regulated by JA (Wallig 2008). There is support for this notion, but there are also reports of aphid resistance related to SA-induced responses (Morkunas et al. 2011; Avila et al. 2012; Louis et al. 2012).

Successful pathogens or herbivores answer by sending effectors into the plant that inhibit the defense reactions. In the case of aphids, several potential aphid salivary effector proteins have been identified that can modulate
plant defense, but only few of them are functionally characterized (Wang et al. 2015). Several studies were done on the C002 protein, which is aphid-specific, and appears to have a role as a salivary effector protein. RNAi-silencing or overexpression of the sequence coding for the C002 protein in *Nicotiana benthamiana* (Domin) decreased aphid fitness or increased aphid reproduction, respectively (Elzinga et al. 2014). Expression of the sequences for two candidate effectors, *Me10* and *Me23*, from potato aphid (*Macrosiphum euphorbiae* Thomas) in *N. benthamiana* increased aphid fecundity (Atamian et al. 2013). A recent study by Wang et al. (2015) suggested that a protein named Armit is another aphid salivary effector protein.

In *R*-gene mediated defense, an *R*-protein recognizes the effector(s) (also called avirulence (*Avr*) gene products) from pathogens or pests. This results in effector-triggered immunity and stronger defense (Fig. 4) (Kaloshian 2004). Plant *R*-genes are classified into 8 groups based on the membrane spanning domain and amino acid motif organization (Gururani et al. 2012). Most of the known *R*-genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins and provide resistance to pathogens and insect herbivores (Jones and Dangl 2006; Hogenhout and Bos 2011).

To date, two aphid *R*- genes have been isolated. The *Mi-1.2* gene was isolated from a wild relative to tomato and confers resistance against certain biotypes of *M. euphorbiae*. The *Vat* gene was found in melon and confers resistance to the melon-cotton aphid (*Aphis gossypii* Glover) (Smith and Chuang 2014). They both belong to the NBS-LRR family of resistance genes.

Other terms for *R*-based resistance are qualitative or vertical resistance, indicating that it gives a very strong specific resistance. The disadvantage is that this type of resistance is only triggered in the interaction with attackers containing avirulence gene products (effectors). Thus, the resistance is not efficient against aphid biotypes without the avirulence protein (Fig.4) (Poland et al. 2009).

Another type of resistance is called horizontal or quantitative. This type of resistance is controlled by several genes each of them adding to the resistance. This type of resistance is more durable, but has as disadvantages that the resistance is not so strong and that it is more difficult to identify the genes adding to the resistance and more difficult to breed for (Poland et al. 2009). The present thesis is focusing on finding genes involved in quantitative resistance.
Breeding methods

To introduce desirable traits into a plant, conventional and transgenic breeding methods may be used. Conventional breeding means the crossing between parental lines and progeny selection. The isolation of homozygous lines carrying the desirable traits might need several rounds of backcrossing, inbreeding and selection, which needs much time and labor. To improve and shorten this process, the methods of marker-assisted breeding and doubled haploid production have been developed.

In a breeding program the superior plants are selected by evaluating the breeding population visually (phenotypic selection) in field trials (e.g. resistance or agronomic traits) or by chemical tests (e.g. grain quality), which needs considerable amounts of time (Collard and Mackill 2008). Marker-assisted selection technology makes it possible to select the superior plants by use of a DNA marker that is linked to the gene of interest and can accelerate the breeding process. In comparison to phenotypic selection, marker-assisted selection saves time, resources and effort, can be carried out at seedling stage and a single plant can be selected based on its genotype. In breeding for *R. padi* resistance, this method would be very useful, since the phenotyping methods require very laborious bio-assays based on aphid growth, development and behavior.

Doubled haploid production includes the cultures of plants from single pollen or microspores (Friedt et al. 2010) and is used in many crops, for example barley, wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rapeseed (*Brassica napus* L.) (Li et al. 2013). By doubled haploid production, complete homozygosity can be obtained in one generation, while it may otherwise take six to eight generations to achieve almost full homozygosity, about three to five years, depending on the crop (Friedt et al. 2010).
Transgenic breeding is a method for transferring desirable agronomic traits into other species or changing the level of expression of available traits in a species. This method has the advantage that one very specific and desirable trait may be changed, whereas using conventional methods of crossing, many undesirable traits are likely to be transferred.

Several aphid-resistant cultivars have been developed by conventional breeding during the last decades in important agricultural and horticultural crops including maize, sorghum, tall fescue, barley, wheat, soybean, barrel medic, lettuce, tomato, apple, melon, peach, peanut, pear, red raspberry, black raspberry (Smith and Chuang 2014). The resistances are either known to be based on classical resistance (R-) genes, or have been mapped to chromosome regions encompassing NBS-LRR sequences, thus with the disadvantage that the resistance may be overcome. So far biotypes virulent to cultivars with R-genes have been found in 17 aphid species. This illustrates the constant need of developing new cultivars with aphid resistance (Smith and Chuang 2014). It has also resulted in an increased interest in searching for quantitative resistance, based on several traits, which is also the focus in this thesis.

**Plant material used in the study**

The barley plant material used in this thesis was a selection of barley breeding lines and their parents, which had been characterized with regard to *R. padi* resistance. The breeding lines had been obtained from a cross between the moderately *R. padi* resistant wild barley accession Hsp5 (*H. vulgare* ssp. *spontaneum*) and cultivar Lina. The first generation (F1) line 660-6:8 was selected as a partially resistant offspring and backcrossed with cultivar Lina and crossed with cultivar Barke that resulted in the first backcross (BC) generations (Fig. 5). Selected resistant offspring lines from the BC1 were further backcrossed to cultivar Lina or cultivar Barke and resulted in BC2 lines. All the BC lines are from doubled haploids. The genotypes were characterized as resistant or susceptible based on an assay of nymphal growth, where 5 nymphs were released in a cage fixed at the basal part of the plant and nymphal weight was evaluated after 4 days (Åhman et al. 2000). The results of the bioassay correlated with quantitative trait loci (QTLs) markers for aphid resistance (Cheung et al. 2010) and predicted well field resistance level (I. Åhman, personal communication).
Fig. 5 Relationships between barley genotypes used in papers I and II. All BC1 and BC2 lines are doubled haploids (bold) and characterised as aphid resistant (R) or aphid susceptible (S).
Main approaches in this thesis

Constitutive gene expression in a large number of barley genotypes with known resistance levels to *R. padi* (Paper I)

In paper I, the constitutive expression of a selection of sequences was explored in a collection of barley breeding lines with different degree of aphid resistance. The aim was to find out if the genes had higher expression in resistant as compared to in susceptible genotypes (or vice versa). This approach is based on the hypotheses (a) that the resistance or susceptibility character is to some extent constitutive and (b) that the transcript abundance of a certain gene sequence gives information about the physiological state of the plant.

Regarding the hypothesis (a) some examples of constitutive differences in trichomes and secondary metabolites relating to aphid resistance have been given above. In addition, recent literature report e.g. constitutive differences in some enzyme activities related to defense (phenyl ammonia lyase, polyphenol oxidase and peroxidase) between *S. avenae*-resistant and -susceptible wheat genotypes (Han et al. 2009) and differences in the constitutive amino acid profiles between soybean genotypes resistant and susceptible towards *A. glycines* (Chiozza et al. 2010). Constitutive differences in the transcript abundance of a number of gene sequences were reported in a comparative study between *R. padi* resistant and susceptible barley genotypes (Delp et al. 2009) and these results were the basis for the study presented here.

Regarding the hypotheses (b) a large number of studies supported the default assumption that higher transcript abundance indicates higher protein levels, but there are also reports that did not support the assumption. For example, the higher transcript level of certain genes involved in the biosynthesis of glucosinolates after Arabidopsis infestation by cabbage aphid (*B. brassicae*) did not relate to glucosinolate level (Mewis et al. 2006). Also, in barley, it was found that higher transcript abundance for a protein involved in gramine biosynthesis, N-methyltransferase (*NMT*), induced by *R. padi* or rose-grain aphid (*Metopolophium dirhodum* Walker) infestation did not lead to higher amounts of the NMT protein (Larsson et al. 2011).

Aphid-induced gene expression of putative susceptibility factors in a large number of barley genotypes with known resistance levels to *R. padi* (Paper II)

In paper II, the constitutive and aphid-induced expression of sequences putatively related to aphid susceptibility were investigated in a collection of barley breeding lines with different degrees of aphid resistance. The hypothesis is that certain sequences which have higher expression in susceptible
compared to in resistant genotypes increase susceptibility to *R. padi*. Breeding for loss of susceptibility is a strategy to develop durable and broad-spectrum resistant cultivars. This method has proven useful in the case of the loss of the *Mlo* gene function in barley, which has given resistance to powdery mildew (Pavan et al. 2010; Zheng et al. 2013). Susceptibility genes are those that code for proteins necessary for or helpful to the pathogen or the pest. They are expected to be expressed in susceptible plants or to be up-regulated in such plants upon infection or infestation. Based on this idea, all gene sequences up-regulated in susceptible plants upon aphid infestation are putative susceptibility genes and those upregulated specifically in susceptible plants (and not resistant plants) are even stronger candidates.

Regarding the hypothesis, in several studies, gene expression and enzyme activity were increased in susceptible plants upon aphid infestation. There is support for the idea that β-glucanases are susceptibility factors in plant-aphid interactions (Botha et al. 2010; Reddy et al. 2013; Anderson et al. 2014). In two separate comparative studies between resistant and susceptible cultivars in barley, several genes were up-regulated only in susceptible lines by *R. padi* and *D. noxia*, respectively (Delp et al. 2009; Gutsche et al. 2009).

This study builds on paper I and an earlier study comparing transcript abundance with and without aphids in two susceptible and two resistant barley genotypes (Delp et al. 2009). Barley β-1,3-glucanases were investigated as putative susceptibility factors in the barley-*R. padi* interaction. β-1,3-glucanases are one of the important groups of hydrolytic enzymes, belonging to the pathogenesis-related proteins family 2 (Balasubramanian et al. 2012) and their transcription increases significantly after pathogen and insect attack in several plant species (van Loon et al. 2006). The exact function of glucanases in plant defense against aphid is unclear (van der Westhuizen et al. 1998, Botha et al., 2014), but it may facilitate aphids probing and feeding by the degradation of callose and cell wall structures (Hao et al. 2008; Anderson et al. 2014).

Higher plants produce several β-1,3-glucanases isoforms with different primary structures and size, cellular localization and pattern of regulation. Based on their amino acid identity they are classified as: class I, II and III. Each of them may have a different biological function. β-1,3-glucanases (commonly found all over the plant kingdom) and β-1,3;1,4-glucanases (reported only in monocots of the Poaceae family) are two structurally related β-glucanases which both have a role in the defense against pathogens, but degrade different linkages in β-glucans present in the cell wall and produce different products (Simmons 1994). β-1,3-glucanases recognize and cleave β-1,3-linkages and β-1,3;1,4-glucanases recognize and cleave β-1,3;1,4-linkages. β-glucan degradation releases fragments from the plant cell walls, which have been shown to be activators of several defense related genes including β-glucanases (Hrmova and Fincher 2001).
**Aphid behavior and performance on transgenic Arabidopsis with a putative aphid resistance sequence from barley (Paper III)**

In paper III and IV, the function of two putative aphid resistance related genes in barley was examined by transformation of the respective cloned cDNA’s into Arabidopisis and barley, followed by study of the effects on aphid biology.

The approach in paper III is based on two hypotheses; (a) that genes up-regulated by *R. padi* in moderately resistant barley and not in susceptible barley may confer increased resistance to *R. padi* and (b) that genes putatively related to resistance towards a monocot specialist aphid, *R. padi* in the monocot barley might confer resistance against a generalist aphid, *M. persicae* in a dicot plant, Arabidopsis.

With regard to hypothesis (a), it was first considered whether there is any previous evidence of a gene being up-regulated in response to an aphid shown later to be conferring resistance against this aphid. Indeed, in Arabidopsis, genes that were up-regulated in response to *M. persicae* encoding for phytoalexin deficient 4 (*PAD4*) and a gene involved in trehalose metabolism (*TPS11*) were shown to provide antibiosis and antixenosis towards *M. persicae* (Louis and Shah 2015). *PAD4* knockout mutants and *TPS11* overexpression resulted in higher and lower aphid population growth in comparison to the wild type Arabidopsis, respectively (Louis and Shah 2015). As a third example, silencing in tomato of an α-dioxygenase that contributes to oxylipin synthesis and that was up-regulated by *M. euphorbiae* enhanced *M. euphorbiae* population growth (Avila et al. 2013).

Regarding hypothesis (b), first it will be considered whether one gene might confer resistance against different (preferably both specialist and generalist) aphids, secondly Arabidopsis-*M. persicae* as a model system in plant-aphid research will be discussed and thirdly, it will be considered whether there are examples of transfer of resistance traits from barley to Arabidopsis.

There is more evidence that a certain gene is active against a specific aphid species, than the opposite, possibly because genes that have been studied are classical *R*-genes, involved in specific interactions. However, the gene *Mi-1* has a very broad organism spectrum and confers resistance against certain nematodes, whiteflies, psyllids and aphids (Kaloshian 2004). Notably, the resistance is specific for certain biotypes of *M. euphorbiae* and it is not active against the generalist *M. persicae*. Furthermore, resistance against specialist spotted alfalfa aphid (*Theriophis trifolii* Monell) and specialist bluegreen aphid (*Acyrthosiphon kondoi* Shinji), in barrel medic (*Medicago truncatula* Gaertn) cultivars were shown to be controlled by separate genes (Klingler et al. 2007). One example of a resistance source active against two different aphids is the case of *D. noxia* resistant barley,
which was shown to reduce the growth of both *D. noxia* and *R. padi*, but it is not known if the resistance to the different aphids is controlled by the same gene or not (Jimoh et al. 2011).

*Arabidopsis thaliana* (L.) is a useful model organism to study plant processes because of its small genome size, short generation time, self-pollination and easy transformation process (Koornneef and Meinke 2010). *M. persicae* is a cosmopolitan, polyphagous herbivore. *M. persicae* primary (winter) host are trees of the genus *Prunus*, mainly the peach tree (*Prunus persica* Batsch) and the aphid can use plants in more than 40 different plant families as its secondary host. The secondary hosts include both dicots (e.g. Brassicaceae, Cucurbitaceae and Fabaceae) and monocots (i.e. Poaceae, Liliaceae and Araceae). The eggs overwinter on the primary host and in the spring the egg hatches. The aphid has several generations on the winter host before it migrates to the secondary hosts. In autumn, adult aphids migrate to *Prunus* species, male and female mate and the eggs overwinter there. *M. persicae* populations can be very dense on the plant and cause water stress, wilting and reduce the plant growth and heavy aphid infestation can reduce the yield of foliage and root crops (Capinera 2008). Arabidopsis and *M. persicae* have a compatible interaction. The Arabidopsis genome is sequenced and preliminary data from the genomic sequence of *M. persicae* are available. In addition, several gene expression profiling studies of their interaction have been done, as well as studies of *M. persicae* performance on many Arabidopsis mutants. All this makes Arabidopsis-*M. persicae* a good model system to study the genes and mechanisms involved in plant response to aphids (AphidBase 2009; Moran et al. 2002; Louis and Shah 2013). In this study, transformation of Arabidopsis made it possible to generate transformants in a relatively short time and to test the effects of our putative aphid resistance sequences.

Whereas Arabidopsis is a good model plant for studying dicots, purple false brome (*Brachypodium distachyon* (L.) Beauv) is now emerging as a model system for studying monocots. There are several reasons, including small stature, self-pollination, short life cycle, small genome and close relation to the important crops wheat and barley. Several molecular resources have been developed for this plant such as germplasm collections, genetic markers, a genetic linkage map, bacterial artificial chromosome libraries, physical maps, mutant collections, microarrays and databases as well as highly efficient transformation protocols (Vogel et al. 2010). *B. distachyon* has good potential as a model in studying plant-pathogen/pest interactions (Fursova et al. 2012; Sandoya and de Oliveira Buanafina 2014). For example it was shown that greenbug did not survive on Arabidopsis and performed poorly on rice whereas it performed well and responded to *B. distachyon* similarly to what was observed in wheat (Azhaguvel et al. 2009). The evaluation of Russian wheat aphid symptoms on *B. distachyon* accessions showed varied levels of resistance to this aphid, enabling search
for QTLs and genes related to this interaction (Sandoya and de Oliveira Buenafina 2014). Similar studies of *R. padi* performance on *B. distachyon* are still waiting to be carried out.

There are several studies where a gene from a monocot was expressed in Arabidopsis and resulted in a phenotypic change. For example, in two separate studies, Arabidopsis with genes responsible for abiotic stresses in wheat (salt stress and hyperosmotic stress) were reported to boost root growth under salt stress and significantly raise tolerance to salt, drought and freezing stress as well as root system improvement under hyperosmotic stress (Ge et al. 2007; Tian et al. 2013). In addition, a gene responsible for heat stress from barley increased thermotolerance when overexpressed in Arabidopsis (Shi et al. 2001).

In this paper, the cDNA encoding a proteinase inhibitor (PI) in barley was cloned and transformed into Arabidopsis. PIs are small proteins. They have a role in plant defense against herbivores and pathogens and some are known as storage proteins (Fan and Wu 2005). PIs are present in seeds and their production is induced in other tissue in response to herbivore attack or wounding (Koiwa et al. 1997). They inhibit digestive enzymes in insect midguts and decrease the essential amino acids for insect growth and development (De Leo et al. 2002). PIs are ubiquitous proteins in plants (Fan and Wu 2005). The first time the possible role of PIs in plant defense was reported was by showing that the larva of red flour beetle (*Tribolium castaneum* Herbst) could not develop normally on soybean flour and grits (Mickel and Standish 1947). The defensive roles of PIs have therefore been studied in many plants. Rearing on artificial diets containing PI caused growth inhibition of *M. persicae* and expressing a gene encoding the same PI in oilseed rape decreased *M. persicae* weight and fecundity (Rahbé et al. 2003). In barley leaves, the levels of two different PIs increased twofold upon *R. padi* infestation in an aphid resistant cultivar and feeding on artificial diet containing the purified PIs decreased *R. padi* survival (Casaretto and Corcuera 1998).

**Aphid behavior and performance on transgenic Arabidopsis and barley with a putative aphid resistance sequence from barley (Paper IV)**

In this paper a barley sequence representing an open reading frame (ORF) encoding a protein with similarity to a part of a kinase was isolated and transformed into Arabidopsis and barley and the interaction with *M. persicae* and *R. padi* were evaluated, respectively.

Plants sense changes in the environment including those causing biotic or abiotic stress. The information from outside of the cell is mediated intracellularly by signal-transducing mechanisms and protein kinases are involved in such transductions (Champion et al. 2004). Protein kinases transfer
phosphoryl groups from ATP to amino acid residues of a protein. Based on their primary sequences and the amino acid residues phosphorylated they are classified into three groups; serine/threonine kinases, tyrosine kinases and histidine kinases (Chevalier and Walker 2005). Some of the known plant resistance genes encode a serine/threonine kinase and confer resistance to pathogens, for example Pto that confers resistance to *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993), Rpg1 that confers resistance to *Puccinia graminis* f. sp. *tritici* (Brueggeman et al. 2006) and Stpk-V that confers resistance to *Blumeria graminis* f. sp. *tritici* (Cao et al. 2011).

Receptor-like serine/threonine kinases play a major role in a variety of processes from growth and development to response to pathogens. They are a family of transmembrane protein with similar structure (Afzal et al. 2008). In Arabidopsis, the *ERECTA* gene, known to be encoding a receptor-like serine/threonine-protein kinase, conferred resistance against bacterial wilt (*Ralstonia solanacearum* Smith) (Godiard et al. 2003). Another example is the *BAK1* gene that encodes a receptor-like serine/threonine-protein kinase involved in resistance against *Pseudomonas syringae* (Van Hall) and *Hyaloperonospora arabidopsidis* (Gäum) (Roux et al. 2011). This gene was also found to be involved in resistance against *M. persicae* in Arabidopsis (Prince et al. 2014). A transcriptomics study on cucumber identified several genes encoding receptor-like serine/threonine-protein kinases as defense factors against *A. gossypii* (Liang et al. 2015).
Main results and discussion

In paper I, the purpose was to investigate the possible relation between the expression of certain genes and aphid resistance characteristics in a selection of barley breeding lines consisting of 23 genotypes. The genes were selected based on the results of a comparative study between aphid resistant and susceptible genotypes after *R. padi* infestation (Delp et al. 2009). Out of nine investigated genes, five were expressed as we predicted. Two gene sequences (thionin (*THIO*) 1570 and *PI*) had higher constitutive transcript abundance in resistant compared to in susceptible genotypes. Two of the sequences (*AOS* and *NMT*) were expressed equally in all the genotypes and lipoxygenase (*LOX*)2 was expressed higher in susceptible genotypes. Three other genes were either not expressed in some of the genotypes (*β*-1,3-glucanase III (*βGLU-GIII*) and the sequence corresponding to contig16360_at (*STK*)) or were not expressed in any of the genotypes (barley chemically induced (*BCI*)-4). Based on the results from paper I, we cannot definitely assign any of the investigated gene sequences as susceptibility or resistance factors towards *R. padi*, but the study gave support to the putative role of *THIO1570*, *LOX2* and *PI* as significant in the interaction between barley and *R. padi* (Fig. 2 and Table 3 in paper I).

In paper II, the role of three *β*-1,3-glucanases in aphid susceptibility was investigated in 15 barley breeding lines with known *R. padi* resistance characteristics based on aphid growth. A time course study of aphid settling was carried out and the transcript abundance of three *β*-1,3-glucanase sequences (contig1636_at, contig1639_at and contig1637_s_at) were examined on the same plant leaf tissue. The previous comparative study on two resistant and two susceptible barley genotypes suggested a susceptibility role for sequences corresponding to contig1636_at and contig1639_at, but showed no difference in the transcript abundance of the sequence corresponding to contig1637_s_at between resistant and susceptible genotypes (Delp et al. 2009). In this study of 15 barley genotypes, ten genotypes expressed contig1636_at (Fig. 2 in paper II). The contig1639_at sequence was induced in 13 genotypes and the contig1637_s_at sequence in 7 genotypes upon *R. padi* infestation (Table 3 in paper II). A comparison between aphid settling and former resistance characterisations of the barley lines based on nymphal growth revealed that in all cases with significant differences in settling, the susceptible genotypes had higher aphid settling than resistant genotypes. Furthermore no resistant genotype had higher transcript
abundance than susceptible genotypes when fed on by *R. padi*. This suggested that certain β-1,3-glucanases might be susceptibility factors in the *R. padi*-barley interaction.

In paper III, the possible effect of a barley proteinase inhibitor gene (*BCI-7*) expected to have a function in *R. padi* resistance (Delp et al. 2009) was investigated by transforming the cDNA into Arabidopsis. The effect was evaluated by studying the behaviour and growth of *M. persicae*. The Arabidopsis plants were transformed with either a constitutive or a phloem specific (ps) promoter. The evaluation showed that the number of aphids was lower on *BCI-7* and *psBCI-7* transformants compared to on control plants after 5 days. In addition, a significant difference was observed between one *BCI-7* transgenic line and controls in choice tests. These results indicated that the expression of *BCI-7* in Arabidopsis increased aphid resistance.

In paper IV, the effect of an ORF from barley, with 55 % similarity to a part of an Arabidopsis putative serine/threonine kinase sequence that was found up-regulated by aphids in resistant barley (Delp et al. 2009) was investigated. The sequence was transformed into Arabidopsis and barley and the effect of transformation on aphid performance was analyzed. There were no differences in *M. persicae* population growth assessments and in choice tests between transformed Arabidopsis and control plants (Fig. 2 and Fig. 3 in paper IV). The barley plants were transformed with a constitutive and a phloem specific promoter, resulting in selected lines named *p*:5, *p*:8 and *ps*:1, respectively. There was no relation between the transcript abundance of the transformed sequence and aphid numbers in a population growth test (Fig. 4 in paper IV). Taken together the results suggest that there was no effect of the transformation and that this might be due to that part of the ORF cloned does not contain domains important in resistance against stresses (Suppl. Fig. 1 in paper IV).
Still based on the idea that the analysis of the transcript abundance of certain genes may give indications of differences between genotypes, there are other possible approaches than the one applied here. Our selection of candidate genes was based on a microarray study (Delp et al 2009), which is a method that gives a broad overview of gene expressions. At the time when the microarray study was carried out, a barley chip was available that only contained about half of the barley genome, thus the study could have missed a number of interesting genes. Nowadays, it is becoming increasingly common to use the RNA sequencing method (RNA-seq) for the quantification of transcriptomes. This method has clear advantages over using the microarray method. Firstly, it does not depend on prior genomic sequence knowledge, which is advantageous for non-model organisms. Secondly, it has a larger dynamic range compared to the microarray method. Very high and very low transcript levels are detectable as well as all the isoforms of a gene. This is a significant advantage compared to the microarray method where a probe might hit just some of the isoforms of a gene and as a result the probe does not show the expression of the whole gene complex. However there are some limitations in RNA-seq as well, e.g. that data analysis is complex, expensive and needs special computers and generate lots of data which is difficult to share and costly to store (Zhao et al. 2014).

Other approaches to study overall differences between genotypes could be to study proteomics and metabolomics. Proteomics is a method to study structure and function of all expressed proteins and provides information about proteins actually being present and possibly interacting with a pathogen or herbivore, which is its advantage over transcriptomics. The disadvantage is that it’s protocol is not as straightforward as transcriptomics and the data analysis is complex (Chandramouli and Qian 2009).

Metabolomics is a method to identify and quantify metabolites. It is a sensitive and rapid method compared to transcriptomics and proteomics, needs little sample preparation and generates a lower number of endogenous molecules relative to the numbers that are analyzed with transcriptomics and proteomics. However, due to the diverse chemical structures with different abundance, there is not a good general method to study all the metabolites. The method needs complex bioinformatic analysis or analytical platforms and is expensive (Roessner and Bowne 2009).
The major reason for our choice of approach was that some candidate sequences for \textit{R. padi} resistance or susceptibility in barley were already identified in the microarray study (Delp et al. 2009) (paper I and II). We aimed to do functional genomic studies by cloning and expression of the candidate genes Arabidopsis and barley (paper III and IV).

In paper I, the transcript abundance of susceptibility- and resistance-related candidate genes were analyzed in a selection of barley breeding lines with different levels of resistance and in paper II, the constitutive and induced transcript abundance of two susceptibility related candidate genes and one additional gene (altogether three \(\beta\)-1,3-glucanase sequences) were investigated in a selection of barley breeding lines with different levels of resistance.

To analyze the transcript abundance of the selected genes, RNA was extracted from frozen leaf tissue and was reverse transcribed to synthesize cDNA and then the transcript levels were assessed using quantitative polymerase chain reaction (qPCR).

A big sample size will increase precision in the statistical analysis (McDonald 2009). In this study, because of logistic reasons the sample size was 3 biological replicates, each consisting of one individual plant. This weakness is somewhat balanced by the usage of a large number of genotypes, which had been characterized with regard to aphid antibiosis.

In papers III and IV, two candidate sequences for \textit{R. padi} resistance identified in the microarray study (Delp et al. 2009) were cloned and transformed to Arabidopsis ecotype Columbia (Col-0) and barley cultivar Golden Promise. Gene silencing and gene addition (or overexpression) are two of several approaches that are used in functional genomics to study the function of a certain gene sequence. It may also be used in applications e.g. to improve crop resistance against pathogens and insects. Gene silencing technology is referring to down-regulation or interruption of the expression of a specific gene. Gene silencing in monocots could be obtained by several methods including RNA-mediated interference (RNAi) (Travella et al. 2006), virus induced gene silencing (VIGS) (Anderson et al. 2014), antisense oligodeoxynucleotides (ODN) (Xie et al. 2014) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Jiang et al. 2013).

All four methods are rapid and inexpensive. On the negative side, it may be noted that they may result in off-target effects and that they are not developed for all plant species. RNAi, VIGS and ODN methods result in partial silencing with variable levels and complete silencing is rare (Gilchrist and Haughn 2010; Dinç et al. 2011).

Using the RNAi method it is possible to knock down multiple homologous genes, in a tissue specific or temporal manner, but some genes are not possible to silence with this method and the method needs transformation (Gilchrist and Haughn 2010). VIGS was used widely in the past few years for characterizing the function of plant genes (Gupta et al. 2013). It is a
rapid method and the results are obtained in 3 to 4 weeks from infection to gene silencing with transient inhibitory effect and unlike the RNAi method does not need transformation (Burch-Smith et al. 2004). The application of this method in plant resistance against fungi, bacteria, nematode and herbivores including aphids were reported (Huang et al. 2012). Negative in this context may be that virus sequences induce defense responses (Unver and Budak 2009).

There are some functional genomic studies on plant-aphid interactions using the silencing methods described above; for example silencing a resistance and a susceptibility gene in wheat by means of VIGS in two separate studies characterized the function of these genes against D. noxia (van Eck et al. 2010; Anderson et al. 2014).

The ODN method is a widely used method in animal research and it has been shown to be an efficient method for gene silencing in plants including wheat and barley. In this method, a single-stranded ODN enters the cell, binds to its complementary RNA and inhibits gene expression (Dinç et al. 2011; Xie et al. 2014). It has as advantages over VIGS and RNAi that ODN design and synthesis are very fast and simple and that the inhibitory effect can be obtained within 24 hours. On the negative side, the inhibitory effect is transient, and the plant tissue needs to be cut and transferred to the solution containing the ODN, thus the plant is not intact which is desirable in studies of plant-aphid interactions (Sun et al. 2005; Dinç et al. 2011).

CRISPR/Cas9 has recently developed as a powerful genome editing tool and can be used to functionally inactivate target genes. In this method a single strand RNA enters the cell and makes a complex with Cas9 protein. The complex binds to complementary DNA and cuts it. This method has been successfully applied to various plant species including N. benthamiana, A. thaliana, rice, wheat and barley (Shan et al. 2013; Lowder et al. 2015; Lawrenson et al. 2015). Successful CRISPR/Cas9 knocks out the target genes whereas other methods mentioned above mediate gene knock down. This method requires clone isolation that makes it time consuming compared to other methods (Lawrenson et al. 2015).

Another approach is gene addition or overexpression. There are several methods to introduce DNA into host plant cells. Direct techniques are based on cell wall penetration physically (e.g. bombardment, electrophoresis, vacuum infiltration) or penetration into protoplasts using e.g. ethylene glycol or calcium phosphate. Indirect techniques are based on biological methods where a plasmid construct is introduced into the target cell by means of microorganisms, e.g. bacteria or viruses (Chung et al. 2006; Rao et al. 2009).

Agrobacterium tumefaciens is a gram-negative bacterium found in soil. It causes crown gall disease in plants and is widely used as a tool to develop stable transformed plants. A. tumefaciens has a tumor-inducing (Ti) plasmid and is able to transfer and integrate a part of the Ti plasmid called T-DNA
into the host plant. *A. tumefaciens* can deliver a desirable sequence with T-DNA to a broad range of plant species (Gordon and Christie 2014).

*Agrobacterium*-mediated plant transformation is the most common method to transform Arabidopsis and barley (Bent 2006; Harwood 2014). Arabidopsis transformation using floral dip method is simple, reliable, with low cost and no needs for tissue culturing that may cause somaclonal variation (Bent 2006). Barley transformation using the immature embryo method is highly efficient and produces transformants with low copy numbers of the transgene, stable transgene expression and rare transgene silencing (Bartlett et al. 2008).
Conclusions

Much effort has been used to develop cultivars resistant against aphids. There are barley cultivars bred for resistance against *S. graminum* and *D. noxia* (Porter et al. 1998; Smith and Chuang 2014). This resistance has been selected based on screening for symptoms and the molecular basis of the resistance is not identified (Mornhinweg et al. 2009). No barley cultivar with resistance against *R. padi* is available. Identifying genes related to susceptibility or resistance against aphids is important to explain plant-aphid interactions and for use in resistance breeding. This thesis contributes to such knowledge with regard to *R. padi* in barley and the results can be summarized as:

- Genes coding for a thionin and a PI were identified as putatively related to resistance and a gene for a lipoxygenase as putatively related to susceptibility against *R. padi*.

- Two β-1,3-glucanase sequences were identified as possible susceptibility factors against *R. padi*.

- The cDNA encoding the PI from barley mentioned above caused increased resistance against *M. persicae* when expressed in Arabidopsis.

- A barley sequence earlier shown to be up-regulated in response to *R. padi* in moderately aphid resistant genotypes and with unknown function, did not increase resistance against *M. persicae* in Arabidopsis nor against *R. padi* in barley upon transformation.
Suggestions for further studies

The transcript abundance of the constitutively expressed candidate gene sequences for aphid resistance or susceptibility identified in paper I could be studied in a time course study with and without aphid (i.e. the plant material used in paper II).

The functional role of the candidate genes from papers I (thionin, proteinase inhibitor \((PI=BCI-7)\) and lipoxygenase) and II (β-glucanases; contig1639_at and contig1637_s_at) could be analyzed by gene silencing and/or overexpression in barley followed by evaluation of \(R.\ padi\) growth and behavior. Transformation of barley for the overexpression of \(BCI-7\) has already been carried out and the evaluation has started. Besides the study of \(R.\ padi\), it would be interesting to study \(M.\ persicae\) on transgenic barley to find out the effects on this generalist aphid.

\(B.\ distachyon\) is a good model plant which can be used instead of barley to do a functional study on the candidate genes. To do that, first it is necessary to study \(R.\ padi\) performance on \(B.\ distachyon\). Studies of the tissue localization of the proteins encoded by the candidate genes would add to the understanding of their functions.

To study the direct effect of the candidate sequences on aphid development and behaviour, the cDNAs can be expressed in \(Escherichia\ coli\), the protein can be purified and the purified protein can then be used in artificial diets. This approach is suitable to examine the effect of the proteinase inhibitor \((PI=BCI-7)\).

As ultimate tests, transgenic barley lines overexpressing or silenced for above candidate genes should be evaluated in field trials with natural or artificial aphid infestations.
Acknowledgements

I sincerely thank everyone who has been helpful in my studies.

To start I would like to thank my supervisors Dr. Lisbeth Jonsson, Dr. Inger Åhman and Dr. Lisa Beste for their kind support during these years.
I am also grateful to Dr. Katharina Pawlowski for her advice, incredible kindness and friendship.
Many thanks to my office-mate Dr. Afaf Hamada and my colleagues Aleksandra, Andrea, Lotta, Marco, Sandra, Sofie, Van and last but not the least Denis, who always was there to answer my questions.
I also want to acknowledge all the present and past members of the Plant Physiology division.

Many thanks to Sara, Behnaz, Afshin, Bahareh and special thanks to Farzad for their friendship, support and kindness. Finally a warm thanks to my beloved parents and my siblings for their love, endless support and encouragement.

This work got financial support from the Swedish Foundation for Strategic Environmental Research (Mistra) via the PlantComMistra program and from CF Lundström Foundation.
I also gratefully acknowledge support in the form of stipends from The Royal Physiographic Society in Lund (the Nilsson Ehle - donations), Helge Ax:son Johnson Foundation and Stockholm University donation funds as well as travel grants from the Department of Ecology, Environment and Plant Sciences.
References


Anderson VA, Haley SD, Peairs FB, van Eck L, Leach JE, Lapitan NLV (2014) Virus-induced gene silencing suggests (1,3;1,4)-β-glucanase is a susceptibility factor in the compatible Russian wheat aphid-wheat interaction. MPMI 27:913-922.


Botha AM, Swanevelder ZH, Lapitan NLI (2010) Transcript profiling of
wheat genes expressed during feeding by two different biotypes of *Diuraphis noxia*. Environ Entomol 39:1206-1231.


Godiard L, Sauviac L, Torii KU, Grenon O, Mangin B, Grimsley NH, Marco Y (2003) *ERECTA*, an LRR receptor-like kinase protein controlling devel-


