An array of responses to insect feeding in *Brassica*

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool EPS (Experimental Plant Sciences)

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff
in het openbaar te verdedigen
op dinsdag 21 oktober 2008
des namiddags te vier uur in de Aula

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PhD thesis, Wageningen University, The Netherlands, 2008
With references - with summaries in English and Dutch

ISBN: 978-90-8504-994-4

Abstract

Plants have developed defence mechanisms to deal with attacks from herbivorous insects. Transcriptional profiling after herbivore feeding reveals, at the molecular level, how plants respond to this type of stress. Differences in transcriptional profiles often underlie phenotypic variation among plants from the same as well as different, related species. Studying intra- and interspecific plant variation on the molecular and the ecological level in an integrated way provides insight into plant defence mechanisms. Intra- and interspecific variation in resistance or susceptibility to herbivores has been widely studied through bioassays. However, few studies link this with a genome-wide transcriptional analysis. Here we take such an approach to study the interaction between cultivated as well as naturally occurring *Brassica* species and two specialist herbivores. Because *Brassica* full genome microarrays are not available, 70-mer oligonucleotide microarrays based on the *Arabidopsis thaliana* genome were used. We analyzed the transcriptional responses of white cabbage cultivars (*Brassica oleracea* var. *capitata*) and the wild black mustard (*Brassica nigra*) after feeding by either the caterpillar *Pieris rapae* or the aphid *Brevicoryne brassicae*.

We show that there is intraspecific variation among *B. oleracea* cultivars with respect to herbivore performance of both *P. rapae* and *B. brassicae*. Relative performance of the latter herbivore on the cultivars was similar in glasshouse and field experiments, suggesting aphid performance to be largely independent of environmental conditions. The transcriptional responses after 24, 48, and 72 hours of *P. rapae* feeding on two white cabbage cultivars that supported different insect performance showed variation in timing and regulation of individual genes. The majority of *P. rapae*-induced genes in both cultivars were jasmonate-dependent. In contrast to *P. rapae*-induced plant responses, *B. brassicae* feeding resulted in the differential regulation of only a small number of genes in the two *B. oleracea* cultivars that supported different insect performance. The genes that were regulated in response to aphid infestation were highly cultivar-specific. We also observed interspecific variation in *B. brassicae* performance as well as in transcriptional responses to feeding by *P. rapae* or *B. brassicae* when comparing *B. oleracea* and *B. nigra*. Temporal patterns of expression of herbivore-responsive genes in the *Brassica* species, together with targeted studies employing *A. thaliana* knock-out mutants revealed a role for a trypsin-and-protease inhibitor in resistance against *P. rapae* as well as *B. brassicae*.

All transcriptomic experiments mentioned above as well as most microarray studies on *A. thaliana* have been performed under carefully controlled environmental conditions in which plants were exposed to a single herbivore. However, it was unclear whether the observed intraspecific variation in transcriptional profiles and herbivore performance in the glasshouse sustain in the field. Therefore, I analysed herbivore occurrence and distribution together with transcriptional profiles of two *B. oleracea* cultivars in the field. Early in the season, no clear differences in herbivore communities and transcriptional profiles were found. Conversely, later in the season herbivore abundance, species richness, and biodiversity differed greatly between the cultivars. These differences can, at least partly, be explained by differences in expression levels of particular genes. In conclusion, the data in this thesis show that inter- and intraspecific variation among plants have a strong impact on their interaction with herbivores both at the molecular and ecological level. This was true under glasshouse as well as field conditions. This thesis forms the basis for further unraveling direct defence mechanisms of white cabbage.

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Chapter 1

General introduction

Colette Broekgaarden

Herbivorous insects and their impact on agriculture

Insect pests cause severe damage to crop production worldwide. On a global level insects take a significant part of the harvest (Figure 1). For example, lepidopteran larvae cause extensive tissue damage by removing whole leaf areas. Other pests that have a great impact on crop production are aphids and whiteflies, which have a more sophisticated way of feeding on plants. They use their piercing mouthparts, the stylets, to probe the plant tissue in order to feed from phloem sieve elements (Pollard, 1973; Tjallingii and Hogen Esch, 1993; Walling, 2000). Aphid feeding may cause chlorosis and leaf curling resulting in disruption of normal plant growth and development. Additionally, feeding by aphids as well as whiteflies can indirectly damage a plant through the transmission of viral diseases (Raybould et al., 1999; Alvarez et al., 2007). The majority of pest insects are specialists, meaning that they feed on one or a few closely related plant species within a plant family. Generalists feed on a wide range of different plant species in different families (Schoonhoven et al., 2005).

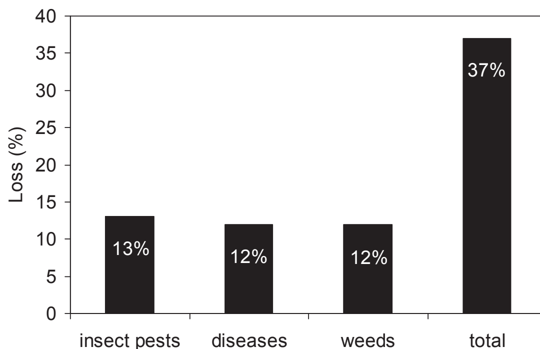

Figure 1. Crop losses due to insect pests, diseases, and weeds in the USA (Pimentel, 1997).

Frequent application of insecticides is used to control herbivorous insects, but is only partly successful and hazardous to the environment and human health. Many insecticides not only kill pest insects, but are also harmful to beneficial insects such as natural enemies of the herbivore and pollinators (Wu et al., 2004; Lin et al., 2007). Moreover, insects develop resistance against insecticides very quickly (Foster et al., 1998; Kranthia et al., 2002; Nauen and Denholm, 2005) resulting in the development of new and often more aggressive insecticides. There is, therefore, a need for a more integrated approach to control herbivorous insects in agriculture. Improving insect resistance in crops will result in better yields in areas with high herbivore pressure. Biotechnology plays an important role in obtaining insect resistant crops by genetic modification. Furthermore, via the use of molecular markers it should be possible to select the desired plant characteristics and obtain resistant varieties through classical marker assisted breeding.

Plant defence

Plants possess effective mechanisms to defend themselves against herbivorous attackers. Constitutive plant defences, which are independent of damage, form the first barrier to herbivorous insects, whereas defences that are induced upon herbivore attack often protect plants from further damage (Kessler and Baldwin, 2002; Schoonhoven et al., 2005). The defences of plants against

herbivores can be divided into direct and indirect defences. Direct defences have a negative effect on the physiology of the attacker, whereas indirect defences promote the effectiveness of natural enemies of the herbivore. Plant morphology features, for example wax layers or trichomes, can function as direct defence by preventing insect herbivores from settling, moving or feeding on a plant (Traw and Dawson, 2002a; Schoonhoven et al., 2005), but also as indirect defence by providing shelter to natural enemies of the herbivore (Schoonhoven et al., 2005). Additionally, secondary metabolites that are produced by plants can function both as direct and indirect defences. Direct defence metabolites can be toxic or repellent, thereby affecting insect behaviour and physiology (Roda and Baldwin, 2003). Chemicals that play a role in direct defence are stored in tissues of the plant that are consumed by herbivores (Van Dam et al., 2000; Harvey et al., 2003). These compounds can alter the physiology of herbivores by reducing their growth rate, adult size and survival probability (Harvey et al., 2003). Proteinase inhibitors, for example, influence herbivore performance by inhibiting insect digestive enzymes (Zavala et al., 2004; Bi et al., 2006). Indirect defence can affect higher trophic levels by enhancing the effectiveness of natural enemies e.g. via the production of secondary metabolites that are volatile (Vet and Dicke, 1992; Pichersky and Gershenzon, 2002; D'Alessandro and Turlings, 2006). Direct and indirect defence mechanisms can function additively against an herbivore. A slower herbivore growth can prolong the time that the herbivore is exposed to a predator or parasitoid (Simms and Fritz, 1990). For example, Kessler and Baldwin (2004) showed that a combination of direct and indirect defence mechanisms of *Nicotiana attenuata* resulted in additional mortality of *Manduca sexta* larvae.

A distinct defence system present in crucifers, including *Brassica* crops as well as the model plant *Arabidopsis thaliana*, is the glucosinolate-myrosinase system. When plant cells are disrupted, glucosinolates are hydrolyzed by myrosinases resulting in the formation of a variety of bioactive compounds such as isothiocyanates, epithionitriles, thiocyanates, and nitriles (Bones and Rossiter, 2006; Grubb and Abel, 2006; Halkier and Gershenzon, 2006; De Vos et al., 2007). Some specialist herbivores have evolved enzyme systems to detoxify glucosinolates as has been shown for the lepidopteran herbivores *Plutella xylostella* and *Pieris rapae* (Ratzka et al., 2002; Wittstock et al., 2004). Some specialists even accumulate intact glucosinolates to use them for their own defence (Schoonhoven et al., 2005; Després et al., 2007). For example, *Brevicoryne brassicae* has evolved its own myrosinase to catalyse the hydrolysis of plant glucosinolates, yielding biologically active products that may have a direct toxic effect on the aphid's natural enemies (Jones et al., 2002; Kazana et al., 2007; Pratt et al., 2008). However, it should be noted that specialists, like generalists, may be susceptible to the toxic effects of secondary metabolites (Adler et al., 1995; Agrawal and Kurashige, 2003; Steppuhn et al., 2004).

Gene expression: the basic process of plant defence

The collection of genes that are expressed, also referred to as the transcriptional profile, is a major determinant of the plant phenotype and, as a consequence, also determines defence mechanisms. Constitutive expression of genes results in preformed defences, whereas the induced expression of genes is responsible for the activation of additional defence mechanisms. DNA microarrays are excellent tools to monitor simultaneously the expression of thousands of genes (Duggan et al., 1999;

Lockhart and Winzeler, 2000; Meyers et al., 2004). The two-colour hybridization strategy is often used with microarrays and involves the co-hybridization of two samples that are labelled with two different fluorescent dyes. By using this strategy it is possible to compare gene expression levels under two different conditions, for example undamaged versus herbivore-damaged plants. Microarray analysis have been used to identify genes responsive to feeding by several herbivorous insects (Rishi et al., 2002; Hui et al., 2003; Korth, 2003; Reymond et al., 2004; Voelckel and Baldwin, 2004; De Vos et al., 2005; Thompson and Goggin, 2006; Smith and Boyko, 2007). Gene expression levels, either constitutive or induced, can also be analysed to compare the transcriptional profiles of different genotypes within a plant species (Becher et al., 2004; Walia et al., 2005; Wang et al., 2008).

In response to herbivore feeding, plants adapt their transcriptional profile by differentially regulating genes. It appears that different attackers can activate different transcriptional responses in plants (Walling, 2000). For example, chewing *P. rapae* larvae elicit a completely different transcriptional response in *A. thaliana* than the phloem-feeding *Myzus persicae*, quantitatively as well as qualitatively (De Vos et al., 2005). Even herbivores with the same feeding strategy can induce different transcriptional changes as shown for *A. thaliana* in response to feeding by aphids (*M. persicae*) and whiteflies (*Bermicia tabaci*) (Kempema et al., 2007). Lepidopteran herbivores elicit changes in the expression of genes involved in glucosinolate metabolism, detoxification, cell survival, and signal transduction (Reymond et al., 2004). Conversely, aphids have been shown to regulate the expression of genes involved in e.g. cell wall modifications, oxidative stress, calcium-dependent signalling, and glucosinolate synthesis (Thompson and Goggin, 2006).

Most plant defence responses are activated by signal-transduction pathways that require jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) as signalling molecules (Kessler and Baldwin, 2002; Pieterse and Dicke, 2007). These plant hormones activate the expression of specific sets of defence-related genes. There appears to be a considerable level of integration between signals from these pathways, either positively or negatively (Rojo et al., 2003; Bostock, 2005; Beckers and Spoel, 2006). The major signal transduction pathway involved in plant responses to herbivores is the JA pathway (Liechti and Farmer, 2002). JA is an oxylipin signalling molecule derived from linolenic acid (Browse, 2005) and accumulates in plants upon wounding and herbivory (Creelman and Mullet, 1997; Reymond et al., 2000; Kessler and Baldwin, 2002; De Vos et al., 2005). JA regulates hundreds of JA-responsive genes that may be involved in defence against herbivores (Reymond et al., 2000; Schenk et al., 2000; Devoto et al., 2005). Studies on *A. thaliana* mutants deficient in JA synthesis or JA perception demonstrated that JA is essential for defence against some insects (McConn et al., 1997; Stotz et al., 2002; Van Poecke and Dicke, 2002; Reymond et al., 2004). Jasmonates activate biosynthetic pathways and result in an increase in total glucosinolate concentration, primarily because of changes in indole glucosinolate concentrations (Kliebenstein et al., 2005). Phloem-feeding insects only briefly puncture cells during their search for the phloem, thereby activating the SA and, to a lesser extent, the JA pathway (Walling, 2000; Moran and Thompson, 2001; Moran et al., 2002; Zhu-Salzman et al., 2004; De Vos et al., 2005; Thompson and Goggin, 2006; De Vos et al., 2007; Kuśnierczyk et al., 2007; Smith and Boyko, 2007). SA is a signalling molecule involved in local defence as well as the induction of systemic resistance (Reymond and Farmer, 1998). Interestingly, aphid bioassays

on *A. thaliana* mutant lines with altered JA or SA signalling suggest that JA-mediated responses limit aphid population growth, whereas SA does not influence or even has a positive effect on aphid performance (Thompson and Goggin, 2006). These results are consistent with the suggestion that phloem-feeding herbivores, such as aphids and whiteflies, manipulate plant responses by activating SA-signalling genes to repress more effective JA-signalling defence genes (Zhu-Salzman et al., 2004; Zhu-Salzman et al., 2005; Thompson and Goggin, 2006; De Vos et al., 2007; Zarate et al., 2007; Gao et al., 2008).

Intraspecific plant variation

Within a species, phenotypic variation among accessions results in differences in for example herbivore performance. Several studies on intraspecific variation link herbivore performance data to metabolomic analysis (Hopkins et al., 1998; Moyes et al., 2000; Kliebenstein et al., 2002). For example, performance and preference of *Mamestra brassicae* was different between two *Barbarea vulgaris* populations that differed in glucosinolate profile (Van Leur et al., 2008). Intraspecific variation in transcription of particular genes is responsible for differences in phenotypic traits (Carroll, 2000) and has been shown to result in differences in herbivore resistance (Gao et al., 2008) or secondary metabolite production (Wu et al., 2008). However, only few studies link intraspecific differences in transcriptional responses on the whole genome level to investigations at the individual or population level of herbivores (Kuśnierczyk et al., 2007; Gao et al., 2008).

Intraspecific variation in plant traits, caused by gene expression, may influence the composition and diversity of herbivore communities on plants grown in the field (Wimp et al., 2005; Whitham et al., 2006). For example, *N. attenuata* plants that were disrupted in the expression of a key gene of the JA pathway harboured larger numbers of herbivores and were infested with a species that was never recorded on control plants before (Kessler et al., 2004). However, nothing is known about the influence of naturally occurring intraspecific transcriptional variation on herbivore community composition in the field.

Interspecific plant variation among cultivated and wild species

Plant phenotypic traits differ more among accessions of different species than among accessions of the same species. Interspecific variation in the performance of herbivores has been well studied, for example for aphids (Ellis et al., 2000; Alvarez et al., 2006; Ranger et al., 2007) and lepidopteran larvae (Gols et al., 2008). Different species from the same plant family can have contrasting life-histories or specific morphological characteristics, which is the case in the Brassicaceae family. For example, *Brassica oleracea* has smooth, waxy leaves and is mostly biennial, whereas *B. nigra* is an annual plant with hairy leaves. These differences may result in the use of different defence strategies against herbivorous insects. Therefore, studying interspecific plant variation can provide useful information to better understand plant-herbivore interactions. Cultivated species and their wild relatives provide good systems to that purpose. Cultivation has given rise to several important brassicaceous crops such as cabbages (*B. oleracea*). Breeding for particular yield- and quality-enhancing traits often resulted in the disruption of original defence strategies that were present in wild progenitors (Rosenthal and Dirzo, 1997). As a consequence, cultivated plants usually have reduced levels of certain secondary

compounds (Evans, 1993). For example, glucosinolate levels in leaves of undamaged plants are higher in wild than cultivated *B. oleracea* (Gols et al., 2008). Interspecific variation among wild and cultivated accessions within a plant family has been shown for herbivore resistance (Ellis et al., 2000; Jensen et al., 2002; Ranger et al., 2007; Gols et al., 2008). However, no studies on these variations in plant defence against herbivores have so far linked whole-genome transcriptional analysis with ecological data. Such an integrated approach will provide new insight into plant-herbivore interactions.

From gene to ecosystem

As mentioned above, intraspecific variation in plant traits may influence herbivore performance. Consequently, the composition and diversity of herbivore communities on plants grown under natural conditions may also be co-determined by phenotypic variation (Wimp et al., 2005; Whitham et al., 2006). To gain insight into the ecological consequences of intraspecific variation in transcriptional profiles, field studies are needed. However, most transcriptomic studies on plant-herbivore interactions have been performed in glasshouses in which plants were grown under carefully controlled conditions and exposed to a single attacker. In their natural environment, plants are exposed to multiple herbivores and pathogens sequentially or simultaneously that may interact through induced plant responses (Agrawal, 2000; Traw and Dawson, 2002b; Kessler and Baldwin, 2004; De Vos et al., 2006b; Halitschke et al., 2008; Zheng and Dicke, 2008). Responses induced by one attacker may affect the behaviour and performance of other species. For example, increased concentrations of secondary metabolites and proteinase inhibitors in tobacco plants attacked by the mired bug (*Tupiocoris notatus*) resulted in reduced performance of the tobacco hornworm (*M. sexta*) (Kessler and Baldwin, 2004). Similarly, root feeding herbivores induced systemic defence responses against shoot herbivores in *B. nigra* (Van Dam et al., 2005). Besides affecting herbivore performance, induced plant responses may also affect host plant selection behaviour of subsequently colonizing herbivores (Shiojiri et al., 2002; Long et al., 2007; Bruinsma et al., 2008) For example, tomato plants damaged by *Macrosiphum euphorbiae* aphids were preferred for oviposition by *Spodoptera exigua* moths, and the larvae gained more weight on aphid-infested plants than on non-induced plants (Rodriguez-Saona et al., 2005). Furthermore, herbivore-induced plant volatiles have been shown to attract certain herbivores to the plant (Bolter et al., 1997; Kalberer et al., 2001).

The step from the glasshouse to the field has not often been made and it is therefore largely unclear whether results obtained from glasshouse experiments are also useful in the field. One of the studies that did take this step showed that disruption of a key gene in the JA pathway of tobacco had a significant effect on herbivore community composition (Kessler and Baldwin, 2004). A few other studies examined global transcriptional responses after experimental manipulation of field-grown plants. These studies monitored differential gene expression after plants had been exposed to methyl jasmonate (Schmidt and Baldwin, 2006), simulated *M. sexta* herbivory (Izaguirre et al., 2003), or the Japanese beetle *Popillia japonica* (Casteel et al., 2008). However, none of these studies investigated intraspecific variation in transcriptional profiles or herbivore community composition.

Research aim and thesis outline

Most transcriptional profiling studies have focussed on the model plant *A. thaliana* for which full-genome microarrays and an extensive mutant collection are available (Pieterse and Dicke, 2007). However, to investigate the effects of gene expression on community ecology, other crucifers are more suitable because *A. thaliana* and many herbivorous insects are active in different time windows (Yano and Ohsaki, 1993). White cabbage (*B. oleracea* var. *capitata*) is an economically important crop that shares about 85% sequence identity with *A. thaliana* (Cavell et al., 1998). This allows the use of the *A. thaliana* genetic toolbox to investigate transcriptomics in white cabbage and other *Brassica* species.

This project is part of a research programme which aims to link variation in plant defence to higher trophic level biodiversity. This programme focussed on integrating (1) transcriptomic, (2) metabolomic, and (3) ecological approaches. In this thesis, I present the data from a transcriptomic and ecological approach to identify and study the expression of plant genes in relation to herbivory and plant defence (Figure 2).

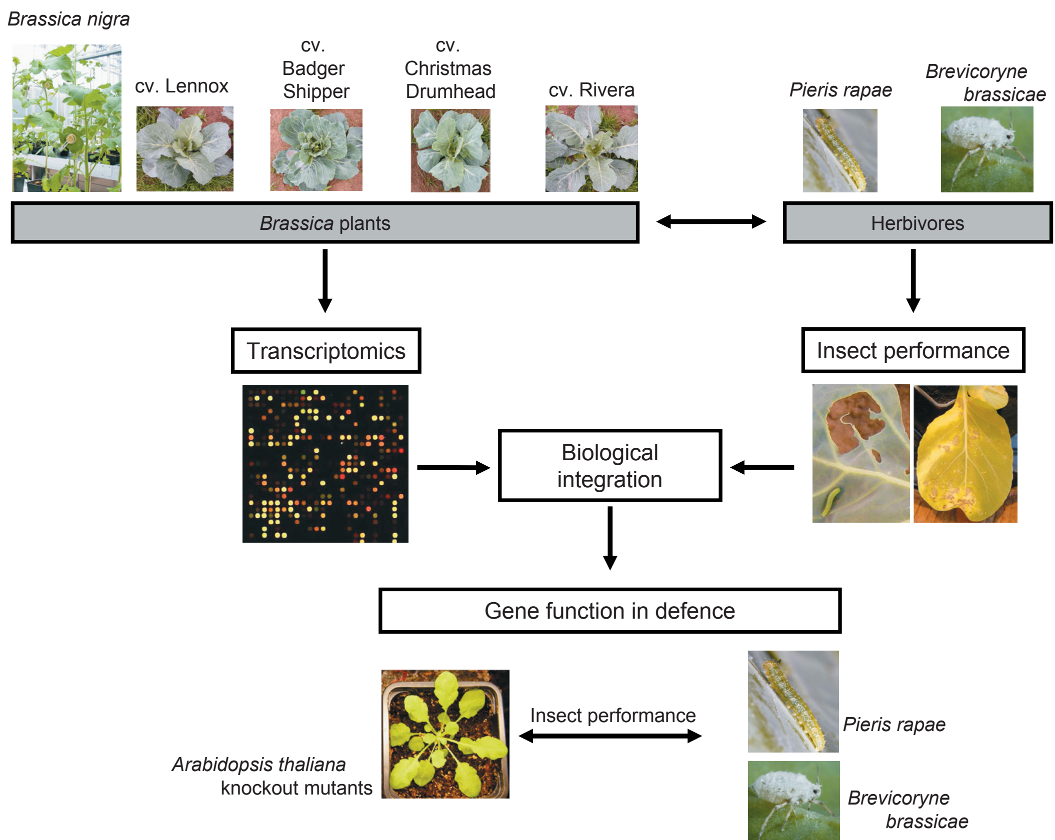

Figure 2. Transcriptomic and ecological approach to identify and study the expression of plant genes in relation to herbivory (foto's cutlivars: Erik Poelman, insecten: Tibor Bukovinszky)

My aim was to identify genes that are involved in the defence of *Brassica* species against *P. rapae* and *B. brassicae* by using the occurrence of intra- and interspecific variation between plants. I characterized phenotypic differences in the susceptibility of cultivated *B. oleracea* accessions and a wild *B. nigra* population and linked that to herbivore-induced transcriptional responses. Genes regulated in response to these herbivores were identified using an *A. thaliana* 70-mer oligonucleotide microarray. This microarray has been demonstrated to be effective for analyzing global gene expression in *B. oleracea* (Lee et al., 2004).

In Chapter 2, the transcriptional responses of the two *B. oleracea* cultivars Rivera and Christmas Drumhead to feeding by larvae of the cabbage white butterfly *P. rapae* are compared in order to identify genes that are potentially involved in inducible direct defence. In addition, the contribution of jasmonate-dependent and jasmonate-independent genes to this response is investigated.

In Chapter 3, I studied the interaction between four *B. oleracea* cultivars and the cabbage aphid, *B. brassicae*. Aphid performance is examined under both glasshouse and field conditions on the cultivars Rivera, Lennox, Christmas Drumhead and Badger Shipper to assess the relative levels of susceptibility to *B. brassicae*. Transcriptional responses to *B. brassicae* infestation are studied in cultivars Rivera and Christmas Drumhead, which supported respectively low and high numbers of aphids. Furthermore, I study the expression behaviour of two *B. brassicae*-responsive genes in all four cultivars and examine their effect on aphid performance in *A. thaliana* T-DNA insertion mutants.

Chapter 4 describes the transcriptional responses of a wild *B. nigra* population to feeding by the two specialist herbivores *P. rapae* and *B. brassicae.* These transcriptional responses are compared to those elicited in the two *B. oleracea* cultivars Rivera and Christmas Drumhead to interpret interspecific variation of inducible responses to specialist herbivores. Additionally, interspecific variation in aphid performance between *B. nigra* and the two *B. oleracea* cultivars is identified.
In Chapter 5, I address the question whether differences in gene expression affect the abundance and composition of herbivores on the *B. oleracea* cultivars Rivera and Christmas Drumhead in the field. Naturally occurring herbivores were monitored on field-grown plants of both cultivars early, i.e. four weeks after transplanting, and nine weeks later in the season. Microarray analyses were performed on material collected from the same plants.

Finally, Chapter 6 summarises the most important results of the studies in this thesis and discusses them with reference to other results from the research programme. Furthermore, future perspectives are discussed in this chapter.

Acknowledgements

I thank Roeland Voorrips, Ben Vosman and Marcel Dicke for valuable suggestions that helped to improve this chapter.

Chapter 2

Genotypic variation in genome-wide transcription profiles induced by insect feeding: *Brassica oleracea* – *Pieris rapae* interactions

Colette Broekgaarden, Erik H. Poelman, Greet Steenhuis, Roeland E. Voorrips, Marcel Dicke & Ben Vosman

BMC Genomics (2007) 8: 239

Abstract

Transcriptional profiling after herbivore attack reveals, at the molecular level, how plants respond to this type of biotic stress. Comparing herbivore-induced transcriptional responses of plants with different phenotypes provides insight into plant defence mechanisms. Here, we compare the whole-genome gene expression patterns induced by *Pieris rapae* caterpillar attack in two white cabbage (*Brassica oleracea* var. *capitata*) cultivars. These two cultivars were shown to differ in their level of direct defence against caterpillar feeding. Because *Brassica* full genome microarrays are not yet available, 70-mer oligonucleotide microarrays based on the *Arabidopsis thaliana* genome were used for this non-model plant.

The transcriptional responses of the two cultivars differed in timing as characterized by changes in their expression pattern after 24, 48 and 72 hours of caterpillar feeding. In addition, they also differed qualitatively. Surprisingly, of all genes induced at any time point only one third was induced in both cultivars. Analyses of transcriptional responses after jasmonate treatment revealed that the difference in timing did not hold for the response to this phytohormone. Additionally, comparisons between *P. rapae*- and jasmonate-induced transcriptional responses showed that this herbivore induced more jasmonate-independent than jasmonate-dependent genes.

The present study clearly shows that whole-genome transcriptional responses in two cultivars of the same plant species in response to insect feeding can differ dramatically. Several of these differences involve genes that are known to have an impact on *P. rapae* performance and probably underlie different mechanisms of direct defence present in the cultivars.

Introduction

In nature, plants are constantly surrounded by herbivorous insects that negatively influence plant fitness. To effectively combat them, plants have evolved direct and indirect defence mechanisms (Karban and Baldwin, 1997; Paré and Tumlinson, 1999; Dicke and Hilker, 2003). Chemical compounds that play a role in direct defence are produced and stored in tissues of the plant that are consumed by herbivores (Van Dam et al., 2000; Harvey et al., 2003). These compounds can alter the physiology of herbivores by reducing their growth rate, adult size, and survival probability (Harvey et al., 2003). Glucosinolates, for example, are well characterized defence compounds of cruciferous plants that are hydrolyzed by specific thioglucosidases called myrosinases. This reaction results in the release of an array of toxic compounds such as isothiocyanates (Rask et al., 2000) that reduce herbivore survival, growth, and development rate (Agrawal and Kurashige, 2003). In contrast to direct defence mechanisms, indirect defence mechanisms promote the effectiveness of the natural enemies of herbivores e.g. through volatile secondary metabolites (Vet and Dicke, 1992; Dicke et al., 2003). Direct and indirect defence mechanisms can function additively against an herbivore. A slower herbivore growth can prolong the time that the herbivore is exposed to a predator or parasitoid (Simms and Fritz, 1990). Kessler and Baldwin (2004) showed that a combination of direct and indirect defence mechanisms of *Nicotiana attenuata* resulted in additional mortality of *Manduca sexta* larvae. Direct and indirect defence mechanisms can be constitutively present or induced upon herbivore attack (Karban and Baldwin, 1997; Baldwin, 1998).

Inducible defence mechanisms involve the activation of a set of genes in response to herbivore attack. DNA microarrays are excellent tools to elucidate the role of these genes in plant defence (Rishi et al., 2002; Korth, 2003). These tools have been extensively exploited to investigate inducible defences in *Arabidopsis thaliana*. *Pieris rapae* feeding on this model plant, for example, induces more than 100 genes that are potentially involved in defence (Reymond et al., 2004). Additionally, similar expression patterns in response to feeding by *P. rapae* and *Spodoptera littoralis* caterpillars have been found (Reymond et al., 2004). Mechanical damage induces a different transcriptional profile than *P. rapae* feeding (Reymond et al., 2000). Attack by the phloem feeding aphid *Myzus persicae* results in the differential expression of many more genes than feeding by the caterpillar *P. rapae*: 2181 versus 186 genes (De Vos et al., 2005).

Despite the availability of several accessions of *A. thaliana*, the studies on *A. thaliana*-insect interactions mentioned above have been performed for only one genotype (Columbia-0). No comparative information is available on the natural variation of global transcriptional responses of different genotypes within one species of the Brassicaceae family.

The most important signal transduction pathway involved in inducible defence mechanisms of plants against chewing-biting insects is the jasmonate pathway (Liechti and Farmer, 2002). Jasmonates are a family of lipid regulators that include jasmonic acid (JA), an oxylipin signalling molecule derived from linolenic acid (Browse, 2005). JA accumulates in response to insect attack, resulting in the regulation of distinct sets of genes (Reymond et al., 2004; De Vos et al., 2005). Studies on *A. thaliana* and tomato mutants deficient in JA synthesis or JA perception demonstrated that JA is essential

for defence against some insects and mites (Howe et al., 1996; McConn et al., 1997; Thaler et al., 2002; Van Poecke and Dicke, 2002; Ament et al., 2004). Accumulation of JA can also be evoked by mechanical wounding alone (Reymond et al., 2000).

Here, we compare the transcriptional responses of two *Brassica oleracea* cultivars upon feeding by larvae of *P. rapae*. Genes regulated in response to this chewing-biting insect were identified using an *A. thaliana* 70-mer oligonucleotide microarray. These microarrays have been demonstrated to be effective for analyzing global gene expression in *B. oleracea* (Lee et al., 2004). We aimed at characterizing genes that are potentially involved in inducible direct defence by comparing transcriptional responses of the *B. oleracea* cultivars Rivera and Christmas Drumhead. In addition, the contribution of jasmonate-dependent and jasmonate-independent genes in the response of *B. oleracea* to *P. rapae* attack was investigated. Our results show the existence of clear genotypic differences in direct defence and in transcriptional responses between cultivars of *B. oleracea*.

Materials and Methods

Plant growth and treatments

Seeds of white cabbage (*Brassica oleracea* var. *capitata*) cultivars Rivera and Christmas Drumhead were germinated in potting compost (Lentse Potgrond®). Seeds of Rivera (an F1 hybrid cultivar) were obtained from Bejo Zaden B.V. (Warmenhuizen, the Netherlands), whereas seeds from the open-pollinated cultivar Christmas Drumhead were obtained from the Centre of Genetic Resources, the Netherlands (CGN). Plants were grown in September. Two-week old seedlings were transferred to 1.45 L pots containing the same potting compost. Plants were cultivated in a greenhouse compartment with a 16 h day and 8 h night period (22 ± 2 ^{o}C). The relative humidity was maintained at 60 to 70 %. Plants were watered every other day. No chemical control for pests and diseases was performed.

Larvae of the small cabbage white butterfly *Pieris rapae* were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv. Cyrus) in a growth chamber with a 16 h day and 8 h night cycle (21 ± 2 ^{o}C, 50-70% relative humidity). Seven-week old plants of Rivera and Christmas Drumhead were infested with *P. rapae* by transferring 10 first-instar larvae to the youngest, fully expanded leaf of each plant using a fine paintbrush. At 6, 24, 48 and 72 h since the start of caterpillar feeding, a disc (diameter 2.3 cm) of the infested leaf from each of 12 individual plants was collected. Leaf discs were pooled and immediately frozen in liquid nitrogen.

An induction treatment with jasmonic acid (JA) was performed by gently rubbing the youngest, fully expanded leaf with 0.5 ml of a solution containing 5 mM JA (Sigma) and 0.1% Triton X-100 (Acros Organics) with a latex-gloved finger. The Triton X-100 was added to facilitate application to the leaf surface and absorption by the cuticle (Bodnaryk, 1994; Ludwig-Müller et al., 1997). Despite the low pH (3.3) of the solution, we did not observe any direct effects on the leaves on which the hormone was applied. Furthermore, we treated a control group of 12 plants with 0.5 ml of 0.1% Triton X-100 (pH 3.3) alone. Material from JA-treated and control plants was collected at 6 h after treatment as described above.

The whole experiment was performed in threefold to obtain 3 biological replicates.

Insect feeding trials

The effect of plant cultivar on *P. rapae* performance was studied using first-instar larvae. Rivera and Christmas Drumhead plants were grown as described above. Ten larvae were placed on individual eight-week old plants. Plants were placed on tablets in a greenhouse compartment (16/8 h day/night period at 22 ± 2 ^{o}C) and isolated from each other by a layer of water on the tablet to prevent larvae from moving to neighbouring plants. After 6 days of feeding, larvae were recollected and weighed separately to the nearest 0.01 mg. After weighing, larvae were placed back on the plants they originated from. They were subsequently monitored for development and time to reach pupation. Once a larva pupated, the date of pupation was recorded, and the pupa was collected and weighed. The whole experiment was performed in tenfold to obtain 10 biological replicates.

Microarray hybridizations

Total RNA was isolated from material of biological replicates separately by using TRIzol reagent (Invitrogen) and purified using the RNaesy MinElute kit (Qiagen). Glass microarray slides carrying 70-mer oligonucleotide probes based on the *Arabidopsis thaliana* genome (obtained from the group of David Galbraith from the University of Arizona, http://www.ag.arizona.edu/microarray) were used in hybridizations. For target labelling, 4 µg of total RNA were linearly amplified in the presence of 5-(3-aminoallyl)-UTP using the MessageAmp™ aRNA kit (Ambion). Cy3 and Cy5 mono-reactive dyes (Amersham) were coupled to the amplified RNA (aRNA) in freshly made 0.2 M sodium carbonate buffer (pH 9.0) for 1 h at room temperature. Labelling of aRNA was monitored by measuring the Cy3 and Cy5 fluorescence emissions using a nanodrop ND-1000 UV-Vis Spectrophotometer (BioRad). Immobilization of the oligonucleotide array elements was performed as described at the manufacturer's website (see above). After applying 80 µl of hybridization mixture containing (heat-denatured) labelled targets (100 pmol Cy3-labeled aRNA from control plants and 50 pmol Cy5-labeled aRNA from treated plants), slides were hybridized for 12 h at 50 ^{o}C and then washed at room temperature down to 0.05x SSC. As a control for the JA treatment, aRNA from JA treated plants (coupled to Cy3) was hybridized to aRNA from Triton X-100 treated plants (coupled to Cy5).

Microarray data analysis

Slides were scanned separately for the two fluorescent dyes using a ScanArray™ Express HT Scanner (PerkinElmer). Median fluorescence intensities for each fluor and each gene were determined using the ScanArray Express program (PerkinElmer). Array images were checked manually to exclude spots with an aberrant shape or spots located in a smear of fluorescence from the data. Median background fluorescence around each spot was calculated and subtracted from each spot. Spots with adjusted intensities lower than half the background were manually raised to half the background to avoid extreme expression ratios. Spots where the difference between spot and background median intensity was below half the background intensity for both dyes were removed from the analysis. The resulting text files were converted by ExpressConverter ver 1.5 to generate co-ordinated MEV and ANN files. MEV files were processed through TIGR-MIDAS ver 2.18. To avoid spatial bias, Lowess (Locfit) normalization was carried out within each slide in such a way that the distribution of $\log_2$ ratios within each subgrid had a median of zero (Yang et al., 2002). Normalized signal intensities were used to calculate expression ratios.

Statistical analyses were carried out using TIGR-MEV ver 3.0.3. A one class Student *t*-test on log_2-transformed expression ratios was conducted for each experimental condition. For all of the experiments, genes with a log_2-transformed expression ratio ≥ 1 or ≤ -1 and a *P*-value < 0.05 were considered significantly induced or repressed. We used the names of *A. thaliana* homologs to identify *B. oleracea* genes.

Quantitative RT-PCR

Quantitative RT-PCR analyses were performed using the same pooled samples used for microarray hybridizations. One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by qRT-PCR using primers of the constitutively expressed gene *GAPDH* (*GAPDH*-LEFT; 5'-AGA GCC GCT TCC TTC AAC ATC ATT-3'; *GAPDH*-RIGHT; 5'-TGG GCA CAC GGA AGG ACA TAC C-3'). Gene-specific primers were designed for five *B. oleracea* genes. The corresponding AGI codes of the *A. thaliana* homologs and primers are At1g27130, LEFT 5'-ATT GGA TCA GTC CAG GTG TTG-3', RIGHT 5'-AGC TGG AAA GCT GAT GGA GA-3', At1g47540, LEFT 5'-CTG AAA GAA TAC GGA GGC AAC-3', RIGHT 5'-AAT ACC GCC ACT TAG AAT CTG G-3'; At1g72290, LEFT 5'-TGG TGA CAA GTA GCT GTG GTG-3', RIGHT 5'-TCC AAG TTA TGG GCA GTG G-3'; At3g45140 (*LOX*), LEFT 5'-CTT TGC TCA CAT ACG GTA GAA GC-3', RIGHT 5'-CCT TTG CAT TGG GCT AGT TC-3' (marker gene for JA pathway); At4g31500, LEFT 5'-CCG GAA TAT CAT AGC CAC CTA TC-3', RIGHT 5'-CCT GAA GCA ATG AAG AAA GCT C-3'. Quantitative RT-PCR analysis was done in optical 96-well plates with a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x IQ SYBR Green Supermix reagent (Bio-Rad, Veenendaal, the Netherlands), 10 ng cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR reactions were performed in duplicate. The following PCR program was used for all PCR reactions: 95 ^{o}C for 3 min; 40 cycles of 95 ^{o}C for 30 sec and 60 ^{o}C for 45 sec. C_T (threshold cycle) values were calculated using Optical System Software, version 2.0 for MyIQ (Bio-Rad, Veenendaal, the Netherlands). Subsequently, C_T values were normalized for differences in cDNA synthesis by subtracting the C_T value of *GAPDH* from the C_T value of the gene of interest. Normalized gene expression was than obtained from $2^{-\Delta CT}$. Normalized gene expression values were used to calculate log_2-transformed expression ratios for each experimental condition. A one class Student *t*-test on log_2 transformed ratios was conducted for each experimental condition using TIGR-MEV version 3.0.3. Quantitative RT-PCR products were resolved on agarose gel and gene identities were confirmed by sequencing.

Results

Larval performance on cultivars Rivera and Christmas Drumhead

The white cabbage (*B. oleracea*) cultivars Rivera and Christmas Drumhead were characterized for larval performance of *P. rapae*. We found that *P. rapae* larvae feeding on Rivera had a significantly lower weight after six days than those feeding on Christmas Drumhead plants (Mann-Whitney U test, $P = 0.001$; Figure 1A), indicating slower growth of *P. rapae* larvae on Rivera. Larvae feeding on Rivera pupated around 2.5 days later than those feeding on Christmas Drumhead plants ($P = 0.005$; Figure 1B). Such retardation in developmental period has large consequences for population growth rates (Birch, 1948). However, larvae feeding on either cultivar did not differ significantly in pupal weight ($P = 0.376$; Figure 1C). The results showed that direct defence against *P. rapae* larvae was more pronounced in Rivera than in Christmas Drumhead plants.

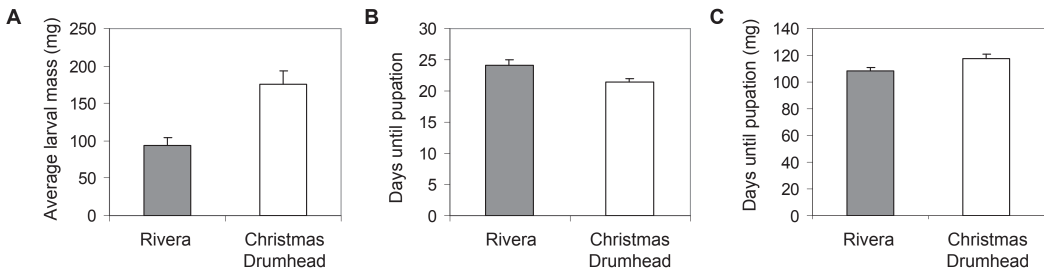

Figure 1 Performance of *P. rapae* larvae on two *B. oleracea* cultivars. A, Larval weight (mean + SE) after 6 days of feeding. B, Time to reach pupation (mean + SE). C, Pupal weight (mean + SE) just after pupation.

Statistical analyses of *P. rapae*-regulated genes in cultivars Rivera and Christmas Drumhead

Because Rivera and Christmas Drumhead displayed different levels of direct defence against *P. rapae* larvae, transcriptional responses to feeding by this insect species were monitored to identify genes that may contribute to inducible direct defence. For this purpose, microarray analyses were performed in which genes were considered to be differentially expressed when they showed an expression ratio ≥ 2-fold or ≤ 0.5-fold with a statistical significance of $P < 0.05$ (Student's *t*-test).

For several genes the induction was highly significant ($P < 0.01$), although their expression change was between 1.5- and 2-fold. On the other hand, a number of genes showed at least a 2-fold change in all three replicates, but a *P*-value above 0.05 because of the large variation between replicates. These genes are potentially interesting candidates that would require careful investigation to determine whether their expression changes have biological relevance. However, these potentially interesting candidates were not considered as differentially expressed in this study.

Transcriptional responses of cultivars Rivera and Christmas Drumhead to *P. rapae* feeding

When comparing unchallenged plants with plants that had been attacked by *P. rapae* for 24 h, 99 genes had at least a 2-fold change in expression level with a *P* value below 0.05 in Christmas Drumhead. Of these 99 genes, 63 were induced and 36 were repressed (Figure 2). Remarkably, no

genes met our selection criteria for induction or repression in Rivera after 24 h of *P. rapae* attack, although two genes showed an expression ratio ≥ 2-fold in two replicates and almost 2-fold (1.9) in the third replicate. These potentially induced genes included *Lipoxygenase 2* (At3g45140) and a gene encoding a trypsin-and-protease inhibitor (At1g72290). Both genes were significantly induced in Christmas Drumhead (Supplemental Table 1). Based on these results, we hypothesized that Rivera has a slower transcriptional response than Christmas Drumhead upon attack by *P. rapae*. To test this hypothesis, we analyzed expression changes in both cultivars after 48 h of *P. rapae* infestation. Indeed, we identified many differentially expressed genes in Rivera at this time point, consisting of 322 induced and 483 repressed genes (Figure 2). Many differentially expressed genes were also identified in Christmas Drumhead after 48 h of *P. rapae* feeding. In this cultivar, 254 induced and 83 repressed genes were identified (Figure 2). After 72 h of *P. rapae* attack, 215 genes were induced and 213 repressed in Rivera (Figure 2). In Christmas Drumhead, the number of differentially expressed genes after 72 h of caterpillar feeding increased to 292 induced and 144 repressed genes (Figure 2). When the larvae had fed for only 6 h, we did not find any genes to be differentially expressed in Rivera according to our selection criteria. In Christmas Drumhead, we only found a gene encoding a trypsin-and-protease inhibitor (At1g72290) to be induced at this time point (Supplemental Table 1). This suggests that after 6 h of larval feeding regulation of expression had not yet started or was not yet strong enough to be detected.

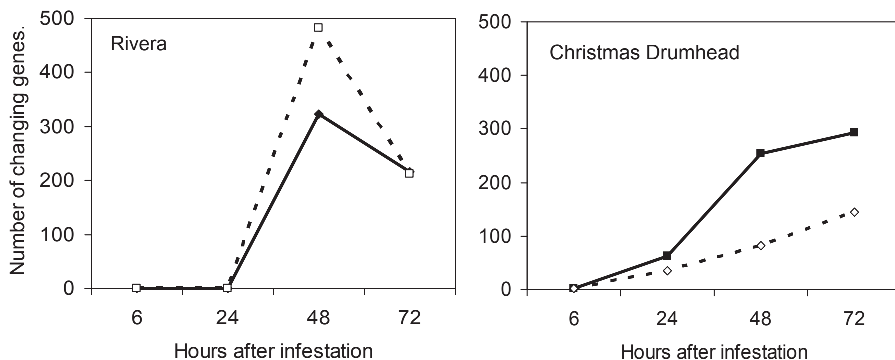

Figure 2 Gene expression changes in cultivars Rivera and Christmas Drumhead after *P. rapae* feeding. Number of expressed genes induced (closed symbols and solid line) and repressed (open symbols and dashed line) more than 2-fold and with $P < 0.05$ at the time points tested.

A comparison of the genes activated at the different time points tested in Rivera showed that 43% of the genes that were induced after 48 h were still induced after 72 h of feeding (Figure 3A). In Christmas Drumhead, 65% of the genes that were induced after 24 h were still up after 48 and even after 72 h of larvae feeding (Figure 3B). This illustrates a relatively long lasting induction for a large proportion of the genes.

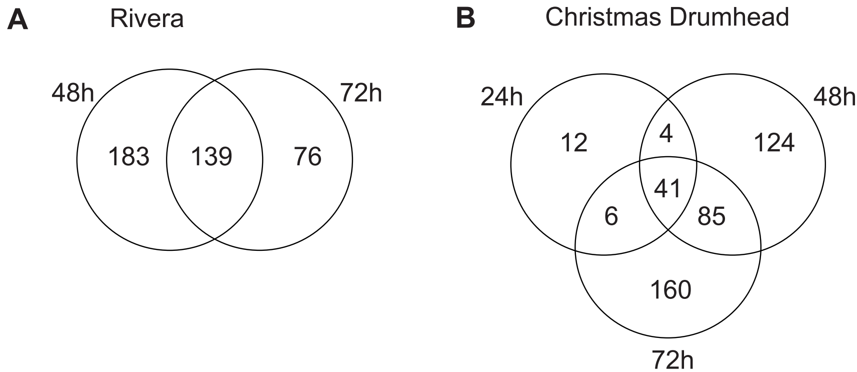

Figure 3 Comparison of gene induction over time after *P. rapae* feeding in cultivars Rivera and Christmas Drumhead. (A) Venn diagram representing the distribution in Rivera of transcripts activated after 48 and 72 h of *P. rapae* challenge. (B) Venn diagram representing the distribution in Christmas Drumhead of transcripts activated after 24, 48, and 72 h of *P. rapae* challenge. The numbers in the overlapping area indicate the shared number of genes in the comparisons and include genes with an average expression ratio ≥ 2-fold and a *P* value < 0.05 in both experiments. Numbers outside the overlapping area represent genes specifically induced at one time point.

The observation that Rivera has a stronger direct defence but a slower transcriptional response after *P. rapae* attack suggests that this cultivar may have a higher level of constitutive direct defence. To study this, we compared gene expression levels in control plants of both cultivars. After hybridizing Rivera against Christmas Drumhead control material, using the same selection criteria as described above, we identified 15 genes with a significantly higher constitutive expression in Rivera (Table 1). However, none of these genes is clearly associated with a higher constitutive level of direct defence.

Table 1 Genes with a higher constitutive expression in Rivera compared to Christmas Drumhead.

Probe identification and Putative Function	AGI Code	Number of Times Higher in Rivera	*P* value
Expressed protein	At1g15230	9.75 ± 1.42	0.010
Kelch repeat-containing F-box family protein	At1g60570	7.67 ± 1.17	0.009
Expansin (EXP1)	At1g69530	2.49 ± 1.26	0.020
La domain-containing protein	At1g79880	4.59 ± 1.31	0.011
60S ribosomal protein L23 (RPL23B)	At2g33370	6.12 ± 1.31	0.007
Expressed protein	At2g34690	2.19 ± 1.19	0.017
Protodermal factor 1 (PDF1)	At2g42840	3.10 ± 1.58	0.050
Acyl-[acyl-carrier-protein] desaturase	At2g43710	2.06 ± 1.10	0.006
Glycosyl hydrolase family 1	At3g18080	2.40 ± 1.39	0.044
Zinc finger (C3HC4-type RING finger) family protein	At4g01023	9.75 ± 1.31	0.005
Expressed protein	At4g01220	6.48 ± 1.47	0.014
Expressed protein	At4g37440	2.41 ± 1.39	0.044
Expressed protein	At5g09980	3.90 ± 1.04	0.013
Germin-like protein (GER3)	At5g20630	2.39 ±1.34	0.035
Expressed protein	At5g20935	5.54 ± 1.08	0.001

Relative difference in constitutive gene expression in Rivera compared to Christmas Drumhead measured in control plants. Mean expression ratios (± SD) were calculated from three biologically independent experiments. The *P* values denote the significant difference of the mean log_2-transformed ratios of unchallenged Rivera over unchallenged Christmas Drumhead plants.

Validation of microarray data

To validate the microarray data, we selected five defence-related genes that showed high expression changes in both cultivars at one or more of the tested time points, to be analyzed with quantitative real-time PCR (qRT-PCR). Figure 4 shows log_2 ratios of the five selected genes in Rivera and Christmas Drumhead as determined by both microarray and qRT-PCR analyses. For all genes, the log_2 ratios were larger using qRT-PCR compared with microarray. Although fold induction of gene expression, especially for low abundant mRNAs, has been shown to differ between the two methods (Czechowski et al., 2004), the qRT-PCR and microarray analyses showed similar expression patterns after *P. rapae* feeding in both cultivars (Figure 4) showing the reliability of the microarray data.

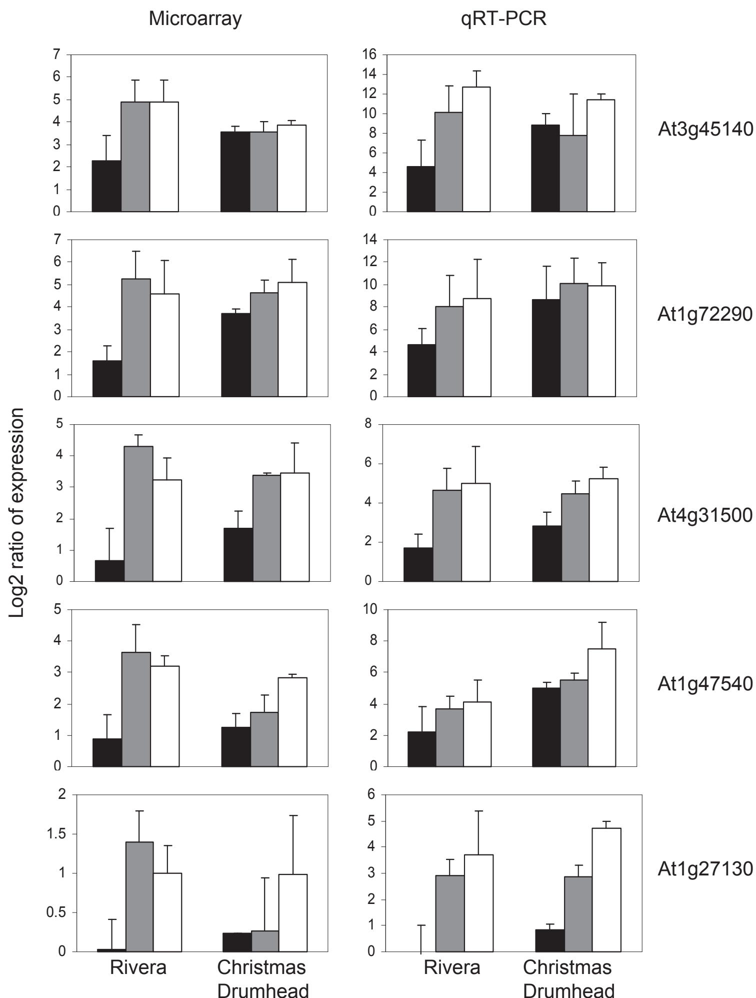

Figure 4 Comparison of microarray and qRT-PCR analysis of five genes. Log_2 ratios of five selected genes (At3g45140, At1g72290, At4g31500, At1g47540, and At1g27130) after infestation of Rivera and Christmas Drumhead by *P. rapae*. On the left, the log_2 ratio patterns from the microarray analysis. On the right, the log_2 ratio patterns from the qRT-PCR analysis. Black, gray and white bars represent log_2 ratios after 24, 48, and 72 h of *P. rapae* feeding, respectively. All bars contain their corresponding standard deviation.

Comparison of transcriptional changes upon *P. rapae* feeding

To investigate which *P. rapae*-induced genes could play a role in direct defence, the overlap in transcriptional responses in Rivera and Christmas Drumhead was analyzed. After 48 h of larval feeding, 64% of the 322 induced genes in Rivera were not induced in Christmas Drumhead. Furthermore, 54% of *P. rapae*-induced genes in Christmas Drumhead were not induced in Rivera at this time point (Figure 5). After 72 h of larvae feeding, 39% of the 215 induced genes in Rivera were not induced in Christmas Drumhead and 55% of *P. rapae*-induced genes in Christmas Drumhead were not induced in Rivera (Figure 5).

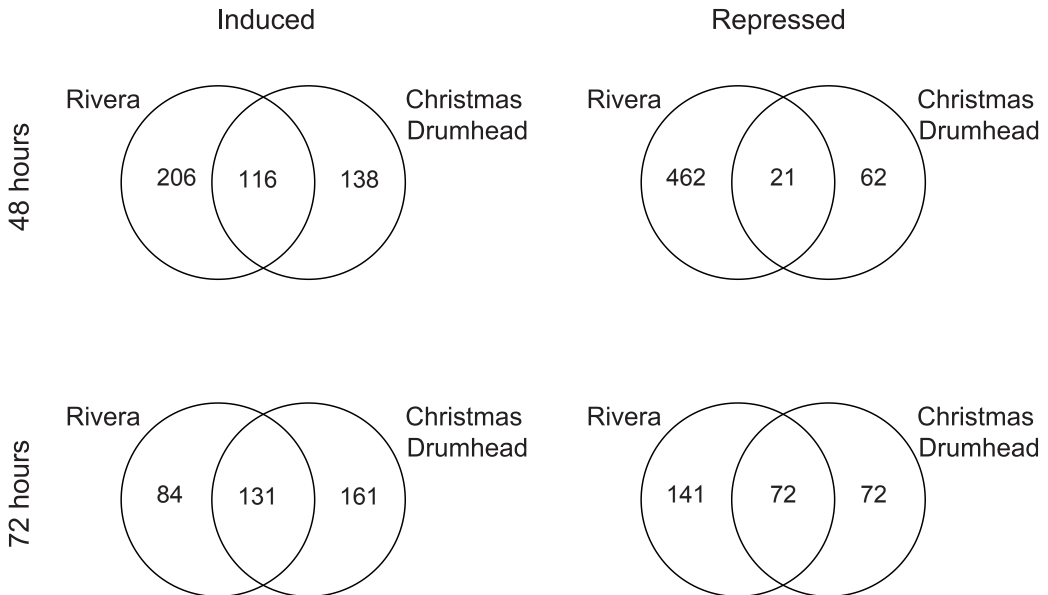

Figure 5 Gene expression in cultivars Rivera and Christmas Drumhead after *P. rapae* feeding. Venn diagrams representing the distribution of induced and repressed genes after 48 and 72 h of *P. rapae* feeding. The numbers in the overlapping areas indicate the shared number of genes in the comparisons and include genes with an average expression ratio ≥ 2-fold or ≤ 0.5-fold and a P value < 0.05 in both experiments. Numbers outside the overlapping area represent genes specifically induced or repressed in one cultivar.

When comparing the overlap between transcriptional responses after combining all tested time points, the data show that 44% of the genes induced in Rivera and 47% of the genes induced in Christmas Drumhead were not induced at any tested time point in the other cultivar (Figure 6). All induced genes were classified according to their putative functional categories. Induced genes that are known to be involved in defence in *A. thaliana* are listed in Table 2. The complete list of *P. rapae*-induced genes is given in Supplemental Table 1.

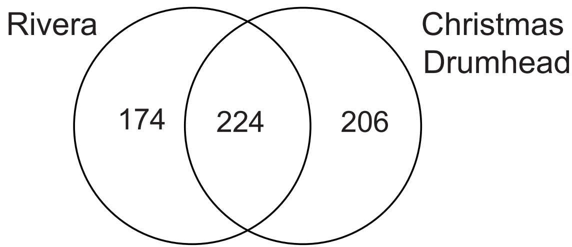

Figure 6 Gene induction in cultivars Rivera and Christmas Drumhead after *P. rapae* feeding. Venn diagram representing the distribution of induced genes when combining all time points tested. The number in the overlapping area indicates the shared number of genes in the comparisons and includes genes with an average expression ratio ≥ 2-fold and a *P* value < 0.05 in both experiments. Numbers outside the overlapping area represent genes specifically induced in one cultivar.

To check whether the overlap between the two cultivars was influenced by the stringency of our selection criteria, we performed statistical analyses using a 1.5-fold cut-off value while keeping the *P* value threshold at 0.05. With the less stringent method, 67% and 25% of *P. rapae*-induced genes in Rivera were only induced in this cultivar after 48 and 72 h, respectively. Based on these less stringent criteria for Christmas Drumhead, 55% and 73% of *P. rapae*-induced genes were induced only in this cultivar after 48 and 72 h, respectively. This indicates that the small overlap in transcriptional responses of the two cultivars is independent of threshold stringency for classifying genes as being induced.

The small overlap between regulated genes in Rivera and Christmas Drumhead does not apply only to induced genes but even more so to repressed genes. After 48 h of larval feeding, 96% of the genes repressed in Rivera were not repressed in Christmas Drumhead and 75% of the repressed genes in Christmas Drumhead were not repressed in Rivera (Figure 5). When larvae had fed for 72 h, 67% of the genes repressed in Rivera were not repressed in Christmas Drumhead and 50% of the repressed genes in Christmas Drumhead were not repressed in Rivera (Figure 5). A large proportion of the repressed genes in both cultivars are involved in photosynthesis and protein metabolism (Supplemental Table 1).

Role of JA in response to *P. rapae*

Several studies in *A. thaliana* have shown that a large percentage of *P. rapae*-inducible genes are under the control of the jasmonate pathway (Reymond et al., 2004; De Vos et al., 2005). To get more insight into the function of *P. rapae*-induced genes and their role in defence in *B. oleracea*, transcriptional responses to *P. rapae* were compared with those triggered by the application of JA. Within the same experiment as that for *P. rapae* induction, seven-week old plants were treated with JA and leaf material was collected after 6 hours. Using the selection criteria described above, we identified 46 genes in Rivera and 80 genes in Christmas Drumhead to be JA-inducible. The complete list of JA-induced genes is given in Supplemental Table 2. Comparison of JA-responsive genes with the *P. rapae*-induced genes revealed that less than 30% of the *P. rapae*-induced genes were responsive to JA in both cultivars. Our results suggest that *P. rapae* induced more jasmonate-independent than jasmonate-dependent genes.

Table 2. Defence-related genes induced after *P. rapae* feeding in cultivars Rivera and Christmas Drumhead.

		Rivera			Christmas Drumhead		
Probe Identification and Putative Function	AGI Code	24h	48h	72h	24h	48h	72h
Genes only induced in Rivera							
Basic endochitinase	At3g12500	1.12	2.54*	2.62	1.06	1.37	1.94
Cup-shaped cotyledon1 protein (CUC1)	At3g15170	1.07	1.63	2.21*	1.11	1.26	1.92
DNA-binding protein	At1g49950	1.35	0.34	2.25*	1.66	1.81	1.50
Glutathione S-transferase	At1g27130	1.02	2.64*	2.01*	1.17	1.21	1.98
Glycosyl hydrolase 1 (BG1)	At1g52400	1.14	2.79	11.00*	2.71	-[1]	6.45
Lectin	At5g35950	0.89	2.51*	1.32	1.02	1.28	1.79
MYB transcription factor	At1g71030	1.16	2.07*	1.60	1.26	1.06	1.79
Telomere repeat-binding protein	At3g46590	0.95	2.48*	1.41	1.24	0.87	1.51
Terpene synthase	At4g16730	1.13	4.15*	2.82*	1.16	1.26	1.97
Trypsin inhibitor	At2g43520	1.19	1.74	3.70*	1.51	-[1]	2.44
Genes only induced in Christmas Drumhead							
Cytochrome P450 71B15 (CYP71B15)	At3g26830	1.01	1.90	-[1]	1.32	1.33	3.51*
ERF domain protein 9 (ERF9)	At5g44210	1.08	1.47	-[1]	1.19	1.23	2.01*
Glutathione S-transferase (ERD9)	At1g10370	1.04	1.55	1.33	1.54	1.77	2.10*
IAA-amino acid hydrolase 3 (IAR3)	At1g51760	1.13	1.76	1.42	1.05	1.23	2.01*
Lectin	At3g16400	1.13	1.86	1.51	1.58	2.03*	1.78
Legume lectin	At1g53070	0.98	1.73	2.03	1.31	1.67	4.21*
MADS-box protein (AGL74)	At1g48150	1.42	0.44	1.40	1.87	2.79*	1.54
Terpene synthase	At5g23960	-[1]	-[1]	-[1]	1.27	1.15	4.29*
Tryptophan synthase β subunit 2 (TSB2)	At4g27070	1.03	1.50	1.19	1.30	2.00	2.94*
Vegetative storage protein 2 (VSP2)	At5g24770	-[1]	1.10	6.96	2.68	3.49	16.20*
Genes induced in both cultivars							
Allene oxide synthase (AOS)	At5g42650	1.71	3.27*	2.46*	1.72	2.08*	3.50*
Coronatine-responsive tyrosine aminotransferase	At4g23600	2.28	28.34*	10.11*	7.70*	7.89*	14.70*
Cysteine proteinase (RD21A)	At1g47128	1.02	2.06*	2.83*	1.83	2.66*	3.96*
Cytochrome b5	At2g46650	1.07	3.02*	1.71	1.18	3.13*	3.68
Cytochrome P450 79B2 (CYP79B2)	At4g39950	1.47	3.23*	4.45*	1.37	1.74	7.18*
Cytochrome P450 83B1 (CYP83B1)	At4g31500	1.59	19.92*	9.38*	3.23*	10.40*	10.99*
Ethylene-responsive element-binding protein	At5g07580	1.99	6.88*	1.89	3.17*	5.73*	7.37*
Glutathione S-transferase 6 (GST6)	At2g47730	1.06	2.03*	1.50	1.44	2.82*	2.40*
Hydroperoxide lyase (HPL1)	At4g15440	1.26	2.88*	2.05*	1.51	2.86*	3.75*
Lectin	At3g16470	1.71	3.67	15.33*	3.36	5.93*	7.21*
Lectin kinase	At3g45410	2.57	14.61*	4.53*	8.38*	3.88*	7.04*
Lipoxygenase (LOX2)	At3g45140	4.74	29.91*	29.27*	11.65*	11.89*	14.53*
MYB transcription factor (MYB49)	At5g54230	1.17	4.36*	1.96	1.13	1.63	6.29*
Myrosinase-associated protein	At1g54020	1.46	4.28*	5.01*	3.06*	2.22*	6.54*
Plant defensin-fusion protein (PDF2.3)	At2g02130	1.11	1.34	2.16*	1.76	2.92*	2.19*
Polygalacturonase inhibiting protein 2 (PGIP2)	At5g06870	1.09	3.37*	5.99*	3.04	5.31*	20.16*
Terpene synthase	At1g61120	1.69	3.48*	5.32*	3.37*	2.44*	3.04*
Trypsin inhibitor	At2g43530	1.59	2.66*	4.34*	3.25	2.11*	4.62*
Trypsin-and-protease inhibitor	At1g72290	3.03	38.70*	23.75*	13.18*	24.37*	34.11*
Tryptophan synthase α subunit (TSA1)	At3g54640	1.19	15.18*	6.72*	2.73	17.34*	12.69*
Tryprophan synthase β subunit 1 (TSB1)	At5g54810	0.94	5.48*	3.43*	1.34	3.91*	4.47

Relative changes in gene expression after challenge with *P. rapae* larvae were measured in Rivera and Christmas Drumhead plants. Mean expression ratios are calculated from three biologically independent replicates. Only genes known to be involved in defence in *A. thaliana* are shown.

*Fold change ≥ 2 with a *P*-value < 0.05.

[1]70-mer oligonucleotide did not hybridize in any of the three replicates.

AGI, Arabidopsis Genome Initiative.

Discussion

Arabidopsis thaliana oligonucleotide microarrays are applicable to _Brassica_ studies

In this study, we aimed at getting insight into the transcriptional responses of two *B. oleracea* cultivars after attack by larvae of the small cabbage white butterfly *P. rapae* by using full genome microarray analyses. *Brassica* is not yet fully sequenced and microarrays based on the *Brassica* genome are not yet available. Because of this, we decided to use microarrays containing 70-mer synthetic oligonucleotides based on the *A. thaliana* genome as these had been shown to be capable of recognizing related DNA sequences of *B. oleracea* (Lee et al., 2004). Overall, 90% of the oligonucleotides present on the microarray showed intensity signals after hybridization. Additionally, for five genes the data obtained from microarray analysis were validated using quantitative real-time PCR and showed to be reliable (Figure 4). In accordance with our results and the studies mentioned above, we expect that all species within the Brassicaceae can be analyzed with *A. thaliana* based oligonucleotide microarrays. Of course, genes specific for *Brassica* will not be detected using these microarrays.

Transcriptional responses differ between _Arabidopsis thaliana_ and _Brassica oleracea_

Given that *A. thaliana* and *B. oleracea* belong to the same plant family and show high sequence identity, we expected to identify a large number of *P. rapae*-induced genes from *A. thaliana* in *B. oleracea*. Reymond and co-workers (2004) performed a study on *A. thaliana* ecotype Col-0 in which they identified 111 *P. rapae*-induced genes (≥ 2-fold induction and P value < 0.05) using a microarray representing around 7200 *A. thaliana* genes. Another study, using the same *A. thaliana* ecotype, identified 128 induced genes with at least a 2-fold induction after both 12 and 24 h of *P. rapae* feeding using a full-genome Affymetrix ATH1 chip (De Vos et al., 2005). Both studies also investigated the transcriptional response upon application of methyl jasmonate (MeJA), a volatile derivative of JA. Interestingly, when comparing the two *A. thaliana* studies, only 9% of the *P. rapae*-induced and 3% of the MeJA-induced genes identified by Reymond and co-workers (2004) were also found to be induced in the study of de Vos and co-workers (2005). The fact that both studies used the same ecotype of *A. thaliana* suggests that the induction of genes is highly dependent on the environmental and experimental conditions used. Factors that might explain the small overlap between the two studies include: (1) different time points after infestation: 3 to 5 h in the study by Reymond and co-workers (2004) versus 12 and 24 h in the study by de Vos and co-workers (2005), and (2) different larval stages: fourth to fifth larval instar in the study by Reymond and co-workers (2004) versus first to second larval instar in the study by de Vos and co-workers (2005).

In comparison with our results, 16% of the *P. rapae*-induced genes identified by Reymond and co-workers (2004) in *A. thaliana* were also induced in *B. oleracea* when combining data for significantly induced genes in Rivera and Christmas Drumhead. Thirteen percent of the genes identified as induced by *P. rapae* in the study by De Vos and co-workers (2005) were also significantly induced in our study. When focusing on the overlap between JA-induced genes in *B. oleracea* and *A. thaliana*, we found that 19% of the JA-induced genes identified by Reymond and co-workers (2004) were also induced in *B. oleracea*. Of the JA-responsive genes in *A. thaliana* identified by de Vos and co-workers (2005),

9% were also induced by JA in *B. oleracea*. In contrast to the application of JA in our study, both *A. thaliana* studies sprayed MeJA to trigger the jasmonate pathway. The use of different derivatives of JA and the difference in application might contribute to the small overlap in induced genes between the studies.

Differences between cultivars Rivera and Christmas Drumhead

We observed differences in performance of *P. rapae* larvae that had fed for 6 days on Rivera and Christmas Drumhead (Figure 1), indicating a higher level of direct defence in Rivera. However, it is not known if this higher level of direct defence is due to constitutive or inducible mechanisms, or a combination of the two. Induced defences in crucifers against herbivorous insects, including *A. thaliana* and *Brassica*, are well documented (Stotz et al., 2000; Van Poecke et al., 2001; Van Poecke and Dicke, 2004; Vuorinen et al., 2004), indicating the presence of inducible components. We performed microarray analyses after challenging Rivera and Christmas Drumhead plants with *P. rapae* larvae and found many differences in the transcriptional response of the two cultivars. For a careful comparison of transcriptional responses, the best approach is to carry out all treatments at the same time under identical conditions. In our experiments, all conditions were kept as constant as possible: biological replicates were performed at the same time, in the same greenhouse, larvae of the same developmental stage from the same rearing batch were used, and the data were analyzed using the same statistical methods. In this way, reliable comparisons can be made between cultivars and treatments.

Timing

Investigation of the transcriptional responses to *P. rapae* feeding showed that both cultivars responded to the herbivore, but the responses differed in timing. The fastest activation of gene expression was found in Christmas Drumhead in which 63, 254, and 292 genes were significantly induced after 24, 48, and 72 h of caterpillar feeding, respectively (Figure 2). Rivera, on the other hand, showed a slower transcriptional response as no genes were significantly induced after 24 h. After 48 h of larval feeding we identified 322 induced genes followed by 215 after 72 h (Figure 2). The slower transcriptional response of Rivera did not hold for the response to JA application. Although JA induced around half the number of genes in Rivera than in Christmas Drumhead at 6 h after treatment, there is a clear induction of gene expression in Rivera. The fact that both cultivars responded to JA application at the same time suggests that the difference in timing is specific for the response to *P. rapae* larvae. However, it can not be excluded that any difference in timing that might exist is obscured by the effect of the high concentration JA used in the experiment. Working with *B. oleracea* lines genetically deficient in JA signalling might be more informative. At present such lines are not available. The observation that larvae grew slower on Rivera and induced a slower transcriptional response, suggests that Rivera has a higher level of constitutive defence. However, when we compared constitutive gene expression between the two cultivars, none of the genes with a higher expression in Rivera is clearly associated with a higher constitutive defence (Table 1).

Overall differences in transcriptional response

The transcriptional response of Rivera differed from that of Christmas Drumhead. The comparison of *P. rapae*-induced transcriptional changes among the two cultivars at 48 h revealed that 64% of the genes induced in Rivera were not induced in Christmas Drumhead and 54% of the genes induced in Christmas Drumhead were not induced in Rivera (Figure 5). After 72 h of caterpillar feeding, 39% of the genes induced in Rivera were not induced in Christmas Drumhead and 55% of the genes induced in Christmas Drumhead were not induced in Rivera (Figure 5). Because the large number of genes only induced in one of the cultivars might be an effect of timing, we also looked at the overlap between transcriptional responses by taking into account all time points. Among the genes induced at one or more of the time points in Rivera, 44% was not induced in Christmas Drumhead at any time point tested. Similarly, 47% of the genes induced after one or more time points in Christmas Drumhead were not induced in Rivera at any time point tested (Figure 6). This shows that the effect of timing does not explain the difference in transcriptional responses. Thus, the two cultivars dramatically differ in transcriptional responses to caterpillar feeding.

Induction of specific defence related genes

Several defence related genes are induced in *B. oleracea* after *P. rapae* feeding (Table 2). Some of these genes were specifically induced in Rivera and might therefore be involved in the stronger direct defence of this cultivar. One of these genes encodes a putative glutathione S-transferase (GST, At1g27130). GSTs are a group of stress response proteins that contribute to cellular survival after oxidative damage (Moons, 2005). Another gene specifically induced in Rivera encodes a putative trypsin inhibitor (At2g43520). Trypsin inhibitors are proteinase inhibitors which provide protection against the proteolytic enzymes of herbivores (Glawe et al., 2003; Telang et al., 2003).

Among the genes that were induced in both cultivars, we found some genes of the lectin family to have a higher level of induction in Rivera than in Christmas Drumhead after *P. rapae* feeding. Lectins are carbohydrate-binding proteins, many of which play a role in plant defence by binding glycoconjugates in the intestinal tract of insects (Peumans and Van Damme, 1995). Among the six *lectin* genes that were induced in both cultivars, three (At1g52070, At3g21380, and At5g35950) showed a significantly higher induction in Rivera than in Christmas Drumhead after 48 h of caterpillar feeding (Between subjects Student *t* test, $P < 0.05$).

Interestingly, a *terpene synthase* (At5g23960) that was induced in Christmas Drumhead after 72 h of *P. rapae* feeding did not hybridize in Rivera at any time point tested (Table 2). Terpene synthases are involved in important regulatory steps in formation of terpenes, which are volatile compounds that could attract natural enemies of the herbivore (Yin et al., 1997; Bohlmann et al., 1998; Bohlmann et al., 2000; Kappers et al., 2005; Schnee et al., 2006). The *A. thaliana* homologue of the terpene synthase induced in Christmas Drumhead has been found to be responsible for the mixture of sesquiterpenes emitted from *A. thaliana* flowers (Tholl et al., 2005). Floral volatiles appear to attract species-specific pollinators, while volatiles emitted from vegetative parts of the plant, especially those released after herbivory, serve as attractants for the enemies of herbivores (Pichersky and Gershenzon, 2002). The induction of At5g23960 in the leaves of Christmas Drumhead and the absence of induction in Rivera suggests that Christmas Drumhead may possess a stronger indirect defence.

The expression of *Lipoxygenase 2* (*LOX2*, At3g45140) and *Allene Oxide Synthase* (*AOS*, At5g42650), which are involved in the synthesis of JA, was increased in both cultivars. The *LOX2* gene is involved in induced indirect defence of *A. thaliana* and mediates the attraction of the parasitic wasp *Cotesia rubecula* that attacks *P. rapae* caterpillars (Van Poecke and Dicke, 2002).

Several genes potentially involved in glucosinolate metabolism were also found to be induced. Genes involved in the biosynthesis of tryptophan (Trp) were induced in both cultivars. *Trp synthase α subunit* (At3g54640) was induced upon *P. rapae* attack in both cultivars but the induction occurred earlier in Rivera than in Christmas Drumhead. *Trp synthase β subunit 1* (At5g54810) was significantly induced in both cultivars, but with a longer lasting induction in Rivera. *Trp synthase β subunit 2* (At4g27070) was mainly induced in Christmas Drumhead. Genes responsible for the subsequent oxidation of Trp to form indole-3-acetaldoxime (*Cytochrome P450 79B2*, At4g39950; *Cytochrome P450 83B1*, At4g31500) were induced in both cultivars. These glucosinolate-related genes were also induced in *A. thaliana* upon *P. rapae* feeding (Reymond et al., 2004). One gene encoding a putative myrosinase-associated protein (At1g54020) was also induced in both cultivars (Table 2).

Conclusions

Taken together, we have demonstrated that global transcriptional responses in two cultivars of the same plant species in response to insect feeding can differ dramatically. Several of these differences involve genes that are known to have an impact on *P. rapae* performance.

Acknowledgements

Authors want to acknowledge the financial support of the Dutch Ministry of Agriculture, Nature, and Food quality. MD was additionally supported by a VICI grant from The Netherlands Organization for Scientific Research, NWO (865.03.002).

Supplemental material

Supplemental Table 1. Mean expression ratios of genes induced and repressed after challenge with Pieris rapae for 6, 24, 48 and 72 h in Brassica oleracea cultivars Rivera and Christmas Drumhead. This table can be found at http://www.biomedcentral.com/content/supplementary/1471-2164-8-239-S1.xls.

Supplemental Table 2. Mean expression ratios of genes induced after Jasmonic acid treatment. This table can be found at http://www.biomedcentral.com/content/supplementary/1471-2164-8-239-S2.xls

Chapter 3

Responses of *Brassica oleracea* cultivars to infestation by the aphid *Brevicoryne brassicae*: an ecological and molecular approach

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Plant, Cell and Environment (2008) published online

Abstract

Intraspecific variation in resistance or susceptibility to herbivorous insects has been widely studied through bioassays. However, few studies have combined this with a full transcriptomic analysis. Here, we take such an approach to study the interaction between the aphid *Brevicoryne brassicae* and four white cabbage (*Brassica oleracea* var. *capitata*) cultivars. Both under glasshouse and field conditions, two of the cultivars clearly supported a faster aphid population development than the other two, indicating that aphid population development was largely independent of the environmental conditions. Genome-wide transcriptomic analysis using 70-mer oligonucleotide microarrays based on the *Arabidopsis thaliana* genome showed that only a small number of genes were differentially regulated and that this regulation was highly cultivar specific. The temporal pattern in expression behaviour of two *B. brassicae*-responsive genes in all four cultivars together with targeted studies employing *A. thaliana* knockout mutants revealed a possible role for a trypsin-and-protease inhibitor in defence against *B. brassicae*. Conversely, a xyloglucan endotransglucosylase seemed to have no effect on aphid performance. Overall, this study shows clear intraspecific variation in *B. brassicae* susceptibility among *B. oleracea* cultivars under glasshouse and field conditions that can be partly explained by certain differences in induced transcriptional changes.

Introduction

Aphids have developed a highly specialized mode of feeding and cause a specific stress to plants. They use their piercing mouthparts, the stylets, to probe the plant tissue in order to feed from phloem sieve elements (Pollard, 1973; Walling, 2000). Aphid feeding may cause chlorosis and leaf curling, of which the latter provides the aphid with a sheltered microenvironment while disrupting normal plant growth and development. Additionally, aphid feeding can indirectly damage a plant through the transmission of viral diseases (Raybould et al., 1999; Alvarez et al., 2007). During probing, aphids move their stylets in between plant cells while making short punctures in epidermal, mesophyll, and parenchymal cells during their search for phloem cells (Tjallingii and Hogen Esch, 1993). Plant responses to aphids are thought to be mainly triggered by this stylet penetration of plant tissues together with the injection of saliva (Goggin, 2007; Will et al., 2007).

Plant morphology, such as wax layers and leaf thickness, can prevent aphids from settling on a plant, but aphid performance can also be influenced by direct defence mechanisms (Schoonhoven et al., 2005). These mechanisms involve the production of compounds that can alter the physiology of aphids resulting in an increased development time, a reduced growth rate, and survival probability. Direct defence mechanisms can be constitutively present or induced upon aphid attack. Proteins and secondary metabolites that have direct defensive effects, such as lectins and protease inhibitors, may have an antibiotic effect on aphids (Goggin, 2007). A distinctive defence system present in cruciferous plants, including *Brassica* crops as well as the model plant *Arabidopsis thaliana*, is the glucosinolate-myrosinase system. Upon tissue damage glucosinolates are hydrolyzed by myrosinases resulting in the formation of toxic products such as isothiocyanates, epithionitriles, thiocyanates, and nitriles (Bones and Rossiter, 2006; Grubb and Abel, 2006; Halkier and Gershenzon, 2006; De Vos et al., 2007). Many herbivores are specialized to feed on a single plant species or family and may have evolved enzyme systems to detoxify glucosinolates as well as other defensive compounds (Ratzka et al., 2002; Wittstock et al., 2004). Some specialist herbivores even accumulate intact glucosinolates and use them for their own defence (Schoonhoven et al., 2005; Després et al., 2007). For example, the specialist aphid *Brevicoryne brassicae* has evolved its own myrosinase to hydrolyze plant glucosinolates that may have a direct toxic affect on natural enemies (Jones et al., 2002; Kazana et al., 2007; Pratt et al., 2008).

DNA microarrays are excellent tools for the analysis of global transcriptional changes in plants and were used to identify genes responsive to aphid feeding (Thompson and Goggin, 2006; Smith and Boyko, 2007). In many plant species, aphid infestation activates genes whose products are involved in cell wall modifications including *expansin*, *cellulose synthase*, *pectin esterase*, and *xyloglucan endotransglucosylase/hydrolase* (*XTH*) (Thompson and Goggin, 2006). Additionally, signal transduction pathways are regulated by aphid infestation resulting in the differential expression of downstream genes. Salicylic acid (SA)-regulated genes are induced and jasmonic acid (JA)-regulated genes are repressed or moderately induced in leaves challenged with aphids (Walling, 2008). Gene expression analysis in *A. thaliana* has shown that transcription of the SA-regulated genes *PR-1* (*pathogenesis-related 1*) and *BGL2* (*ß-1,3-glucanase*), and the JA/ethylene-regulated gene *PDF1.2* (*plant defensin 1.2*) were induced after both *Myzus persicae* and *B. brassicae* feeding (Moran and Thompson, 2001;

Moran et al., 2002). Moreover, genes involved in oxidative stress, calcium-dependent signalling, and glucosinolate and auxin synthesis were induced after *M. persicae* attack in *A. thaliana* (Moran et al., 2002; Kuśnierczyk et al., 2007). Interestingly, aphid bioassays on *A. thaliana* mutant lines with altered JA or SA signalling suggest that JA-mediated responses limit aphid population growth, whereas SA does not influence or even has a positive effect on aphid performance (Thompson and Goggin, 2006). These results are consistent with the suggestion that piercing-sucking herbivores, such as aphids and whiteflies, manipulate plant responses by activating SA-signalling genes to repress more effective JA-signalling defence genes (Zhu-Salzman et al., 2004; Zhu-Salzman etal., 2005; Thompson and Goggin, 2006; De Vos et al., 2007; Gao et al., 2008; Zarate et al., 2007).

Several studies have addressed intraspecific variation in performance of phloem-feeding insects (Ellis et al., 2000; Alvarez et al., 2006; Ranger et al., 2007; Mooney and Agrawal, 2008; Wang et al., 2008). However, few studies on intraspecific variation so far link whole-genome transcriptomic analysis with investigations at the individual or population level of aphids (Kuśnierczyk et al., 2007). Such an integrated approach linking molecular genetics with population ecology will allow the understanding of intraspecific variation in plant defence at different levels of biological integration (Zheng and Dicke, 2008). Here, we take such an approach for the non-model plant *Brassica oleracea* and the aphid *B. brassicae*.

Plants are members of complex communities and defences to some community members may facilitate or compromise the defence against other community members (Kessler and Baldwin, 2004; De Vos et al., 2006b; Halitschke et al., 2008; Poelman et al., 2008a; Zheng and Dicke, 2008). Studies on *Pieris rapae* (Lepidoptera: Pieridae) performance showed differences in susceptibility among *B. oleracea* cultivars (Poelman et al., 2008b). Based on these results, we selected four *B. oleracea* cultivars: two with relatively poor *P. rapae* performance (Rivera and Lennox) and two with relatively good performance of this herbivore (Badger Shipper and Christmas Drumhead). These four cultivars were used to study *B. oleracea*-*B. brassicae* interactions. We examined aphid performance under both glasshouse and field conditions to assess the relative levels of susceptibility to *B. brassicae*. Transcriptional responses upon *B. brassicae* infestation were studied in Rivera and Christmas Drumhead, cultivars supporting relative slow and fast *B. brassicae* population growth respectively. To this purpose, we used a 70-mer oligonucleotide microarray representing the whole genome of *A. thaliana*. This microarray proved to be a good tool to study transcriptional response in *B. oleracea* as it recognizes related RNA sequences of *B. oleracea* (Lee et al., 2004; Broekgaarden et al., 2007), and shows intensity signals for 90 % of the oligonucleotides present on the microarray (Broekgaarden et al., 2007). Furthermore, we studied the expression behaviour of two *B. brassicae*-responsive genes in all four cultivars and examined their effect on aphid performance using *A. thaliana* T-DNA insertion mutants. The results of the present study show clear differences in *B. brassicae* performance between cultivars of *B. oleracea* that can be partly linked to induced changes at the molecular level.

Materials and methods

Aphid rearing and cultivation of *B. oleracea* plants

Cabbage aphids (*Brevicoryne brassicae*) originated from the stock rearing of the Laboratory of Entomology, Wageningen University. They were maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in an acclimatized room with a 16 h day and 8 h night cycle (21 ± 2 ºC, 50-70% relative humidity).

Seeds of white cabbage (*B. oleracea* var. *capitata*) cultivars Rivera and Lennox (F1 hybrid cultivars) were obtained from Bejo Zaden B.V. (Warmenhuizen, the Netherlands), and seeds from the open-pollinated cultivars Christmas Drumhead and Badger Shipper were obtained from the Centre of Genetic Resources, the Netherlands (CGN). Seeds were germinated in potting compost (Lentse Potgrond®) and two-week old seedlings were transferred to 1.45 L pots containing the same potting compost. Plants were grown in a glasshouse compartment with a 16 h day and 8 h night period (22 ± 2 ºC, 60-70 % relative humidity). All plants were watered every other day. No chemical control for pests or diseases was applied.

Aphid performance no-choice experiments on *B. oleracea* cultivars

One new-born aphid nymph was placed on each of the five youngest leaves of each five-week old *B. oleracea* plant. Individual plants were covered with nets to keep the aphids from escaping. The experiment was set up in a randomized design with 18 biological replicates per cultivar. Nymphs were monitored daily to estimate the development time (number of days between birth and first reproduction) and mortality was scored on day 11, the day by which almost all individuals had reproduced. From 11 days onwards, population size was recorded twice a week. Statistical analyses were performed using SPSS version 12.0.1. Nymph mortality percentages were arcsine square root-transformed and development time data were log-transformed to obtain normal data distributions. Transformed data were analyzed with one-way analysis of variance (ANOVA). If a significant cultivar effect was present, then differences between means for ANOVA were compared with least significant difference tests (LSD, $\alpha = 0.05$). Population size was corrected for the number of *B. brassicae* nymphs that started the population and corrected data were subsequently log-transformed. General linear model (GLM) repeated measures ANOVA (SPSS version 12.0.1) was used to assess the impact of different *B. oleracea* cultivars on the number of aphids over the entire experimental time course. Day was considered a within-subjects factor and cultivar a between-subjects factor. If a significant cultivar effect was present, then differences between means were compared with LSD tests ($\alpha = 0.05$).

Aphid populations under field conditions on *B. oleracea* cultivars

The experimental site for field monitoring was located in the neighbourhood of Wageningen, the Netherlands. Thirty two plots (each 6 x 6 m) with a monoculture of one of the four cultivars (eight plots per cultivar) were established using a randomized design. Five-week-old seedlings were transplanted to the field in week 19 (9 May) of 2005, planting 49 plants per plot in a square of 7 x 7 plants with a spacing of 75 cm between plants. An isolation area of 6 m with a grass mixture of *Lolium* and *Poa* species separated the different plots from each other. The central 9 plants of each plot were monitored

weekly from week 23 (6 June) until week 37 (16 September) for the presence of *B. brassicae*. Obtained data were log transformed to obtain normalized distribution of the residuals. Repeated measures ANOVA was performed using the Mixed Model procedure in the SAS statistical package (Kowalchuk *et al*. 2004) to evaluate population dynamics of *B. brassicae.* The model included the fixed effects of cultivar, week, the cultivar-week interaction, and the random effects of plot. If a significant interaction was present, then differences between means were compared using a Post Hoc multiple comparison test with a Tukey correction.

Aphid infestation for gene expression analyses in *B. oleracea* cultivars

Six-week-old, glasshouse-grown plants were infested with twenty wingless aphids of assorted life stages. Aphids were confined to the adaxial surface of the youngest, fully expanded leaf using clip cages. Control plants received empty clip cages. Leaf discs (diameter 2.3 cm) were taken next to the clip cage from infested and control plants after 48 hours of *B. brassicae* infestation, immediately flash frozen in liquid nitrogen, and stored at -80 ºC until use. Leaf discs of twelve plants were pooled for each biological replicate before freezing in liquid nitrogen. One biological replicate was performed in January 2005, whereas the other two replicates were performed in March 2005.

In a second experiment, plants were infested as described above. Material from different plant groups was collected after 24, 48, and 72 h of aphid feeding. Leaf discs of five plants were pooled. All leaf material was collected at the same time of the day. All experiments were set up in a randomized block design in such a way that we obtained three biological replicates.

Microarray hybridization and analysis

Pooled leaf samples were ground in liquid nitrogen and total RNA was extracted with TRIzol reagent (Invitrogen) followed by a purification using the RNeasy Plant Mini kit (Qiagen). Four µg of total RNA were linearly amplified using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). Control and herbivore-infested samples were labelled respectively with Cy3 and Cy5 monoreactive dye (Amersham). Amplified RNA was labelled in freshly made 0.2 M sodium carbonate buffer (pH 9.0) for 1 h at room temperature. Dye incorporation was monitored by measuring the Cy3 and Cy5 fluorescence emissions using a nanodrop ND-1000 UV-Vis Spectrophotometer (BioRad). Microarrays containing 70-mer oligonucleotides based on the genome of *A. thaliana* were obtained from the group of David Galbraith from the University of Arizona (http://www.ag.arizona.edu/microarray). Immobilization of the array elements was performed according to the manufacturer's website (see above). The hybridization mixture contained 100 pmol of the Cy3-labeled sample, 50 pmol of the Cy5-labeled sample, 2X SSC, 0.08% SDS, and 4.8 µl Liquid Block (Amersham) in a final volume of 80 µl. The solution was incubated at 65 ºC for 5 min before application to the microarray covered with a lifterslip (Gerhard Menzel). The microarray was placed in a hybridization chamber (Genetix) and incubated at 50 ºC. After 12 h the microarray was washed for 5 min in 2X SSC/0.5% SDS at 50 ºC, followed by a 5 min wash in 0.5X SSC at room temperature, and a final 5 min wash in 0.05X SSC at room temperature. The microarray was immediately dried by centrifugation for 4 min at 200 rpm.

Hybridized microarrays were scanned with a ScanArray Express HT Scanner (PerkinElmer). Mean fluorescent intensities for Cy3 and Cy5 were determined using the ScanArray Express software (PerkinElmer). Each image was overlaid with a grid to assess the signal intensities for both dyes from each spot. Background fluorescence was subtracted and spots with adjusted intensities lower than half the background were manually raised to half the background to avoid extreme expression ratios. Spots were excluded from the analysis when: (1) showing signal intensities less than half the background for both dyes; (2) showing aberrant shape; (3) located in a smear of fluorescence. Lowess (locfit) normalization was carried out within each slide using TIGR MIDAS version 2.19 to avoid spatial bias. Normalized expression ratios for each individual spot and the mean of the three replicate spots were calculated. A Student *t* test on $\log_2$ transformed expression ratios was conducted for each experimental condition using TIGR MEV version 3.1. Genes with a $\log_2$ expression ratio ≥ 1 (expression ratio 2-fold) or ≤ -1 (expression ratio 0.5-fold) in combination with a P value < 0.05 were considered significantly different. We used the names of *A. thaliana* homologs to identify *B. oleracea* genes and examined the potential function of differentially regulated genes according to gene ontology (GO) terms from The Arabidopsis Information Resource (http://www.arabidopsis.org).

Quantitative RT-PCR

Quantitative RT-PCR was used to technically validate the microarray results of selected genes by using the same RNA pools as used for microarray analysis. In a separate experiment, qRT-PCR analysis was used to examine the transcript levels of selected genes at three different time points. One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (BioRad). Gene-specific primers were designed for *B. oleracea* genes based on sequences obtained by a BLAST search in the TIGR *B. oleracea* database. The primer sequences are shown in Table 1. Primers were tested for gene specificity by performing melt curve analysis on a MyIQ Single-Color Real-Time PCR Detection System (BioRad). PCR products were sequenced to confirm amplification of the gene of interest. Sequence results were checked by a BLAST search in the *B. oleracea* as well as in the *A. thaliana* TIGR database. RT-PCR analysis was done in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR were performed in duplicate. The following PCR program was used for all PCR reactions: 3 min 95 ^{o}C; 40 cycles of 30 sec 95 ^{o}C (denaturation) and 45 sec 60 ^{o}C (annealing and elongation). Threshold cycle (Ct) values were

Table 1. Sequences of B. oleracea-derived primers used in quantitative real-time PCR analyses.

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
GAPDH	AGAGCCGCTTCCTTCAACATCATT	TGGGCACACGGAAGGACATACC
XTH6	GGTGGGACAGGATACCTTGACTTG	GGTGGGAAGGTACCGCTTATCAGT
CAT2	GCTTCAGACCCGTGTCTTCT	GATACTTCTCAGCATGACGAACC
STO	GCCCTCCATCTCAAACTCTC	CCCAGTGGCTAAGAACCTCT
PDX1	ACCGGCGGCGAACTGAACGA	GAAGGCGCGGCGATGATTAGGAC
TPI	TGGTGACAAGTAGCTGTGGTG	TCCAAGTTATGGGCAGTGG

calculated using Optical System software, version 2.0 for MyIQ (BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) from the Ct value of the gene of interest. *GAPDH* was proven to be a good housekeeping gene in *B. oleracea* (Zheng *et al*. 2007) and is frequently used as reference gene in expression studies (Carraro *et al*. 2005). The absolute expression levels of *GAPDH* were similar for all samples in our study (data not shown). Normalized gene expression was then obtained from the equation $2^{-\Delta Ct}$. Normalized gene expression values were used to calculate log_2-transformed expression ratios for each experimental condition. Statistical analysis was performed using SPSS version 15.0.

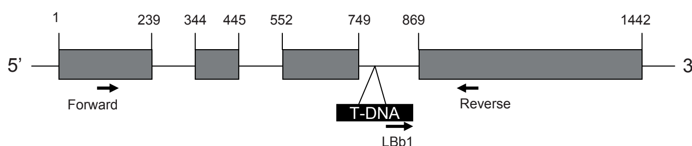
Cultivation of *A. thaliana* T-DNA mutant lines

Seeds of *A. thaliana* accession Col-0, knockout mutant *xth6* (T-DNA insertion line SALK_121671), and knockout mutant *tpi* (T-DNA insertion line SALK_009681) were obtained from the SIGnAL collection (Alonso et al., 2003; http://signal.salk.edu). For T-DNA confirmation experiments, plants were sown in autoclaved potting compost (Lentse Potgrond®). Two-week-old seedlings were transferred to 60 ml pots containing the same autoclaved potting compost. Plants were cultivated in a growth chamber with an 8 h day (200 µE.m^{-2}.sec^{-1}) and 16 h night cycle at 20 ± 2 ºC and 60-70 % relative humidity. All plants were watered every other day. No chemical control for pests or diseases was performed.

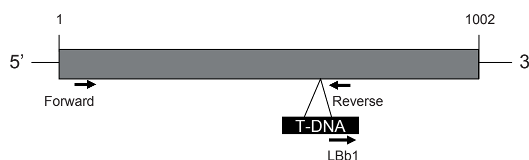
Identification of homozygous *xth6* and *tpi A. thaliana* T-DNA mutant lines

For genomic DNA isolation, 20-40 mg fresh leaf material from individual plants was harvested in 2 ml safe-lock microcentrifuge tubes containing 3 mm steel beads. Tissue was ground in a TissueLyser (Qiagen) for 30 sec at 30 Hz and genomic DNA was extracted using the DNeasy Plant Mini kit

A *XTH6* (At5g65730)



TPI (At1g72290)



B

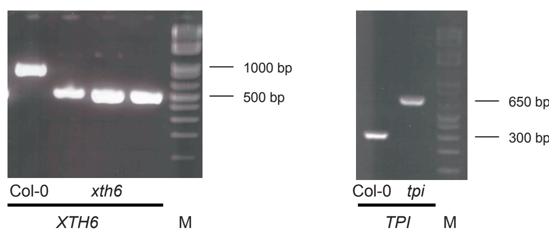


Figure 1. Molecular analysis of the *xth6* and *tpi* mutation lines. (A) Structure of the *AtXTH6* and *AtTPI* genes and positions of the T-DNA insertion in the silenced mutants. Exons are indicated as grey boxes with their start and end nucleotide numbers above. The primers used for the verification of the T-DNA insertion are indicated by arrows (Forward, Reverse, and LBb1). (B) To verify the T-DNA insertion, PCR amplification on genomic DNA of Col-0, *xth6* and *tpi* plants was performed. M, 100 bp DNA ladder.

(Qiagen). A PCR reaction was carried out to confirm the presence of the T-DNA insertion in the target gene. The T-DNA insertions are located 747 and 602 bp downstream of the translation start site for *xth6* and *tpi*, respectively (Figure 1A). Gene-specific primers were designed up- and downstream of the T-DNA insertion site (~600 bp upstream *XTH6*-forward 5'-AAT CTC ACA TCC GTC AAA TGG-3', ~300 bp downstream *XTH6*-reverse 5'-CCA GGA ACA GGA CAA CCT TC-3'; ~550 bp upstream *TPI*-forward 5'-GTG AAG GAT ACA GCC GGA AA-3', ~100 bp downstream *TPI*-reverse 5'-ATT AAG CCT GAG ACT CGT CCA T-3'). These primers were used in combination with a T-DNA left border primer (LBb1 5'-GCG TGG ACC GCT TGC TGC AAC T-3'). The following PCR program was used: 94 °C for 3 min; 30 cycles of 94 °C for 30 sec, 60 °C for 45 sec, and 72 °C for 1 min. Wild-type plants yielded a single band of ~900 (amplified from primers *XTH6*-forward and *XTH6*-reverse) or ~650 bp (amplified from primers *TPI*-forward and *TPI*-reverse). Plants containing a T-DNA insertion yielded a single band of ~500 (amplified from primers LBb1 and *XTH6*-reverse) or ~350 bp (amplified from primers LBb1 and *TPI*-reverse) (Figure 1B). No obvious differences in development or morphology between wild type and mutant plants were observed.

Aphid performance no-choice experiments on *A. thaliana* mutants

For the aphid performance experiments with the *A. thaliana* wild type and mutant lines, five new-born aphid nymphs were randomly placed on five-week-old plants. The experiment was set up in a randomized design with 20 biological replicates per line. After 10 days, the total number of aphids on each plant was counted. Significance of both wild type-mutant contrasts was tested with independent student *t*-tests using SPSS version 12.0.1 software.

Results

Aphid performance on *B. oleracea* cultivars in a no-choice glasshouse experiment

The four *B. oleracea* cultivars Rivera, Lennox, Badger Shipper, and Christmas Drumhead were evaluated for their relative susceptibility to *B. brassicae* in a no-choice performance test under glasshouse conditions. The percentage of dead nymphs after 11 days differed significantly among the cultivars (ANOVA $F = 2.52$, $P < 0.05$, Figure 2A). Significant (LSD, $P < 0.05$) contrasts were found between Lennox (47% mortality) versus Christmas Drumhead (32%) and Badger Shipper (26%). The development time of the aphids, which is the number of days between birth and first reproduction, also differed significantly among the four cultivars (ANOVA $F = 7.60$, $P < 0.001$, Figure 2B) ranging from 11.2 ± 0.2 days (mean ± SE) on Christmas Drumhead to 12.3 ± 0.2 days on Lennox. Population development was recorded per plant and corrected for the number of *B. brassicae* individuals that actually started reproducing. A general linear model based on repeated measurements over the complete course of the development showed that population size increased significantly over time ($F = 3354.18$, $P < 0.001$) and was significantly different for the cultivars ($F = 3.86$, $P = 0.007$, Figure 2C). At the end point, populations of *B. brassicae* were 1.3 times smaller on Rivera and Lennox than on Christmas Drumhead.

Plants of all four cultivars that harboured large numbers of aphids (> 500 individuals) started to display leaf curling, chlorosis, and necrosis at the end of the experiment.

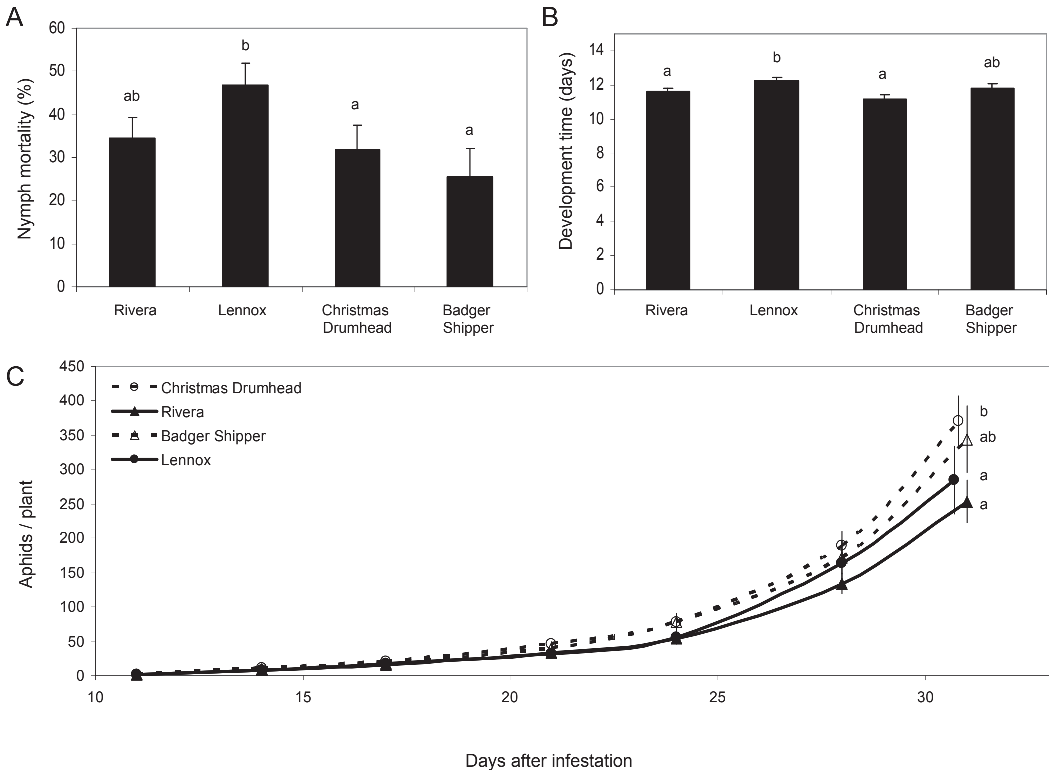


Figure 2. Performance of *B. brassicae* aphids on four *B. oleracea* cultivars in a no-choice, glasshouse experiment. (A) Nymph mortality in percentages after 11 days of feeding (mean + SE), (B) Development time in days (mean + SE), (C) Population increase over the first 31 days after infestation (mean ± SE). Bars or lines marked with a common letter do not differ significantly (α = 0.05).

Aphid populations on *B. oleracea* cultivars under field conditions

The greenhouse experiment described above was performed under controlled no-choice conditions. To investigate if the results found in this experiment represent the situation under field conditions, population development of *B. brassicae* on Rivera, Lennox, Badger Shipper, and Christmas Drumhead was monitored throughout the season in an experimental field setup. A mixed model based on repeated measurements constructed over the complete course of aphid population development revealed that population sizes were different over time for the four cultivars (F = 54.76, P < 0.001; Figure 3). Aphid populations started developing after week 25, reached a maximum around week 29, and disappeared after week 36. In week 29, population size was 1.6 times larger on Badger Shipper than on Rivera or Lennox (Figure 3). Additionally, *B. brassicae* population size on Christmas Drumhead was 1.5 times larger than on Badger Shipper and 2.5 times larger than on Rivera and Lennox (Figure 3).

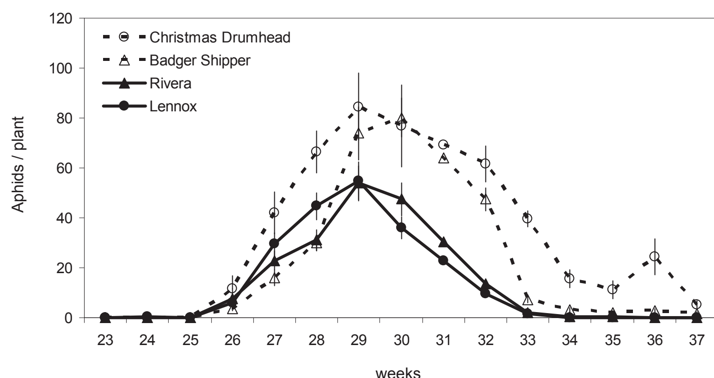


Figure 3. Population development of *B. brassicae* on four *B. oleracea* cultivars under field conditions. Mean (± SE) number of aphids per plant monitored over 15 weeks during the season.

Transcriptional responses of cultivars Rivera and Christmas Drumhead to *B. brassicae* feeding

To study the molecular responses underlying the differences in *B. brassicae* performance we analyzed the transcriptional changes of two contrasting cultivars: one cultivar (Rivera) supporting slower aphid population development than the other (Christmas Drumhead). Microarrays based on the *A. thaliana* genome were used to compare non-challenged plants with plants that had been attacked by *B. brassicae* for 48 h. This time point was chosen based on the transcriptional responses observed in other studies (Voelckel, Weisser & Baldwin 2004; Zhu-Salzman *et al*. 2004). In Rivera 28 genes were differentially expressed among which 12 were induced and 16 repressed (Table 2). In Christmas Drumhead we identified 27 induced and 20 repressed genes upon *B. brassicae* feeding (Table 2). Remarkably, there was very little overlap between transcriptional responses in Rivera and Christmas Drumhead. Only one of the induced genes, *xyloglucan endotransglucosylase* (*XTH6*), was induced in both cultivars (Table 2). The small overlap of induced genes between Rivera and Christmas Drumhead also holds for repressed genes as only one gene, encoding a 29 kDa ribonucleoprotein of unknown function, was repressed in both cultivars (Table 2). To test whether the small overlap of transcriptional responses between the two cultivars was due to our stringent selection criteria, we examined the overlap of all genes that were significantly regulated (student *t*-test, $P < 0.05$). Of all the 1582 genes that matched this criterion, only 3.5 % were commonly regulated in both cultivars (Supplemental Figure S1). Among the genes that were only induced in Rivera, we found several transport-related genes of which two encode aquaporins (*PIP2.2B*, *RD28*). We also found induced expression for the oxidative stress response genes *Catalase 2* (*CAT2*) and *Pyridoxal phosphate synthase* (*PDX1.3*) in Rivera (Table 2). A gene encoding a protein with endopeptidase inhibitor activity, *Trypsin-and-protease inhibitor* (*TPI*), was almost significantly induced in Rivera ($P = 0.091$) and not in Christmas Drumhead ($P = 0.244$). Genes only induced in Christmas Drumhead after *B. brassicae* feeding included genes involved in terpene biosynthesis (*terpene synthase*), oxidative stress response (*glutathione peroxidase 1; GPX1*), and glucosinolate metabolism (*glycosyl hydrolase; TGG2*). Repressed genes in Rivera included genes involved in development and general metabolism. Among the repressed genes in Christmas Drumhead were genes involved in general metabolism, photosynthesis, and cell organization (Table 2).

Table 2. Expression level of *B. brassicae*-responsive genes in cultivars Rivera and Christmas Drumhead.

		Rivera		Christmas Drumhead		
Probe identification	AGI code	Log_2 Ratio	*P* value	Log_2 Ratio	*P* value	Process category
Genes induced in both cultivars						
Xyloglucan endotransglucosylase (XTH6)	At5g65730	1.89	0.045	1.71	0.021	Cell wall metabolism
Genes induced only in Rivera						
Trypsin and protease inhibitor (TPI)	At1g72290	1.95	0.091	1.59	0.244	Defence
Dolichyl-phosphate beta-D-mannosyltransferase	At1g20575	1.12	0.024	0.66	0.152	Protein metabolism
Inorganic phosphate transporter	At2g29650	1.29	0.019	1.09	0.106	Stress response
Catalase 2 (CAT2)	At4g35090	2.06	0.026	1.03	0.156	Stress response
Stress-responsive protein (PDX1)	At5g01410	1.21	0.030	0.71	0.127	Stress response
Aquaporin PIP2.2 (PIP2B)	At2g37170	1.67	0.026	0.95	0.188	Transport
Aquaporin PIP2.3 (RD28)	At2g37180	1.46	0.010	1.20	0.081	Transport
Pentatricopeptide (PPR) repeat-containing protein	At1g64310	1.20	0.009	0.19	0.292	Unknown
T-complex protein 11	At4g09150	1.05	0.050	0.37	0.294	Unknown
Expressed protein	At4g18335	1.13	0.043	0.12	0.761	Unknown
Expressed protein	At4g32860	1.06	0.028	0.83	0.023	Unknown
Expressed protein	At5g20935	1.10	0.024	0.61	0.282	Unknown
Genes induced only in Christmas Drumhead						
Glycosyl hydrolase family 1	At1g61820	*		2.40	0.015	Carbohydrate metabolism
Expressed protein	At5g40940	0.08	0.802	1.02	0.022	Cell adhesion
Hydroxyproline-rich glycoprotein (PRP2)	At2g21140	0.00	0.991	1.30	0.019	Cell wall metabolism
Terpene synthase	At1g61120	0.23	0.236	1.11	0.023	Defence
Glycosyl hydrolase family 1 protein (TGG2)	At5g25980	-0.36	0.252	1.36	0.036	Defence
Ribose-phosphate pyrophosphokinase 2 (PRS2)	At1g32380	0.05	0.936	1.14	0.012	Metabolism
Squalene monooxygenase 1.1 (SQP1)	At5g24150	1.20	0.096	1.05	0.001	Metabolism
FtsH protease	At1g06430	0.68	0.263	1.00	0.047	Photosynthesis
Protein prenyltransferase alpha subunit-related	At1g10095	-0.04	0.959	1.26	0.044	Protein metabolism
Protein kinase family protein	At5g55560	-0.29	0.415	1.10	0.004	Protein metabolism
Isoprenylcysteine carboxyl methyltransferase	At5g08335	0.90	0.114	1.00	0.034	Signal transduction
Salt-tolerance protein (STO)	At1g06040	0.82	0.017	1.07	0.026	Stress response
Phospholipid hydroperoxide glutathione peroxidase (GPX1)	At2g25080	0.07	0.766	1.93	0.022	Stress response
Expressed protein	At1g04030	-0.15	0.787	1.13	0.048	Unknown
Pentatricopeptide (PPR) repeat-containing protein	At1g06140	0.17	0.147	1.18	0.034	Unknown
Expressed protein	At1g07020	-0.31	0.331	1.11	0.017	Unknown
F-box family protein-related	At1g47350	0.08	**	1.12	0.035	Unknown
Expressed protein	At1g65270	-0.25	0.844	3.31	0.040	Unknown
Glycine-rich protein	At1g66820	-0.37	0.661	3.27	0.028	Unknown
Expressed protein	At3g11000	0.44	0.158	1.16	0.007	Unknown
Photosystem II 5 kD protein	At3g21055	-0.56	0.506	1.95	0.015	Unknown
F-box family protein	At3g44080	-0.48	0.532	1.37	0.004	Unknown
Expressed protein	At3g51510	0.85	0.006	1.05	0.039	Unknown
Cinnamyl-alcohol dehydrogenase (CAD)	At5g19440	0.33	0.188	1.23	0.045	Unknown
Expressed protein	At5g54170	0.08	0.826	1.72	0.002	Unknown
Expressed protein	At5g55880	0.04	0.612	1.15	0.011	Unknown

Table 2. (continued)

Probe identification	AGI code	Rivera Log$_2$ Ratio	Rivera *P* value	Christmas Drumhead Log$_2$ Ratio	Christmas Drumhead *P* value	Process category
Genes repressed in both cultivars						
29 kDa ribonucleoprotein (CP29)	At3g53460	-1.68	0.033	-1.26	0.028	Unknown
Genes repressed only in Rivera						
Histone H1-3 (HIS1-3)	At2g18050	-1.08	0.035	-0.04	0.861	Cell organization & biogenesis
Magnesium-protoporphyrin O-methyltransferase	At4g25080	-1.02	0.045	0.84	0.012	Chlorophyll biosynthesis
Expressed protein	At2g15890	-3.50	0.037	-0.40	0.334	Development
4-aminobutyrate aminotransferase (POP2)	At3g22200	-1.07	0.007	-0.45	0.203	Development
Glycine-rich RNA-binding protein (GRP2)	At4g13850	-1.18	0.045	-1.04	0.253	Development
60S ribosomal protein L13 (RPL13C)	At3g48960	-1.63	0.028	0.69	0.391	Protein metabolism
40S ribosomal protein S7 (RPS7C)	At5g16130	-1.06	0.014	-0.73	0.074	Protein metabolism
DNAJ heat shock N-terminal domain-containing protein	At5g23240	-1.60	0.001	-0.83	0.199	Protein metabolism
Alpha-amylase (AMY1)	At4g25000	-1.50	0.032	-0.85	0.018	Response to gibberellin & ABA
AP2 domain-containing transcription factor	At3g25890	-1.12	0.044	-0.91	0.202	Transcription
Proteasome family protein	At3g02200	-1.10	0.019	-0.40	0.334	Unknown
Expressed protein	At3g45320	-1.18	0.007	-0.64	0.086	Unknown
Bundle-sheath defective protein 2	At3g47650	-1.09	0.047	-0.84	0.124	Unknown
Expressed protein	At5g02160	-1.17	0.030	-0.51	0.340	Unknown
Expressed protein	At5g25460	-1.38	0.012	-1.15	0.123	Unknown
Genes repressed only in Christmas Drumhead						
Histone H2A	At5g02560	-0.08	0.724	-1.02	0.011	Cell organization & biogenesis
Cysteine synthase	At3g22460	-0.01	0.972	-1.05	0.026	Cysteine biosynthesis
CCR4-NOT transcription complex protein	At1g15920	-0.30	0.571	-1.23	0.012	Metabolism
Carbon-nitrogen hydrolase (NLP1)	At2g27450	-0.81	0.161	-1.41	0.006	Metabolism
Chlorophyll A-B binding protein (LHB1B2)	At2g34420	-0.18	0.715	-1.21	0.019	Photosynthesis
60S ribosomal protein L7 (RPL7B)	At2g01250	-0.60	0.019	-1.14	0.040	Protein metabolism
Calcineurin B-like protein 1 (CBL1)	At4g17615	-0.12	0.800	-1.07	0.007	Signal transduction
Stress-responsive protein (KIN2)	At5g15970	-0.60	0.345	-1.30	0.006	Stress response
PHD finger family protein	At3g11200	-0.42	0.391	-1.21	0.043	Transcription
Zinc finger (GATA type) family protein	At5g66320	0.11	0.838	-1.18	0.028	Transcription
Basic helix-loop-helix (bHLH) family protein	At1g66470	0.36	0.517	-1.14	0.002	Transcription
Myosin family protein (XIF)	At2g31900	-2.31	0.097	-1.49	0.05	Transport
Nodulin MtN21 family protein	At1g01070	0.07	**	-1.48	0.037	Unknown
Pentatricopeptide (PPR) repeat-containing protein	At1g56690	0.02	0.743	-2.01	0.016	Unknown
Expressed protein	At2g25990	0.14	0.900	-1.19	0.005	Unknown
Expressed protein	At3g08030	-0.87	0.045	-1.07	0.041	Unknown
Proline-rich family protein	At3g20850	-0.20	0.544	-1.02	0.045	Unknown
Expressed protein	At4g14270	-1.10	0.087	-1.24	0.035	Unknown
RNA and export factor-binding protein	At5g59950	-0.08	0.904	-1.16	0.039	Unknown

Relative changes in gene expression after challenge with *B. brassicae* were measured in Rivera and Christmas Drumhead leaves. Mean $\log_2$ expression ratios and *P*-values are calculated from three independent biological replicates.
*70-mer-oligonucleotide did not hybridize in any of the three replicates;
**70-mer oligonucleotide only hybridized in one of the three replicates.
AGI, Arabidopsis Genome Initiative.

Validation of microarray data

Since we used an *A. thaliana*-based microarray to detect transcriptional responses in *B. oleracea*, we performed quantitative real-time PCR (qRT-PCR) to technically validate five genes. These genes were selected on the basis of their induction level on the microarray and were induced either in both cultivars (*XTH6*), only in Rivera (*CAT2, STO*; encoding a salt-tolerance protein, and the almost significant *TPI*), or only in Christmas Drumhead (*PDX1.3*). In contrast to the microarray, we used *B. oleracea*-derived primers to perform qRT-PCR. Figure 4 shows $\log_2$ expression ratios of the five selected genes in Rivera and Christmas Drumhead as determined by both types of analyses. For both cultivars, the transcript levels of the selected genes were consistent for microarray and qRT-PCR analysis in either the induction or the lack of induction (Figure 4). Four of the selected genes (*XTH6, CAT2, PDX1.3,* and *STO*) were significantly induced in one or both cultivars using both types of analyses (one sample *t*-test, $P < 0.05$). The induced expression of *TPI* was almost significant in Rivera for microarray ($P = 0.091$) and qRT-PCR ($P = 0.072$), whereas no *TPI* induction was shown in Christmas Drumhead (microarray, $P = 0.244$; qRT-PCR, $P = 0.158$).

Statistical comparisons revealed no significant differences between values obtained from microarray and qRT-PCR analyses (paired-sample *t*-test, $P > 0.1$), except for *CAT2* in Rivera ($P = 0.03$). Quantitative RT-PCR showed a 1.5 times higher level of *CAT2* induction than the microarray. Overall, microarray and qRT-PCR analyses show similar patterns of gene expression regulation after *B. brassicae* feeding, confirming the reliability of the microarray data.

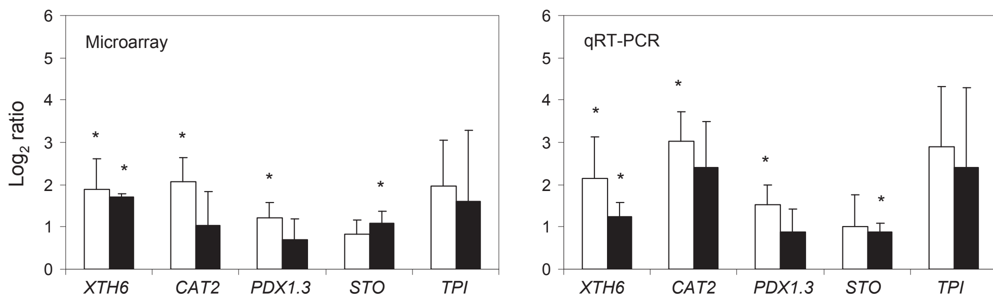


Figure 4. Comparison of microarray and qRT-PCR analysis of four genes. Log_2 ratios of transcript levels of four selected genes after infestation of Rivera (black bars) and Christmas Drumhead (white bars) by *B. brassicae*. Genes were selected based on their induction level in the microarray analysis. *XTH6* (*xyloglucan endotransglu-cosylase*) was induced in both cultivars, *CAT2* (*Catalase 2*) and *STO* (salt tolerance protein) were only induced in Rivera, and *PDX1* (stress-responsive protein) was only induced in Christmas Drumhead. *TPI (Trypsin-and-protease inhibitor* was almost significant only in Rivera in the microarray analysis. All bars are shown with their corresponding standard deviation. Values marked with an asterisk are significantly regulated according to student t-tests ($P < 0.05$).

Gene expression changes in four *B. oleracea* cultivars

The expression behaviour of the gene that was commonly induced in Rivera and Christmas Drumhead, *XTH6*, was examined over time in all four cultivars after *B. brassicae* feeding in a separate experiment with independent RNA pools for three different time points. The expression of *XTH6* was significantly induced after 48 h of *B. brassicae* feeding in Rivera (log_2 ratio 0.91; one-way ANOVA $P = 0.004$; Figure 5), which is consistent with the microarray results. In Lennox, the other cultivar supporting relatively slow *B. brassicae* population increase, *XTH6* expression was induced after 72 h of aphid feeding (log_2 ratio 0.8, $P = 0.016$; Figure 5). In Badger Shipper, a cultivar with relatively fast aphid population growth, the expression of this gene was induced after 48 and 72 h (48 h: log_2 ratio 0.8, $P = 0.004$; 72 h: log_2 ratio 0.65, $P = 0.014$; Figure 5A). In this experiment, no significant changes of *XTH6* expression were detected in Christmas Drumhead (Figure 5).

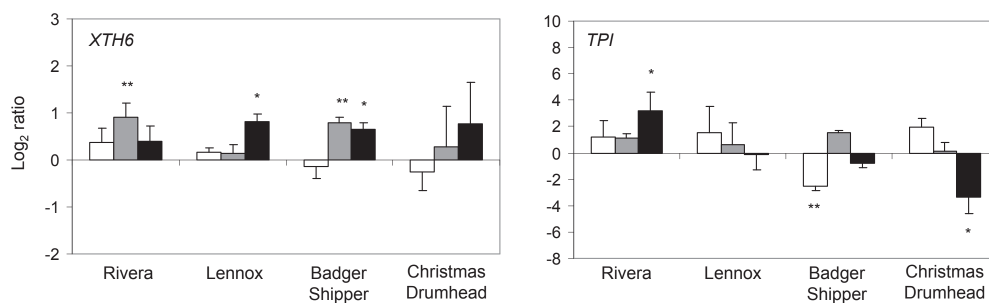


Figure 5. Time course of expression changes for two *B. brassicae*-responsive genes in four *B. oleracea* cultivars using qRT-PCR. Mean log_2 ratio (+ SD) are shown for *XTH6* and *TPI* after 24 (white bar), 48 (grey bar), and 72 (black bar) hours of *B. brassicae* feeding. Bars within cultivars marked with one or more asterisks differ significantly (student *t*-test, one asterisk: $P < 0.05$; 2 asterisks: $P \leq 0.01$).

We also analyzed the expression of the *TPI* gene, which encodes for a protein with endopeptidase inhibitor activity, suggesting a possible role for this gene in defence against *B. brassicae*. In the microarray analysis, its expression was almost significantly induced in Rivera ($P = 0.091$) and not in Christmas Drumhead ($P = 0.244$) (Table 2). In a second experiment, using qRT-PCR with *B. oleracea TPI* specific primers, the expression was found to be significantly induced in Rivera after 72 h (log_2 ratio 3.16, $P = 0.021$; Figure 5), whereas it was significantly repressed in Christmas Drumhead after 72 h (log_2 ratio -3.34, $P = 0.025$; Figure 5) and in Badger Shipper after 24 h of *B. brassicae* feeding (log_2 ratio -2.54, $P = 0.003$). In Lennox, no significant expression changes could be detected for *TPI* (Figure 5).

Effects of *XTH6* and *TPI* on population growth of *B. brassicae*

It is difficult to study the role of specific genes in defence against aphids when no mutants are available as is the case for *B. oleracea*. Therefore, we took advantage of the availability of *A. thaliana* mutants. We examined *B. brassicae* performance on *A. thaliana* mutant plants with a T-DNA insertion in either the *XTH6* (encoding a xyloglucan endotransglucosylase) or *TPI* gene (encoding a trypsin-and-protease inhibitor). *B. brassicae* reproduced similarly on wild type and *xth6* mutant plants (independent sample *t*-test, *P* = 0.39; Figure 6). Interestingly, the number of *B. brassicae* aphids was 1.4 times higher on *tpi* mutant than on wild-type plants (independent sample *t*-test, *P* = 0.015; Figure 6).

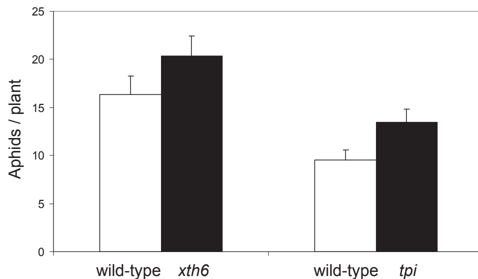


Figure 6. Number of *B. brassicae* aphids after 10 days on wild-type, *xth6* insertion mutant, and tpi silenced mutant plants in no-choice feeding experiments. Bars represent means + SE.

Discussion

B. brassicae performance differed on *B. oleracea* cultivars

A no-choice glasshouse experiment on aphid performance showed intraspecific variation in plant resistance: the performance of the aphid *B. brassicae* was dependent on the cultivars used. We found small but significant differences between cultivars for nymph mortality after 11 days of infestation and for development time. Both effects are likely to influence aphid population development as aphids have high intrinsic rates of population increase, meaning that even small effects on development time have important consequences for population development (Sabelis 1985). Indeed, Lennox supported slower aphid population growth when compared to Badger Shipper or Christmas Drumhead (Figure 2C). Based on the aphid performance tests, the four cultivars can be divided into two groups: (1) Rivera and Lennox supporting slower population growth, and (2) Badger Shipper and Christmas Drumhead supporting faster population growth.

To assess whether the results on aphid performance from the glasshouse experiment represent the field situation, we monitored aphid population dynamics at an experimental field site in the Netherlands. The results of the field experiment, where plants were exposed to naturally occurring populations of *B. brassicae*, matched the results of the glasshouse experiment and showed again that cultivars Rivera and Lennox support slower aphid population increase than Badger Shipper and Christmas Drumhead. These results show that relative aphid performance on these cultivars in the glasshouse and field experiments is similar and therefore largely independent of the environmental conditions.

B. brassicae feeding leads to induced transcriptional responses in Rivera and Christmas Drumhead

We observed differences in *B. brassicae* performance on four *B. oleracea* cultivars, indicating differences in susceptibility to this phloem-feeding herbivore. It has been shown that some inducible mechanisms are triggered upon aphid feeding that correlate either positively or negatively with aphid performance (Sauge et al., 2006; Dugravot et al., 2007; Kuśnierczyk et al., 2007). We analyzed transcriptional changes in Rivera and Christmas Drumhead, cultivars respectively supporting relative slow and fast *B. brassicae* population increase, to study molecular responses underlying the differences in aphid performance. Both cultivars responded to feeding by *B. brassicae* through the induction and repression of a number of genes. We identified 28 and 47 *B. brassicae*-responsive genes in Rivera and Christmas Drumhead, respectively (Table 2). Feeding by *Myzus persicae* on *A. thaliana* resulted in a much higher number of differentially regulated genes, namely 2181 (De Vos et al., 2005). However, it should be noted that this study used only one biological replicate and identified differentially regulated genes on the basis of fold-changes (De Vos et al., 2005). More recently, a study on the *M. persicae-A. thaliana* interaction found only 27 genes with altered expression as analyzed from six biological replicates (Couldridge et al., 2007), pointing out the importance of standardizing the experimental design. A study on the interaction between *B. brassicae* and three *A. thaliana* ecotypes identified between 93 and 164 differentially regulated genes (ratio > 2-fold, $P < 0.05$) (Kuśnierczyk et al., 2007). This higher number of *B. brassicae*-responsive genes may be due to differences between plant species and/or the 90% hybridization efficiency of our microarray study (Broekgaarden et al., 2007).

Both cultivars induced genes responsive to oxidative stress, which supports previous findings that aphid induce oxidative stress (Ni et al., 2001; Moran et al., 2002; De Ilarduya et al., 2003; Divol et al., 2005; Park et al., 2006; Kuśnierczyk et al., 2007). As the induction occurs in both cultivars, these are possibly secondary effects that do not influence aphid performance. Genes that were repressed in one or both *B. oleracea* cultivars were mostly involved in photosynthesis, general metabolism and development (Table 2), reflecting the reallocation of resources from general growth and development to defence (Herms and Mattson, 1992; Baldwin, 1998; Kaloshian and Walling, 2005).

The transcriptional response of Rivera and Christmas Drumhead to *B. brassicae* feeding was relatively mild compared to the responses of these cultivars to feeding by *P. rapae* caterpillars (Broekgaarden et al., 2007). After 48 h we identified 28 and 47 *B. brassicae*-responsive genes in Rivera and Christmas Drumhead, respectively (Table 2). Conversely, 48 h of *P. rapae* feeding resulted in the differential expression of 805 genes in Rivera and 337 genes in Christmas Drumhead (Broekgaarden et al., 2007). Less pronounced transcriptional changes in response to aphid feeding compared to tissue-damaging herbivores have been found in other studies as well (Heidel and Baldwin, 2004; Voelckel et al., 2004) and may be due to minimal tissue damage (Kaloshian and Walling, 2005).

Differences in *B. brassicae* performance may partly be explained by induced transcriptional responses

To examine if there is a correlation between aphid performance and inducible mechanisms in the *B. oleracea* cultivars, we compared *B. brassicae*-induced transcriptional responses of Rivera and Christmas Drumhead. We found many differences in the transcriptional response of the two cultivars and only two commonly regulated genes. Interestingly, one of the two commonly induced genes in both cultivars, *XTH6*, encodes a xyloglucan endotransglucosylase. Genes encoding XTH were also induced in other aphid-infested plant species (Voelckel et al., 2004; Divol et al., 2005). XTHs remove and re-attach oligosaccharides thereby modifying hemicelluloses to strengthen cell walls (Campbell and Braam, 1999). Cell wall modifications in response to aphid feeding may deter these insects both locally and systemically by strengthening barriers against probing and feeding (Thompson and Goggin, 2006). To further investigate the role of *XTH6* in defence against *B. brassicae*, we studied the expression behaviour of this gene in all four cultivars at several time points after aphid feeding. This second experiment revealed an induction of *XTH6* in Rivera and Lennox, cultivars supporting relative slow *B. brassicae* population increase. However, *XTH6* was also significantly induced in Badger Shipper, which supports relative fast aphid population growth. These observations suggest that *XTH6* does not explain the differences in *B. brassicae* performance between the cultivars. Indeed, aphid performance on an *A. thaliana xth6* knockout mutant was similar (P = 0.73) to that on wild type *A. thaliana* plants (Figure 6). However, several members of the XTH family have high homology to *XTH6* (Divol et al., 2007) and may take over its role when *XTH6* expression is absent. Furthermore, XTHs may influence aphid preference as a mutation in the *XTH33* gene in *A. thaliana* increased *M. persicae* preference (Divol et al., 2007).

We identified *TPI* as almost significantly induced only in Rivera (P = 0.091) in the microarray analysis. In a qRT-PCR time-course experiment, *TPI* was found to be significantly induced in Rivera, one of the cultivars supporting relative slow aphid population increase. Furthermore, *TPI* expression was repressed in Christmas Drumhead after 72 h and in Badger Shipper after 24 h of *B. brassicae* feeding. Although no changes in *TPI* expression could be detected in Lennox, which also supported relative slow aphid population growth, we hypothesized a possible role for this gene in defence. Indeed, higher numbers of *B. brassicae* individuals were found on an *A. thaliana tpi*-mutant than on wild type plants of this species after 10 days of population development (Figure 6). This suggests a role for *TPI* in defence against this phloem-feeding herbivore. However, *B. oleracea TPI* silenced mutants are needed to confirm the function of this gene in white cabbage.

Five genes were significantly induced in Rivera only, but literature searches revealed that the functions of these genes are unknown to date. Whether these genes are involved in resistance against aphids therefore remains to be investigated.

Two genes encoding aquaporins (*PIP2.2*, *RD28*) were also induced in Rivera. The induction of one of these genes, *RD28*, was almost significant (P = 0.081) in Christmas Drumhead. The induction of aquaporins may be linked to an altered source-sink transition in the plant due to aphid feeding. From a plant's perspective, aphids are sinks for sugars and nutrients (Douglas, 2003), similar to newly expanding leaves. The induction of aquaporins could increase nutrient concentrations in the phloem that benefit the aphid.

One of the genes that were only induced in Christmas Drumhead, *TGG2*, encodes a myrosinase that hydrolyzes glucosinolates to form toxic products (Xu et al., 2004). Since aphid feeding does not result in extensive tissue damage, glucosinolates may not come into contact with the induced TGG2 myrosinase (De Vos et al., 2007). This is supported by the observation that reproduction of *B. brassicae* on *A. thaliana* is not affected by a *tgg2* mutation (Barth and Jander, 2006).

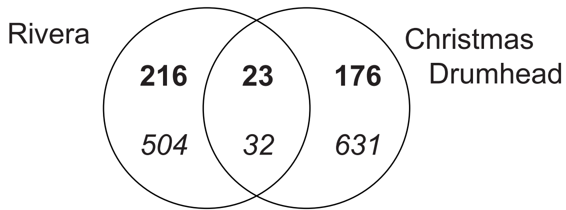
Conclusions

We have addressed intraspecific plant variation and its effects on the aphid *B. brassicae* at different levels of biological integration under glasshouse and field conditions. We demonstrated that *B. brassicae* performed differently on four cultivars of *B. oleracea*, both in the glasshouse and in the field. Based on these results, the cultivars could be divided into a group supporting relatively fast and a group supporting relatively slow aphid population growth. Transcriptional responses to aphid feeding in two cultivars, one from each group, were relatively small; a low number of genes were differentially regulated. The genes that did show significant regulation were highly cultivar-specific. One of the genes that was only induced in one of the cultivars supporting slow aphid population growth and repressed in both cultivars with high aphid population growth, i.e. *TPI,* possibly plays a role in defence against *B. brassicae*.

Acknowledgements

This research was supported by the Dutch ministry of Agriculture, Nature, and Food quality. MD was additionally supported by a VICI grant from the Netherlands Organization for Scientific Research, NWO (865.03.002). We thank David Galbraith for supplying microarrays, David Gonidec for assistance in the field; Ana Escribano Cumba for assistance in the glasshouse, Leo Koopman, André Gidding, and Frans van Aggelen for insect rearing; CGN and Bejo Zaden for providing seeds of the cultivars; Unifarm for maintenance of the plants and field site, and two anonymous reviewers for constructive comments on an earlier version of the manuscript.

Supplemental material



Supplemental Figure 1. Venn diagram representing the number of genes significantly regulated ($P < 0.05$) in *B. oleracea* cultivars Rivera and Christmas Drumhead. Induced and repressed genes are represented in bold and italic, respectively. Numbers in the overlapping area represent genes regulated in both cultivars.

Chapter 4

Transcriptional response of wild and cultivated *Brassica* to two specialist insect herbivore species

Colette Broekgaarden, Roeland E. Voorrips, Marcel Dicke & Ben Vosman

Abstract

Plants show phenotypic changes when challenged with herbivorous insects. The mechanisms underlying these changes include the activation of transcriptional responses. Comparing transcriptional responses of wild and cultivated members of the same plant family may contribute to the understanding of plant-herbivore interactions. Previously, we showed that white cabbage (*Brassica oleracea* var. *capitata*) cultivars exhibit intraspecific variation in susceptibility and transcriptional responses to feeding by the caterpillar *Pieris rapae* or the aphid *Brevicoryne brassicae*. Here, we study interspecific variation in susceptibility and transcriptional responses to herbivore infestation between a wild black mustard population (*Brassica nigra*) and white cabbage cultivars. We analyzed gene expression changes of *B. nigra* after infestation with either *P. rapae* or *B. brassicae* by using an *Arabidopsis thaliana* whole-genome microarray. The results show that *P. rapae*- and *B. brassicae*-regulated genes are highly insect-specific. Comparing the transcriptional responses of *B. nigra* and *B. oleracea* cultivars after *P. rapae* feeding suggests that certain lines of defence in *B. nigra* are absent in the cultivated material and vice versa. A targeted study employing an *A. thaliana* knockout mutant revealed that a trypsin-and-protease inhibitor, of which its gene expression was only induced in *B. oleracea* cultivars, negatively influences *P. rapae* performance. Additionally, we observed interspecific variation in *B. brassicae* performance between *B. nigra* and *B. oleracea* cultivars that can be partly explained by differences in transcriptional responses. Overall, this study shows clear differences in susceptibility and transcriptional responses to herbivore feeding between cultivated and wild *Brassica* species that provide more insight into plant-insect interactions.

Introduction

In their natural environment, plants are under constant threat of herbivorous insect attack. Despite the fact that plants can not avoid these herbivores by simply moving away, the Earth is generously covered with flora. Thus, plants clearly possess effective defence mechanisms to prevent or overcome herbivore attack. Plant defence mechanisms are based on a combination of physical and chemical features that can be constitutively present or induced upon herbivore attack (Kessler and Baldwin, 2002; Schoonhoven et al., 2005). The defence mechanisms of plants can be classified as direct defences that negatively affect herbivore growth and survival, or indirect defences that enhance the effectiveness of natural enemies of herbivores. Morphological factors that interfere with feeding or oviposition, such as trichomes or leaf toughness, provide a first barrier to herbivores (Schoonhoven et al., 2005). Secondly, the production of toxic or repellent secondary metabolites affects insect behaviour and physiology (Roda and Baldwin, 2003). Finally, the production and release of volatile compounds affects higher trophic levels by functioning as cues for predators and parasitoids that enable them to locate their herbivorous victims (D’Alessandro and Turlings, 2006; Pichersky and Gershenzon, 2002).

Glucosinolates comprise a group of secondary metabolites that are almost exclusively found in species of the Brassicaceae family, which include *Brassica* crops, native *Brassica* species as well as the model plant *Arabidopsis thaliana.* Herbivore attack, particularly by chewing insects, initiates myrosinase-catalyzed glucosinolate breakdown in plants, leading to the generation of a variety of bioactive compounds such as isothiocyanates, thiocyanates, or nitriles (Grubb and Abel, 2006). The glucosinolate/myrosinase system serves as a defence against generalist insects, whereas many specialists have adapted to this system (Agrawal, 2000; Després et al., 2007; Kliebenstein et al., 2005). Specialist herbivores have evolved enzyme systems to detoxify glucosinolates (Ratzka et al., 2002; Wittstock et al., 2004) or are even able to accumulate intact glucosinolates to use them for their own defence (Després et al., 2007; Kazana et al., 2007; Müller et al., 2001; Pratt et al., 2008). However, specialists may be susceptible to high concentrations of secondary metabolites (Adler et al., 1995; Agrawal and Kurashige, 2003; Steppuhn et al., 2004).

Insect herbivores activate plant defences via signalling pathways in which the plant hormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) play important roles (Pieterse and Dicke, 2007). Accumulation of these hormones results in the activation of defence-related genes followed by the production of various metabolic defences. Cross-talk between signal-transduction pathways can occur that further shapes the final response. Both positive and negative interactions between pathways have been reported (Beckers and Spoel, 2006; Bostock, 2005; Rojo et al., 2003). Depending on the type of herbivore, different plant hormones can be induced resulting in different plant responses (De Vos et al., 2005) and thus in different plant phenotypes. Lepidopteran larvae, for example, cause extensive tissue damage thereby eliciting JA and ET induction (De Vos et al., 2005; Kessler and Baldwin, 2002). Conversely, phloem feeding insects such as aphids only briefly puncture cells during their search for the phloem and may therefore elicit different plant responses than chewing insects (De Vos et al., 2005; Walling, 2000). Microarray analyses of the transcriptional changes induced by herbivory have identified genes responsive to damage inflicted by several herbivorous insects

(De Vos et al., 2005; Hui et al., 2003; Reymond et al., 2004; Thompson and Goggin, 2006; Voelckel and Baldwin, 2004), but few studies directly compared the transcriptional responses of a plant to specialist chewing and phloem-feeding insects (Heidel and Baldwin, 2004; Mewis et al., 2006).

Herbivorous insects are a worldwide problem in agroecosystems and this has promoted the study of insect-plant interactions in crop plants. However, cultivated plants have been under intense selection for particular yield- and quality-enhancing traits and original defence strategies that were present in wild progenitors may have been disrupted or lost (Rosenthal and Dirzo, 1997). Breeding has often resulted in reduced levels of certain secondary compounds (Evans, 1993). A comparative analysis using cultivated species and their wild relatives can provide useful information to better understand plant-herbivore interactions. Several studies showed interspecific variation in herbivore resistance among wild and cultivated accessions within a plant family (Benrey et al., 1998; Ellis et al., 2000; Gols et al., 2008; Harvey et al., 2007). However, no study on interspecific variation in plant defence so far has linked transcriptional analysis with herbivore performance data.

Previously, we studied intraspecific variation in transcriptional responses of white cabbage (*Brassica oleracea*) cultivars to feeding by the specialist herbivores *Pieris rapae* and *Brevicoryne brassicae* (Broekgaarden et al., 2007; 2008). Furthermore, intra- and interspecific variation in *P. rapae* performance on cultivated *B. oleracea* and the wild *Brassica nigra* revealed that this insect performed best on the latter species (Poelman *et al.* 2008b). *B. nigra* and *B. oleracea* possess contrasting life histories and morphological characteristics that may result in the use of different defence strategies against herbivorous insects. Here, we characterize transcriptional responses of plants from this *B. nigra* population to feeding by *P. rapae* or *B. brassicae.* For this purpose, we used a 70-mer oligonucleotide microarray based on the *A. thaliana* genome. This microarray has been successfully used to study the transcriptional responses of *B. oleracea* (Broekgaarden et al., 2007; 2008) and is therefore expected to be a useful tool to investigate the transcriptional responses of *B. nigra* too. Furthermore, we compare *P. rapae*- and *B. brassicae*-induced transcriptional responses in *B. nigra* to those elicited in the two *B. oleracea* cultivars Rivera and Christmas Drumhead (Broekgaarden et al., 2007; 2008). Our results show clear interspecific variation in inducible transcriptional responses of plants to specialist herbivores that can partly explain the observed differences in insect performance.

Materials and Methods

Plant cultivation and insect rearing

Seeds of *Brassica nigra* were collected in 2000 from a wild population near Heteren, The Netherlands. Seeds were germinated on peat soil (Lentse Potgrond®, No. 4) and transferred to 1.45 L pots containing the same soil after two weeks. Plants were cultivated in a greenhouse compartment with a 16 h day and 8 h night period (22 ± 4 °C). The relative humidity was maintained at 60 to 70 %. All plants were watered every other day without chemical control for pests and diseases.

Seeds of *Arabidopsis thaliana* accession Col-0 and knockout mutant *tpi* (T-DNA insertion line SALK_009681) were obtained from the SIGnAL collection (Alonso et al., 2003; http://signal.salk.edu). Plants

were sown in autoclaved potting compost (Lentse Potgrond®) and two-week old seedlings were transferred to 60 ml pots containing the same autoclaved potting compost. Plants were cultivated in a growth chamber with an 8 h day (200 μE.m^{-2}.sec^{-1}) and 16 h night cycle at 20 ± 2 ^{o}C and 65 ± 10 % relative humidity. All plants were watered every other day. No chemical control for pests or diseases was performed.

Larvae of the small cabbage white butterfly *Pieris rapae* and cabbage aphids (*Brevicoryne brassicae*) originated from the stock rearing of the Laboratory of Entomology, Wageningen University. They were individually maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in a growth chamber with a 16 h day and 8 h night cycle (21 ± 2 ^{o}C, 50-70% relative humidity).

Insect infestations

Insects were placed on the adaxial surface of the youngest fully expanded leaf of seven-week-old *B. nigra* plants. We infested plants by confining either ten first instar caterpillars or 20 wingless aphids of assorted life stages in a clip cage on the leaf. Leaf discs (diameter 2.3 cm) taken next to the clip cage were collected, immediately flash frozen in liquid nitrogen, and stored at -80 ^{o}C until use. Material was collected after 24, 48, and 72 h of caterpillar feeding from different plant groups or after 48 h of aphid feeding. Leaf discs of twelve plants were pooled. Control material for caterpillar infestation was collected at the start of the experiment from a different plant group, whereas control plants for aphid infestation received empty clip cages and material was collected at the same time as from infested plants. In a second experimental setup, plants were infested with aphids as described above. Leaf discs were collected after 48 h of aphid feeding. Leaf discs of five plants were pooled. Control material was collected at the start of the experiment. All experiments were set up in a randomized block design in such a way that we obtained three independent biological replicates.

Microarray hybridization and analysis

Pooled leaf samples were ground in liquid nitrogen and total RNA was extracted with TRIzol reagent (Invitrogen) followed by a purification using the Rneasy Plant Mini kit (Qiagen). Four μg of total RNA were linearly amplified using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). Control and herbivore-infested samples were labelled respectively with Cy3 and Cy5 monoreactive dye (Amersham). Amplified RNA was labelled in freshly made 0.2 M sodium carbonate buffer (pH 9.0) for 1 h at room temperature. Dye incorporation was monitored by measuring the Cy3 and Cy5 fluorescence emissions using a nanodrop ND-1000 UV-Vis Spectrophotometer (BioRad). Microarrays containing 70-mer oligonucleotides based on the genome of *A. thaliana* were obtained from the group of David Galbraith from the University of Arizona (http://www.ag.arizona.edu/microarray). Immobilization of the array elements was performed according to the protocol on the manufacturer's website (see above). The hybridization mixture contained 100 pmol of the Cy3-labeled sample, 50 pmol of the Cy5-labeled sample, 2X SSC, 0.08% SDS, and 4.8 μl Liquid Block (Amersham) in a final volume of 80 μl. The solution was incubated at 65 ºC for 5 min before application to the microarray covered with a lifterslip (Gerhard Menzel). The microarray was placed in a hybridization chamber (Genetix) and incubated at 50 ºC. After 12 h the microarray was washed for 5 min in 2X SSC/0.5% SDS at 50 ºC, followed by a 5 min wash in 0.5X SSC at room temperature, and a final 5 min wash in 0.05X SSC at room temperature. The microarray was immediately dried by centrifugation for 4 min at 200 rpm.

Hybridized microarrays were scanned with a ScanArray Express HT Scanner (PerkinElmer). Mean fluorescent intensities for Cy3 and Cy5 were determined using the ScanArray Express software (PerkinElmer). Each image was overlaid with a grid to assess the signal intensities for both dyes from each spot. Background fluorescence was subtracted and spots with adjusted intensities lower than half the background were manually raised to half the background to avoid extreme expression ratios. Spots were excluded from the analysis when: (1) showing signal intensities less than half the background for both dyes; (2) showing aberrant shape; (3) located in a smear of fluorescence. Lowess (locfit) normalization was carried out within each slide using TIGR MIDAS version 2.19 to avoid spatial bias. Normalized expression ratios for each individual spot and the mean of the three replicate spots were calculated. A student *t* test on log_2 transformed expression ratios was conducted for each experimental condition using TIGR MEV version 3.1. Genes with a log_2 expression ratio ≥ 1 (expression ratio 2-fold) or ≤ -1 (expression ratio 0.5-fold) in combination with a P value < 0.05 were considered significantly different. We used the names of *A. thaliana* homologs to identify *B. nigra* genes and examined the potential function of differentially regulated genes according to gene ontology (GO) terms from The Arabidopsis Information Resource (http://www.arabidopsis.org).

Quantitative RT-PCR analysis

One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (BioRad). Gene-specific primers were designed for *B. nigra* genes based on sequences obtained by a BLAST search in the TIGR *B. oleracea* database. The primer sequences are shown in Table 1. Primers were tested for gene specificity by performing melt curve analysis and PCR products were sequenced to confirm amplification of the gene of interest. Sequence results were checked by a BLAST search in the *B. oleracea* as well as in the *A. thaliana* TIGR database. Quantitative RT-PCR analysis was done in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR analyses were performed in duplicate. The following PCR program was used for all PCR reactions: 3 min 95 °C; 40 cycles of 30 sec 95 °C and 45 sec 60 °C. Threshold cycle (Ct) values were calculated using Optical System software, version 2.0 for MyIQ (BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) from the Ct value of the gene

Table 1. Sequences of *B. nigra* gene specific primers used for quantitative real-time PCR analyses.

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
GAPDH	AGAGCCGCTTCCTTCAACATCATT	TGGGCACACGGAAGGACATACC
LOX2	CAGAGTTGTCAAAGCTGTTGCT	ACCATAAACCGCAGGGTCT
CTR1	AAATCAGCGGTTCCTCCAC	GCTCACGAGGCATGTACCTT
PR1	TCCACCATTGTTACACCTTGC	GGCCTTATGGAGAGAACTTGG
Trypsin inhibitor	CTGAAAGAATACGGAGGCAAC	AATACCGCCACTTAGAATCTGG
TPI	TGGTGACAAGTAGCTGTGGTG	TCCAAGTTATGGGCAGTGG
CYP83B1	CCGGAATATCATAGCCACCTATC	CCTGAAGCAATGAAGAAAGCTC

of interest. Normalized gene expression was then calculated as $2^{-\Delta Ct}$. Normalized gene expression values were used to compute log_2-transformed expression ratios for each experimental condition.

Identification of homozygous *tpi A. thaliana* T-DNA mutant plants

For genomic DNA isolation, 20-40 mg fresh leaf material from individual plants was harvested in 2 ml safe-lock microcentrifuge tubes containing 3 mm steel beads. Tissue was ground in a TissueLyser (Qiagen) for 30 sec at 30 Hz and genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen). A PCR reaction was carried out to identify the T-DNA position within the target gene. The T-DNA insertion was located 602 bp downstream of the translation start site. Gene-specific primers were designed ~550 bp up- and ~100 bp downstream of the predicted T-DNA insertion site (*TPI*-forward 5'-GTG AAG GAT ACA GCC GGA AA-3', *TPI*-reverse 5'-ATT AAG CCT GAG ACT CGT CCA T-3'). These primers were used in combination with a T-DNA left border primer (LBb1 5'-GCG TGG ACC GCT TGC TGC AAC T-3'). The following PCR program was used: 94 °C for 3 min; 30 cycles of 94 °C for 30 sec, 60 °C for 45 sec, and 72 °C for 1 min. Wild-type plants yielded a single band of ~650 bp (amplified from primers *TPI*-forward and *TPI*-reverse), whereas plants containing a T-DNA insertion yielded a single band of ~350 bp (amplified from primers LBb1 and *TPI*-reverse). No obvious differences in phenotype between wild type and mutant plants were observed.

Larval performance on *A. thaliana* mutant plants

For the caterpillar performance experiment with *A. thaliana* lines, two first-instar *P. rapae* larvae were placed on six-week-old plants. The experiment was set up in a randomized design with 20 biological replicates per line. Larvae were allowed to feed on plants for 6 days before being collected and weighed. Larval fresh weight was determined using a precision balance (Sartorius, isoCAL). Significance was tested with independent sample *t*-tests.

Aphid performance no-choice experiment on *B. nigra*

One new-born *B. brassicae* nymph was placed on each of the five youngest leaves of each five-week-old *B. nigra* plant. Individual plants were covered with gauze to prevent the aphids from escaping. The experiment was integrated in a previously described aphid performance no-choice experiment including four *B. oleracea* cultivars (Broekgaarden et al., 2008). Eighteen biological replicates (18 plants) were included for *B. nigra*. Development time (number of days between birth and first reproduction) was estimated by daily recording of the nymphs. Nymph mortality was scored 11 days after infestation, which was the day that almost all individuals had reproduced. From 11 days onwards, population size was recorded twice a week. Nymph mortality percentages were arcsine square root-transformed and development time data were log transformed to obtain normal data distributions. Transformed data were analyzed with one-way analysis of variance (ANOVA) tests. If a significant cultivar effect was present, then differences between means for ANOVA were compared with least significant difference tests ($\alpha = 0.05$). Population size was corrected for the number of *B. brassicae* nymphs that started the population and corrected data were subsequently log transformed. Resulting population development curves of *B. brassicae* were analyzed with the SPSS general linear model (GLM) procedure (Field, 2005) using repeated measurements ANOVA. Day was considered a within-subjects factor and cultivar a between-subjects factor. If a significant cultivar effect was present, then differences between means were compared with least significant difference tests ($\alpha = 0.05$).

Results

Transcriptional responses of *B. nigra* plants to *P. rapae* and *B. brassicae* feeding

Using *A. thaliana* full-genome microarrays, we investigated the transcriptional responses of *B. nigra* plants to feeding by either *P. rapae* for 24 h or *B. brassicae* for 48 h. We found 34 induced and 22 repressed genes after 24 h of *P. rapae* feeding (Table 2). Forty-eight hours of *B. brassicae* feeding caused the induction of 18 and the repression of 23 genes (Table 3). Defence-related genes were induced, whereas genes involved in protein metabolism were repressed after feeding by both insects. *P. rapae* also induced several transcription-related genes and repressed several genes involved in photosynthesis, whereas *B. brassicae* also regulated several development-related genes. Furthermore, we did not find any commonly regulated genes after *P. rapae* and *B. brassicae* feeding. To check whether the lack of overlap between the herbivore-induced specific genes was due to the stringency of our selection criteria, we examined the overlap of all genes that were significantly regulated (student *t*-test, $P < 0.05$). Of all 2134 genes that were significantly regulated, only 2 % were commonly regulated by both insects (Supplemental Figure 1), indicating that the small overlap in transcriptional responses elicited by *P. rapae* and *B. brassicae* is independent of threshold stringency to identify regulated genes.

Microarray validation

Quantitative real-time PCR (qRT-PCR) analysis was used to confirm the microarray data for one *P. rapae*-induced gene (*GSTU18*) and two *B. brassicae*-induced genes (*XTH6* and *CAT2*). The *P. rapae*-induced gene was technically validated whereas material from a second, independent experiment was used to confirm the expression of the *B. brassicae*-induced genes. In contrast to the microarray experiment, we used *B. nigra*-specific primers to perform qRT-PCR. All three genes were significantly regulated (one sample *t*-test, $P < 0.01$), showing the consistency between microarray and qRT-PCR analysis (Figure 1). Furthermore, statistical comparisons between the two types of analysis revealed no significant differences between the expression ratios for each of the three genes (paired sample *t*-test, $P > 0.05$).

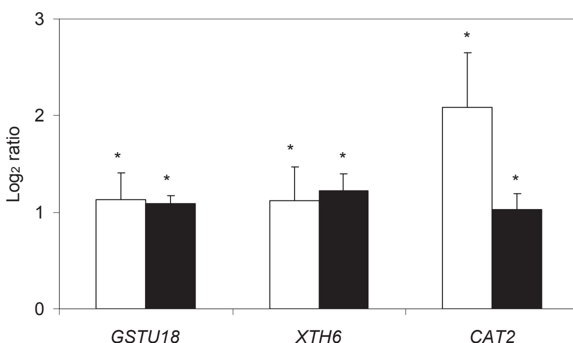


Figure 1. Comparison of microarray and qRT-PCR analysis of the induced expression levels of three herbivore-induced genes. Bars represent $\log_2$ ratios of expression levels of three genes in *B. nigra* after feeding by *P. rapae* (*Glutathione S-transferase, GSTU18*) or *B. brassicae* (*Xyloglucan endotransglucosylase, XTH6* and *Catalase 2, CAT2*). Bars represent mean $\log_2$ expression ratios (+ SD) calculated from three biological replicates obtained from microarray (white bars) or qRT-PCR (black bars) analysis. Bars marked with an asterisk are significantly regulated (student t-test, $P < 0.05$). Values obtained from the two types of analysis were not significantly different from each other (paired sample *t*-test, $P > 0.05$).

Table 2. List of genes regulated after 24 h of *P. rapae* feeding in *B. nigra*

Probe identification	AGI code	Log_2 Ratio	*P* value	Process category
Induced genes				
Hypothetical protein	At4g23090	1.22	0.019	Bioluminescence
Glutathione S-transferase (GSTU18)	At1g10360	1.13	0.019	Defence
Lectin	At1g52070	1.22	0.035	Defence
Epithiospecifier protein (ESP)	At1g54040	1.10	0.041	Defence
Terpene synthase	At1g61120	1.30	0.037	Defence
Lectin	At5g38540	1.70	0.032	Defence
Haloacid dehalogenase-like hydrolase	At2g32150	1.14	0.004	Metabolism
CAAX amino terminal protease	At2g03140	1.06	0.004	Protein metabolism
Peptidase M16	At3g19170	1.19	0.016	Protein metabolism
Ribonuclease III	At4g15417	1.07	0.040	RNA processing
DNA mismatch repair protein (MSH7)	At3g24495	1.04	0.027	Stress response
Starch phosphorylase	At3g46970	1.51	0.019	Stress response
Homeobox-leucine zipper transcription factor (PRS)	At2g28610	1.04	0.038	Transcription
Zinc finger (B-box type) family protein	At2g47890	1.18	0.034	Transcription
Zinc finger (C2H2 type) family protein	At3g10470	1.06	0.022	Transcription
Expressed protein	At3g16680	1.19	0.042	Transcription
Expressed protein	At5g25475	1.02	0.029	Transcription
Nitrate/chlorate transporter (NRT1.1)	At1g12110	1.43	0.002	Transport
F-box family protein-related	At1g47350	1.15	0.005	Unknown
Expressed protein	At1g70230	1.39	0.003	Unknown
Heat shock protein 70 (HSP70)	At1g79920	1.48	0.021	Unknown
CSL zinc finger domain-containing protein	At2g15910	1.00	0.028	Unknown
Zinc-binding protein-related	At2g17785	1.42	0.041	Unknown
Expressed protein	At3g06547	1.26	0.047	Unknown
Proline-rich family protein	At3g06870	1.09	0.002	Unknown
Expressed protein	At3g12870	1.03	0.026	Unknown
Tesmin/TSO1-like CXC domain-containing protein	At3g16160	1.64	0.031	Unknown
Complex 1 family protein	At3g62810	1.40	0.038	Unknown
Expressed protein	At4g01080	1.24	0.029	Unknown
F-box family protein	At4g05010	1.04	0.026	Unknown
Hypothetical protein	At4g18150	1.12	0.019	Unknown
Pre-mRNA cleavage complex-related	At5g11010	1.14	0.008	Unknown
Expressed protein	At5g45660	1.01	0.005	Unknown
Zinc finger (C3HC4-type RING finger) family protein	At5g55970	1.06	0.022	Unknown
Repressed genes				
Disease resistance protein (CC-NBS-LRR class)	At5g43730	-1.04	0.009	Defence
Flavin-containing monooxygenase family protein	At1g12200	-1.06	0.029	Electron transport
Peroxiredoxin Q	At3g26060	-1.31	0.006	Electron transport
Chlorophyll A-B binding protein (LHCB4.3)	At2g40100	-1.12	0.002	Photosynthesis
Carbonic anhydrase 1 (CA1)	At3g01500	-1.05	0.040	Photosynthesis
Ribulose bisphosphate carboxylase small chain 3B	At5g38410	-1.09	0.036	Photosynthesis
Protein kinase	At5g41260	-1.23	0.033	Protein metabolism
Leucine-rich repeat transmembrane protein kinase	At5g49770	-1.46	0.040	Protein metabolism
DNAJ heat shock N-terminal domain-containing protein	At5g62780	-1.34	0.042	Protein metabolism
Amidophosphoribosyltransferase	At4g38880	-1.20	0.044	Purine base synthesis
Peptidyl-prolyl cis-trans isomerase (ROC4)	At3g62030	-1.23	0.001	Signal transduction
Superoxide dismutase (FSD1)	At4g25100	-1.23	0.020	Stress response
Hypothetical protein	At2g24340	-1.49	0.032	Transcription
Heavy-metal-associated domain-containing protein	At1g22990	-1.46	0.031	Transport
Aquaporin PIP2.1 (PIP2A)	At3g53420	-1.13	0.002	Transport
Expressed protein	At1g56420	-1.51	0.049	Unknown
Expressed protein	At1g79510	-1.02	0.011	Unknown
Receptor-like protein kinase-related	At3g21960	-1.29	0.023	Unknown
Hypothetical protein	At3g51760	-1.01	0.010	Unknown
C2 domain-containing protein	At4g15740	-1.15	0.012	Unknown
Arabinogalactan-protein	At4g16980	-1.08	0.047	Unknown
Phosphatidylethanolamine-binding family protein	At5g01300	-1.11	0.027	Unknown

Relative changes in gene expression after 24 h of *P. rapae* feeding were measured in *B. nigra* leaves. Mean log_2 expression ratios and *P*-values are calculated from three independent biological replicates.
AGI, Arabidopsis Genome Initiative.

Table 3. List of genes regulated after 48 h of *B. brassicae* feeding in *B. nigra*

Probe indentification	AGI code	Log_2 Ratio	*P* value	Process category
Induced genes				
Zeaxanthin epoxidase (ABA1)	At5g67030	1.10	0.031	ABA synthesis
Xyloglucan endotransglucosylase (XTH6)	At5g65730	1.12	0.030	Cell wall metabolism
Cytochrome P450 79 F1 (CYP79F1)	At1g16410	1.53	0.018	Defence
Expressed protein (VTC2)	At4g26850	1.15	0.023	Defence
Late embryogenesis abundant 3	At1g02820	1.04	0.029	Development
Ubiquitin-specific protease	At3g11910	1.02	0.018	Protein metabolism
Protein kinase	At3g57120	1.13	0.036	Protein metabolism
Salt-tolerance zinc finger protein (STZ)	At1g27730	1.20	0.026	Stress response
Catalase 2 (CAT2)	At4g35090	2.08	0.024	Stress response
Endonuclease/exonuclease/phosphatase family protein	At4g36050	1.07	0.020	Stress response
Dehydrin (RAB18)	At5g66400	1.64	0.050	Stress response
protease inhibitor/seed storage/lipid transfer protein	At4g12510	1.99	0.022	Transport
Kinesin motor protein-related (ATK4)	At5g27000	1.00	0.009	Transport
Calcium-binding protein	At2g46600	1.07	0.037	Unknown
Dicarboxylate diiron protein	At3g56940	1.05	0.003	Unknown
Expressed protein	At5g15860	1.90	0.007	Unknown
Chaperone protein dnaJ-related	At5g43260	1.02	0.010	Unknown
Expressed protein	At5g55620	1.56	0.048	Unknown
Repressed genes				
Histone H3	At1g13370	-1.08	0.023	Cell organization & biogenesis
Protochlorophyllide reductase A (PORA)	At5g54190	-1.34	0.012	Chlorophyll synthesis
Leucine-rich repeat family protein (AIR9)	At2g34680	-1.45	0.007	Development
Glycine-rich RNA-binding protein (GRP2)	At4g13850	-1.21	0.040	Development
O-methyltransferase family 2 protein	At1g33030	-1.22	0.022	Lignin synthesis
40S ribosomal protein S7 (RPS7C)	At5g16130	-1.15	0.031	Protein metabolism
Subtilase family protein	At5g45650	-1.26	0.010	Protein metabolism
29 kDa ribonucleoprotein	At2g37220	-1.34	0.034	Stress response
Dehydration-induced protein (ERD15)	At2g41430	-1.77	0.003	Stress response
Glucan phosphorylase	At3g29320	-2.05	0.034	Stress response
Hydrophobic protein	At4g30650	-2.07	0.031	Stress response
Germin-like protein (GER3)	At5g20630	-2.75	0.040	Stress response
ATPase	At5g62670	-1.28	0.020	Transport
Expressed protein	At1g11850	-1.08	0.024	Unknown
Dormancy-associated protein (DRM1)	At1g28330	-2.93	0.042	Unknown
Expressed protein	At1g54920	-1.05	0.035	Unknown
Expressed protein	At1g74830	-1.20	0.013	Unknown
Dormancy/auxin associated	At2g33830	-2.31	0.046	Unknown
Photosystem II 5 kD protein	At3g21055	-1.32	0.025	Unknown
29 kDa ribonucleoprotein (CP29)	At3g53460	-1.21	0.000	Unknown
Expressed protein	At4g14270	-1.06	0.043	Unknown
Acid phosphatase class B family protein	At5g44020	-1.10	0.022	Unknown
Expressed protein	At5g57760	-1.53	0.001	Unknown

Relative changes in gene expression after 48 h of *B. brassicae* feeding were measured in *B. nigra* leaves. Mean log_2 expression ratios and *P*-values are calculated from three independent biological replicates.
AGI, Arabidopsis Genome Initiative.

Comparing transcriptional responses of *B. nigra* and *B. oleracea* to *P. rapae* feeding

In previous studies, we used the same *A. thaliana* microarray to study transcriptional responses after either *P. rapae* or *B. brassicae* feeding in the two *B. oleracea* cultivars Rivera and Christmas Drumhead (Broekgaarden et al., 2007). The experiments with *B. nigra* described here were integrated in these experiments with *B. oleracea* cultivars, thus allowing a direct comparison of the results. Larval feeding resulted in the differential regulation of a lower number of genes in *B. nigra* than in *B. oleracea* cultivar Christmas Drumhead, but more than in cultivar Rivera as the latter cultivar showed no significantly regulated genes after 24 h of *P. rapae* feeding (Broekgaarden et al., 2007). A comparison of the responses showed that 26 % (9/34) of the genes induced in *B. nigra* were also induced in Christmas Drumhead (Figure 2) including two genes encoding lectin, a *terpene synthase*, a *starch phosphorylase*, and several genes of unknown function (Supplemental Table 1).

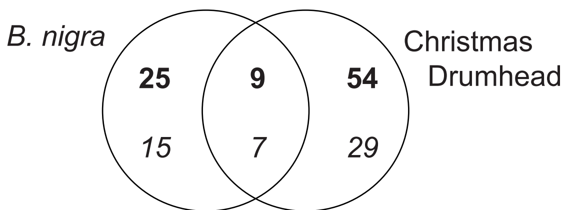


Figure 2. Venn diagram representing the number of genes showing common or differential regulation in response to *P. rapae* feeding in *B. nigra* and the *B. oleracea* cultivar Christmas Drumhead. Induced and repressed genes are shown in bold and italic, respectively.

The defence-related gene *ESP*, which encodes an epithiospecifier protein, was induced in *B. nigra* only. On the other hand, several defence-related genes were induced in Christmas Drumhead that did not show an expression change in *B. nigra* (Supplemental Table 1). Quantitative RT-PCR with *Brassica*-specific primers was used to further analyse four of these defence-related genes: *lipoxygenase 2* (*LOX2*), *trypsin inhibitor*, *trypsin-and-protease inhibitor* (*TPI*), and *cytochrome P450 B1* (*CYP83B1*). Relative expression levels were measured in control leaves and leaves challenged with *P. rapae* for 24, 48 or 72 h. Log_2 expression ratios were then calculated and compared to log_2 expression ratios in Rivera and Christmas Drumhead (Broekgaarden et al., 2007). The *LOX2* and *trypsin inhibitor* genes showed a quantitatively similar response in *B. nigra* compared to the two *B. oleracea* cultivars (Figure 3). Both genes were significantly induced after 72 h of *P. rapae* feeding (student *t* test, $P < 0.01$). The expression of *TPI* and *CYP83B1* did not change after *P. rapae* feeding in *B. nigra*, whereas the expression of these genes was induced in Rivera and Christmas Drumhead at all time points tested (student *t*-test, $P < 0.05$; Figure 3).

Fourteen percent (7/51) of the genes repressed in *B. nigra* were also repressed in the *B. oleracea* cultivar Christmas Drumhead (Figure 2), including genes involved in protein metabolism, photosynthesis, carbon utilization, and several genes of unknown function. In contrast to *B. nigra*, two development-related genes were repressed in Christmas Drumhead (Supplemental Table 1).

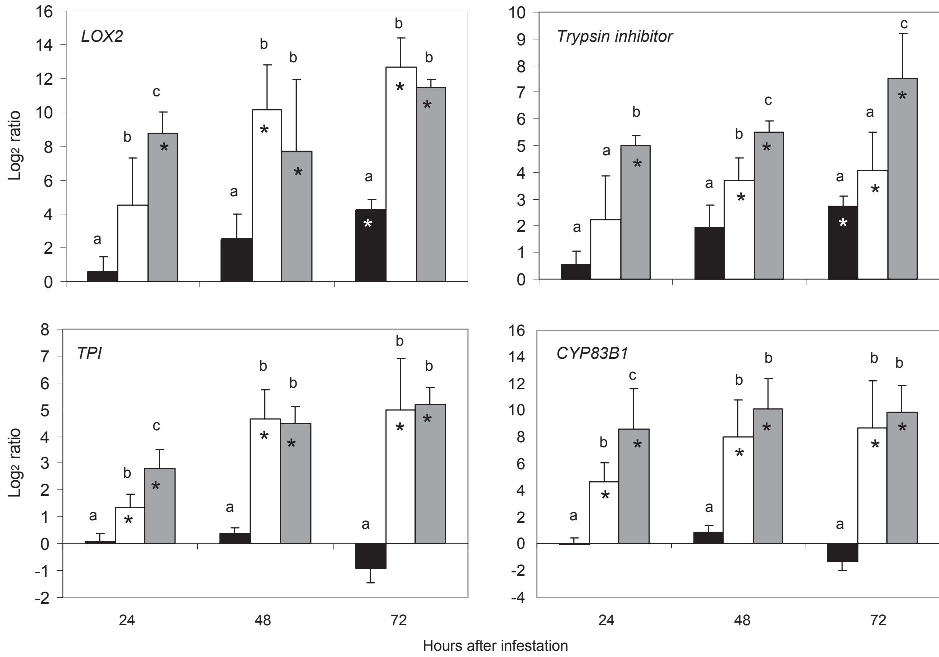


Figure 3. Time course of expression changes in *B. nigra* (black bars), *B. oleracea* cultivar Rivera (white bars), and *B. oleracea* cultivar Christmas Drumhead (grey bars) after *P. rapae* feeding. Quantitative RT-PCR data are shown for *Lipoxygenase 2* (*LOX2*), *Trypsin inhibitor*, *Trypsin-and-protease inhibitor* (*TPI*), and *Cytochrome P450 83 B1* (*CYP83B1*). Values are the mean (+ SD) of three biological replicates and marked with an asterisk when significantly regulated (student *t*-test, $P < 0.05$). Bars marked with different letters are significantly different (one-way ANOVA, LSD, $P < 0.05$).

Effect of *TPI* on *P. rapae* feeding behaviour

Silenced mutants are good tools to study the role of a particular gene in defence, but unfortunately no *Brassica* mutants are available yet. In order to study the effect of *TPI* on *P. rapae* performance, we used an *A. thaliana* mutant line that has a T-DNA insertion in the *TPI* gene. We allowed *P. rapae* larvae to feed on *tpi* mutant and wild type plants. *P. rapae* larvae were significantly heavier after 6 days of feeding on the *tpi* mutant than on wild-type plants (independent sample *t*-test, $P < 0.05$; Figure 4).

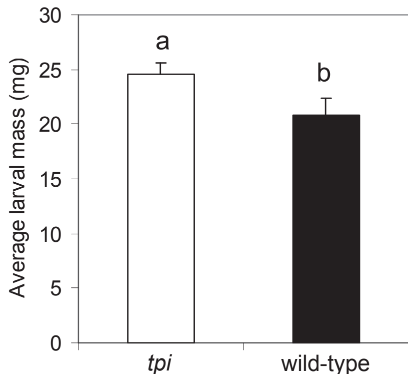


Figure 4. Growth of *P. rapae* caterpillars on *tpi*-insertion mutant and wild-type *A. thaliana* plants. Weight gain (fresh weight) of newly hatched larvae after six days of feeding. Values represent means (n = 20) + SE. Bars are marked with different letters indicating a significant difference according to an independent sample *t*-test ($P < 0.05$).

Comparing transcriptional responses of *B. nigra* and *B. oleracea* to cabbage aphid and aphid performance on these two *Brassica* species

To examine interspecific variation among *Brassica* species with respect to *B. brassicae* performance we tested nymph mortality, development time, and population development of this aphid on *B. nigra* in a no-choice experiment in a glasshouse. The experiment was integrated in an experiment previously reported in which *B. brassicae* performance was tested on several *B. oleracea* cultivars (Broekgaarden et al., 2008). In this way, we were able to compare aphid performance on *B. nigra* and the *B. oleracea* cultivars Rivera and Christmas Drumhead under identical conditions. Eleven days after placing neonate nymphs on the plant, 29% of the nymphs had died on *B. nigra* plants, a mortality similar to that on *B. oleracea* cultivars Rivera and Christmas Drumhead (ANOVA $P > 0.05$, Figure 5A). The development time, i.e. the number of days between birth and first reproduction, was significantly shorter on *B. nigra* than on the *B. oleracea* cultivars ($P < 0.05$, Figure 5B). Population size increase, expressed as the number of aphids per plant over the different time points, was significantly different among the *Brassica* species (GLM repeated measurements ANOVA $P = 0.007$, Figure 5C). At the end point, populations of *B. brassicae* were 2 times larger on *B. nigra* than on Rivera.

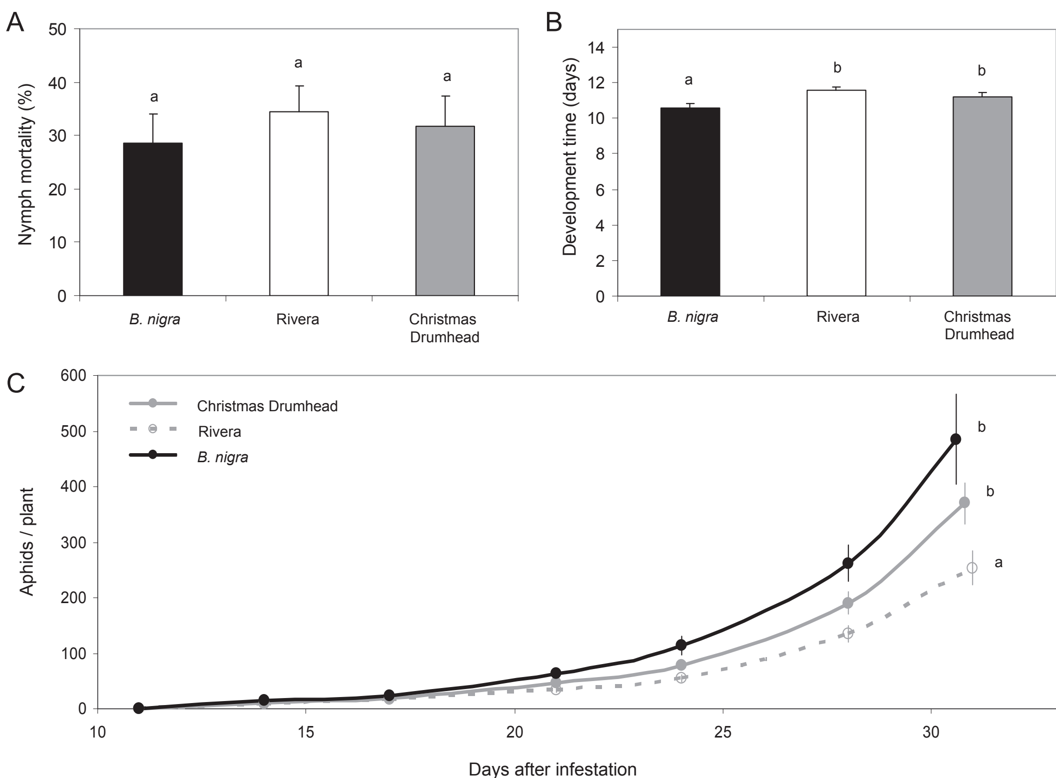


Figure 5. Performance of *B. brassicae* aphids on *B. nigra* and the two *B. oleracea* cultivars Rivera and Christmas Drumhead. (A) Percentage nymph mortality 11 days after infestation, (B) Number of days between birth and first reproduction (C) population development resulting from the infestation with a single first instar nymph during 31 days. Values represent means ± SE of 18 (*B. nigra*) or 20 (*B. oleracea* cultivars) plants. Bars marked with different letters are significantly different (one-way ANOVA, LSD, $P < 0.05$).

Transcriptional responses after *B. brassicae* feeding in *B. nigra* showed a similar number of regulated genes as identified in the *B. oleracea* cultivars Rivera and Christmas Drumhead (Broekgaarden et al., 2008), but the responses were highly specific to the host plant (Figure 6). Only one gene, encoding a protein involved in cell wall metabolism (xyloglucan endotransglucosylase, XTH6), was found to be commonly induced in the *Brassica* species. *Catalase 2*, which is involved in oxidative stress, was induced both in Rivera and in *B. nigra*. Genes significantly induced in Rivera include mainly genes of unknown function (Supplemental Table 2). Quantitative RT-PCR showed that one defence-related gene (*TPI*) was induced by *B. brassicae* in Rivera (Broekgaarden et al., 2008), whereas its expression did not change in Christmas Drumhead (Chapter 3) and *B. nigra* (data not shown).

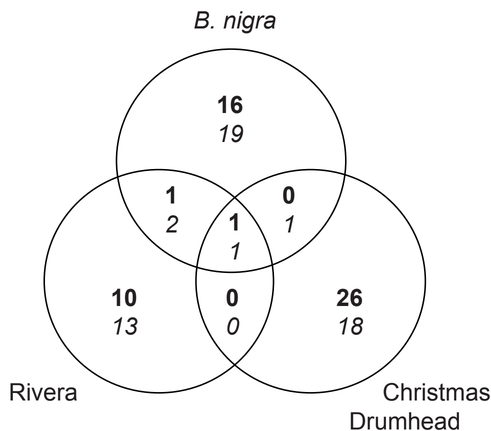


Figure 6. Venn diagram representing the number of genes showing common or differential regulation in response to *B. brassicae* feeding in *B. nigra* and the *B. oleracea* cultivars Rivera and Christmas Drumhead. Induced and repressed genes are shown in bold and italics, respectively.

Discussion

Induced transcriptional responses in *B. nigra* is highly insect-specific

We analyzed the transcriptional responses of *B. nigra* to insect feeding using a 70-mer oligonucleotide microarray based on the genome of *A. thaliana.* This microarray has previously been successfully used to study transcriptional responses in *B. oleracea* (Broekgaarden et al., 2007; 2008) and was, therefore, expected to be a good tool to study *B. nigra* responses as well. Indeed, data obtained from the present microarray analysis using material from *B. nigra* were validated using qRT-PCR and were shown to be reliable (Figure 1). We found that 24 h of *P. rapae* feeding and 48 h of *B. brassicae* feeding resulted in the induction of defence-related genes in *B. nigra*, indicating the activation of certain defence mechanisms. Several photosynthesis- and/or development-related genes were repressed upon *P. rapae* or *B. brassicae* feeding. Since defence activation has been shown to be costly (Baldwin, 1998; Herms and Mattson, 1992), it is likely that plants have reallocated resources for defence at the expense of growth and/or photosynthesis. Although both insects regulated the expression of genes from similar process categories, the induced transcriptional responses were highly insect-specific. No differentially expressed genes ($\log_2$ expression ratio ≥ 1 or ≤ -1, $P < 0.05$) were found to be regulated after *P. rapae* as well as after *B. brassicae* feeding

and the overlap of all significantly regulated genes (*P* < 0.05) was very small. Large differences in transcriptional responses induced after feeding by insects with different feeding strategies were also found in other studies (De Vos et al., 2005; Heidel and Baldwin, 2004; Voelckel et al., 2004). The difference in transcriptional responses to *P. rapae* and *B. brassicae* feeding may result from the different feeding modes of these insects. For example, *P. rapae* feeding causes activation of genes associated with JA biosynthesis, such as *LOX2* (Figure 3; Reymond et al., 2004; Zheng et al., 2007), resulting in the accumulation of JA (Bell et al., 1995). Conversely, no *LOX2* expression was induced after feeding by *B. brassicae* (Table 2). This corresponds with the observed differences in JA accumulation in *A. thaliana* in response to *P. rapae* and *Myzus persicae* feeding (De Vos et al., 2005).

Transcriptional responses after *P. rapae* feeding differ between *B. nigra* and *B. oleracea*

In wild plant populations, such as the *B. nigra* studied here, plant defence mechanisms have evolved under natural selection pressures from biotic and abiotic origin. *B. oleracea* cultivars, on the other hand, are the result of artificial selection for certain plant traits and original defence mechanisms may have been disrupted or lost (Evans, 1993; Rosenthal and Dirzo, 1997). The results of this study revealed that *B. nigra* differentially regulated fewer genes than the *B. oleracea* cultivar Christmas Drumhead after 24 h of *P. rapae* feeding. This may be due to differences in hybridization efficiency of the *A. thaliana* microarray as 70% of the oligonucleotides present on the microarray showed intensity signals after hybridizing with *B. nigra* material compared to 90% for *B. oleracea* material (Broekgaarden et al., 2007). However, this does not influence the overlap of responsive genes since only two differentially regulated genes in Christmas Drumhead did not show intensity signals after hybridization with *B. nigra* material. When comparing the responses of individual genes we found a small overlap in transcriptional responses of *B. nigra* and Christmas Drumhead after *P. rapae* feeding, but most of the genes were induced in a host-specific way. No comparison could be made between *B. nigra* and the *B. oleracea* cultivar Rivera as no significant changes in gene expression were detected in Rivera at that time point (Broekgaarden et al., 2007). In the field, *B. nigra* plants harboured less individuals of *P. rapae* than the *B. oleracea* cultivars Rivera and Christmas Drumhead (Poelman et al., 2008c), suggesting that certain defence mechanisms present in *B. nigra* are lacking in the *B. oleracea* cultivars. One of the genes that were only induced in *B. nigra* after 24 h of *P. rapae* feeding encodes an epithiospecifier protein (ESP). Transgenic *A. thaliana* plants overexpressing *ESP* have shown to be less attractive to ovipositing *P. rapae* females (De Vos et al., 2008; Mumm et al., 2008). Interestingly, no-choice experiments performed in a glasshouse showed better *P. rapae* performance on *B. nigra* than on the *B. oleracea* cultivars (Poelman et al., 2008b). These observations suggest that *B. nigra* uses ESP together with other strategies to survive in nature, for example completing its life cycle shortly after herbivores become active in the field (Poelman et al., 2008c). Furthermore, *B. nigra* may cause butterfly egg mortality through the induction of a hypersensitive response as has been observed in some *B. nigra* populations (Shapiro and DeVay, 1987).

The observed differences in *P. rapae* performance between the *Brassica* species (Poelman et al., 2008b) suggests that certain defence traits are present in the *B. oleracea* cultivars that are lacking in *B. nigra*. The induced expression of *LOX2* in *B. nigra* was observed later and at a lower level than in the *B. oleracea* cultivars (Figure 3). This suggests that *B. nigra* accumulated less JA, as *LOX2*

expression is required for JA biosynthesis (Bell et al., 1995). Lower JA concentrations may result in better *P. rapae* performance since blocking JA-mediated responses in *A. thaliana* plants increased *P. rapae* performance (Reymond et al., 2004). The expression pattern of *trypsin inhibitor* was similar to that of *LOX2* as it also showed a later and lower level of induction after *P. rapae* feeding than in the two *B. oleracea* cultivars (Figure 3). This difference in expression behaviour may also contribute to the difference in *P. rapae* performance between the *Brassica* species as trypsin *inhibitor* encodes a proteinase inhibitor that inhibits insect digestive proteases thereby reducing insect performance (Ryan, 1990). However, more studies are needed to determine the exact role of this gene in direct defence against *P. rapae.* Another gene that encodes a proteinase inhibitor, *TPI,* showed no induction in *B. nigra* after *P. rapae* feeding, whereas its expression was induced in the *B. oleracea* cultivars (Figure 3). The finding that *P. rapae* performed better on the *A. thaliana tpi* knock-out mutant (Figure 4), suggests a role for *TPI* in defence of *Brassica* against this chewing herbivore. However, *Brassica* mutants that are silenced in *TPI* expression are needed to support this suggestion. The defence-related gene *CYP83B1*, which plays a crucial role in the production of indole glucosinolates (Bak et al., 2001; Hansen et al., 2001) showed no changes in expression in *B. nigra* after *P. rapae* feeding whereas it was induced in both *B. oleracea* cultivars (Figure 3; Broekgaarden et al., 2007). This suggests that *B. nigra* does not induce indole glucosinolates after *P. rapae* feeding, which is supported by a study of glucosinolate induction in *B. nigra* under glasshouse conditions (Poelman et al., 2008b). However, differences in accumulation of glucosinolates probably do not explain differences in *P. rapae* performance (Poelman et al., 2008b), most likely because specialist herbivores such as *Pieris* spp. have evolved mechanisms to detoxify glucosinolates (Wittstock et al., 2004).

Interspecific variation in transcriptional responses may partly explain differences in *B. brassicae* performance

Under no-choice conditions, cabbage aphids performed better on *B. nigra* than on the *B. oleracea* cultivar Rivera. Additionally, no difference in performance was found between aphids feeding on *B. nigra* and Christmas Drumhead (Figure 5). When comparing the induced responses after *B. brassicae* feeding of *B. nigra* and the two *B. oleracea* cultivars, we found that a small number of genes were differentially regulated in all three plants and that regulation was highly host specific. Quantitative RT-PCR revealed that the expression of the defence-related gene *TPI* was not regulated in *B. nigra* and Christmas Drumhead (Broekgaarden et al., 2008) after feeding by *B. brassicae*, whereas its expression was induced in Rivera (Broekgaarden et al., 2008). Disrupting the expression of this gene in *A. thaliana* resulted in better *B. brassicae* performance compared to wild-type plants (Broekgaarden et al., 2008). The lack of regulation of other defence-related genes after *B. brassicae* feeding suggests that constitutive factors also negatively affect aphid performance.

Although *B. brassicae* has developed a mechanism to benefit from glucosinolates (Kazana et al., 2007), higher glucosinolate concentrations can have a negative effect on aphid numbers per plant (Mewis et al., 2005). The higher constitutive glucosinolate concentrations in *B. nigra* compared to the *B. oleracea* cultivars (Poelman et al., 2008b) may, therefore, contribute to the differences in *B. brassicae* performance. Not only defence mechanisms, but also different nutritional values may account for differences in *B. brassicae* performance (Awmack and Leather, 2002). A recent study shows that a higher nutritional quality of wild than of cultivated *Brassica* resulted in higher aphid densities (Bukovinszky et al., 2008).

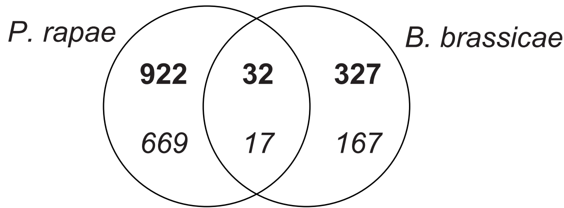
Conclusion

We have compared the response of *B. nigra* to specialist herbivores with different feeding strategies and found that they induce different transcriptional responses. The defence-related gene *ESP* was only induced in *B. nigra* after 24 h of *P. rapae* feeding, indicating the absence of certain lines of defence in the cultivated material. However, our results also suggest that certain defence traits are present in the cultivars that are lacking in *B. nigra*. Several genes that were induced in the *B. oleracea* cultivars but not in *B. nigra* may be involved in direct defence against *P. rapae* as *B. nigra* has previously been shown to be a better host (less resistant) than the *B. oleracea* cultivars for this herbivore under no-choice conditions. One of these genes, e.g. *TPI,* may play a role in defence against *P. rapae* in *A. thaliana.* Additionally, we observed differences in the performance of the cabbage aphid between *B. nigra* and the *B. oleracea* cultivars that can be partly explained by *B. brassicae*-induced gene expression.

Acknowledgements

This research was supported by the Dutch ministry of Agriculture, Nature, and Food quality. MD was additionally supported by a VICI grant from the Netherlands Organization for Scientific Research, NWO (865.03.002). We thank Merlijn van den Berg for assistance in the laboratory; Greet Steenhuis for assistance in all glasshouse experiments; Ana Escribano Cumba and Erik Poelman for help with counting aphids; Leo Koopman, André Gidding, and Frans van Aggelen for insect rearing; Erik Poelman for providing seeds of *B. nigra* and Unifarm for maintenance of the plants.

Supplemental material



Supplemental Figure S1 Venn diagram representing the overlap of genes significantly regulated (student *t*-test, $P < 0.05$) after feeding by *P. rapae* or *B. brassicae*.

Supplemental table S1. List of genes regulated by *P. rapae* in *B. nigra* compared to regulation in the *B. oleracea* cultivar Christmas Drumhead. This table can be found at page 124.

Supplemental table S2. List of genes regulated by *B. brassicae* in *B. nigra* compared to regulation in the *B. oleracea* cultivars Rivera and Christmas Drumhead. This table can be found at page 126.

Intraspecific variation in herbivore community composition and transcriptional profiles in field-grown *Brassica oleracea* cultivars

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Abstract

The composition of herbivore communities on field-grown plants may be influenced by intraspecific variation in plant traits. Differences in transcriptional profiles often underlie phenotypic variation among plants from the same species. Most studies on transcriptional responses of plants to herbivorous insects have been carried out under controlled conditions in the laboratory or greenhouse and only few examine intraspecific transcriptional variation. Here, we address intraspecific variation in herbivore community composition and transcriptional profiles between two *Brassica oleracea* cultivars grown in the field. Early in the season no differences were found for naturally occurring herbivores, whereas cultivars differed greatly in the abundance, species richness, and herbivore community later in the season. Genome-wide transcriptomic analysis using an *Arabidopsis thaliana* oligonucleotide microarray showed clear differences for the expression levels of 51 genes between the two cultivars later in the season. Several defence-related genes showed higher levels of expression in the cultivar that harboured the lowest numbers of herbivores. Our study shows that two *B. oleracea* cultivars grown in the field differently develop their phenotype throughout the season resulting in intraspecific variation in herbivore community composition. The differences in herbivore communities can be, at least partly, explained by differential expression of particular defence-related genes.

Introduction

Intraspecific variation in plant traits may influence the composition and diversity of herbivore communities on plants grown under natural conditions (Wimp et al., 2005; Whitham et al., 2006; Poelman et al., 2008c). Plant traits that affect herbivores include morphological factors, such as wax layers, and secondary metabolites, such as toxins and digestibility reducers (Schoonhoven et al., 2005). Differences in the transcription of particular genes have been shown to control intraspecific variation in phenotypic traits (Carroll, 2000). Studies on different populations of the same species have revealed that variation in transcription of particular genes is responsible for variation in secondary metabolite production (Wu et al., 2008) and herbivore resistance (Kuśnierczyk et al., 2007; Gao et al., 2008). Disrupting the expression of a key gene in the jasmonic acid (JA) pathway, which plays a role in the induction of defence against leaf chewing herbivores, had a significant effect on the composition of the herbivore community on tobacco plants (Kessler and Baldwin, 2004). However, nothing is known about the influence of naturally occurring intraspecific variation in gene expression on herbivore communities in the field.

Plant traits can be constitutively present, but plants can also alter their phenotype in response to herbivory (Kessler and Baldwin, 2002). For example, changes in leaf surface composition and concentrations of defence-related secondary metabolites have been observed after herbivore damage (Agrawal, 2000; Traw and Dawson, 2002a; Inbar and Gerling, 2008). Depending on their feeding strategy, herbivores differentially induce plant responses (Heidel and Baldwin, 2004; Voelckel et al., 2004; De Vos et al., 2005) and the changed plant phenotypes may affect the performance of the initial herbivore as well as that of subsequently colonizing species (Agrawal, 2000; Traw and Dawson, 2002b). In *Brassica oleracea* plants, experimentally introducing *Pieris rapae* caterpillars early in the season modifies the plants phenotype in such a way that it affects herbivore community composition (Poelman et al., 2008a; 2008d). Induced plant responses not only affect the performance of subsequent herbivores feeding on the plant, but may also affect their host plant preference (Shiojiri et al., 2002; Long et al., 2007; Poelman et al., 2008c). Herbivores may be differentially affected by induced plant responses depending on their host plant range. Induced secondary metabolites that have a negative effect on generalist herbivores may act as feeding stimulants or can be detoxified by specialists (Agrawal, 2000; Ratzka et al., 2002; Wittstock et al., 2004; Kliebenstein et al., 2005; Després et al., 2007). Specialists may even be able to accumulate certain defence-related secondary metabolites to use them for their own defence (Després et al., 2007; Kazana et al., 2007). Differences between generalists and specialists can also be found with regard to attraction: generalist herbivores often avoid induced plants, whereas some specialists may prefer these plants (Bolter et al., 1997; Kaplan and Denno, 2007; Long et al., 2007; Poelman et al., 2008c). However, it should be noted that specialists may still be susceptible to the toxic effects of secondary metabolites (Adler et al., 1995; Agrawal and Kurashige, 2003; Steppuhn et al., 2004).

Signal transduction pathways underlie induced defences in which the plant hormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) play important roles (Kessler and Baldwin, 2002; Pieterse and Dicke, 2007). The model plant *Arabidopsis thaliana* is frequently used to investigate mechanisms of induced defences through a molecular genetic approach (Reymond et al., 2004; De Vos et al.,

2005; Thompson and Goggin, 2006) because full-genome microarrays, extensive mutant collections, and ample information on signal transduction pathways are available (Pieterse and Dicke, 2007). Most of these studies have been performed under carefully controlled environmental conditions in the glasshouse in which plants are exposed to a single attacker. In natural habitats however, plants can be exposed to multiple herbivores simultaneously and under a variety of conditions. It is unclear whether differences in gene expression observed in the glasshouse sustain in the field. Until now, transcriptional responses in field-grown plants have been studied after exposure to methyl jasmonate (Schmidt et al., 2005), induction by *Manduca sexta* herbivory (Izaguirre et al., 2003), or Japanese beetles (*Popillia japonica*) (Casteel et al., 2008). None of these studies investigated intraspecific variation in gene expression nor did they monitor the presence of naturally occurring herbivorous insects.

Intraspecific variation in secondary metabolite content of four *B. oleracea* cultivars (Rivera, Lennox, Christmas Drumhead, and Badger Shipper) has been shown to influence herbivore community composition in the field (Poelman et al., 2008c). Two of these *B. oleracea* cultivars, Rivera and Christmas Drumhead, induced different transcriptional responses to herbivory by caterpillars of the Small Cabbage White *P. rapae* and the cabbage aphid *Brevicoryne brassicae* feeding under glasshouse conditions (Broekgaarden et al., 2007; 2008). Here, we address the question whether differences in herbivore community composition in the field between the two *B. oleracea* cultivars Rivera and Christmas Drumhead can be related to intraspecific variation in gene expression. To our knowledge, this is the first study that links herbivore community composition and whole-genome gene expression.

Material and methods

Plant growth

Seeds of the F1 hybrid white cabbage (*Brassica oleracea* var. *capitata*) cultivar Rivera and the open-pollinated cultivar Christmas Drumhead were obtained from Bejo Zaden B.V. (Warmenhuizen, the Netherlands) and the Centre of Genetic Resources, the Netherlands (CGN) respectively. Seeds were directly sown in peat soil cubes containing potting compost (Lentse Potgrond®) and allowed to germinate in a glasshouse compartment (22-26 °C/16 h light; 18-22 °C/8 h night; 40-70% relative humidity). Prior to being transplanted into the field site, trays with peat soil cubes containing three-week-old seedlings were placed outside the glasshouse during the day for 2 weeks.

Field site

The experimental field site was located in the neighbourhood of Wageningen, the Netherlands. Eighteen plots (6 x 6 m) with a monoculture of one of the two cultivars (ten plots for Rivera and eight plots for Christmas Drumhead) were established using a randomized design. Five-week-old plants were transferred with their peat soil cubes to the field in week 19 (7 May) of 2007. Plots contained 49 plants in a square of 7 x 7 plants with a spacing of 85 cm between plants. A strip of 6 m sown with a grass mixture of *Lolium* and *Poa* species isolated the plots.

Collection of material

In week 23 (6 June) and week 32 (6 August), i.e. four and 13 weeks after plants had been transferred to the field, respectively, material was collected from 18 plots (10 for Rivera and 8 for Christmas Drumhead). The two time points were selected based on data on herbivore population development in 2005 (Poelman et al., 2008c) and 2006 (Poelman et al., 2008d) (Figure 1). One leaf disc (diameter 2.3 cm) was harvested from a young leaf of nine separate plants in each plot, and the leaf discs were pooled to create a single sample per plot. Upon harvesting, samples were immediately flash-frozen in liquid nitrogen and stored at -80 °C. After collecting leaf discs, the same plants were completely harvested in plastic bags to monitor the presence of naturally occurring insects. Bags were stored at 4 °C until plants were monitored. All plants were monitored within 5 days. Besides monitoring the naturally occurring herbivores, we also weighed all the plants individually. Plants remained in their plastic bag until they were weighed and the number of leaves per plant was counted afterwards.

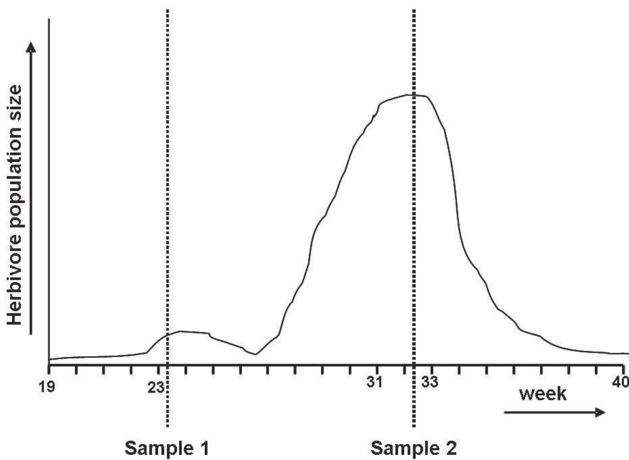

Figure 1. Representation of sampling times for gene expression analysis and monitoring of herbivore numbers. Graph represents the total herbivore population development based on results obtained in 2005 (Poelman et al., 2008c) and 2006 (Poelman et al., 2008d)

Microarray hybridization and analysis

Leaf samples from two plots were pooled per cultivar and three biological replicates were analyzed per cultivar. Total RNA was extracted with TRIzol reagent (Invitrogen) followed by a purification using the RNeasy Plant Mini kit (Qiagen). Four µg of total RNA were linearly amplified using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). Rivera and Christmas Drumhead samples were labelled respectively with Cy3 and Cy5 monoreactive dye (Amersham). Amplified RNA was labelled in freshly made 0.2 M sodium carbonate buffer (pH 9.0) for 1 h at room temperature. Dye incorporation was monitored by measuring the Cy3 and Cy5 fluorescence emissions using a nanodrop ND-1000 UV-Vis Spectrophotometer (BioRad). Microarrays containing 70-mer oligonucleotides based on the genome of *Arabidopsis thaliana* were obtained from the group of David Galbraith from the University of Arizona (http://www.ag.arizona.edu/microarray). Immobilization of the array elements was performed according to the manufacturer's website (see above). The hybridization mixture contained 100 pmol of

the Cy3-labeled sample, 50 pmol of the Cy5-labeled sample, 2X SSC, 0.08% SDS, and 4.8 µl Liquid Block (Amersham) in a final volume of 80 µl. The solution was incubated at 65 ºC for 5 min before application to the microarray covered with a lifterslip (Gerhard Menzel). The microarray was placed in a hybridization chamber (Genetix) and incubated at 50 ºC. After 12 h the microarray was washed for 5 min in 2X SSC/0.5% SDS at 50 ºC, followed by a 5 min wash in 0.5X SSC at room temperature, and a final 5 min wash in 0.05X SSC at room temperature. The microarray was immediately dried by centrifugation for 4 min at 200 rpm.

Hybridized microarrays were scanned with a ScanArray Express HT Scanner (PerkinElmer). Mean fluorescent intensities for Cy3 and Cy5 were determined using the ScanArray Express software (PerkinElmer). Each image was overlaid with a grid to assess the signal intensities for both dyes from each spot. Background fluorescence was subtracted and spots with adjusted intensities lower than half the background were manually raised to half the background to avoid extreme expression ratios. Spots were excluded from the analysis when: (1) showing signal intensities less than half the background for both dyes; (2) showing aberrant shape; (3) located in a smear of fluorescence. Lowess (locfit) normalization was carried out within each slide using TIGR MIDAS version 2.19 to avoid spatial bias. Normalized expression ratios for each individual spot and the mean of the three replicate spots were calculated. A Student's *t* test on $\log_2$ transformed expression ratios was conducted for each experimental condition using TIGR MEV version 3.1. Genes that showed a significant difference in expression level ($P < 0.05$) and a $\log_2$ expression ratio ≥ 1 or ≤ -1 were considered higher expressed in Rivera or Christmas Drumhead, respectively. We used the names of *A. thaliana* homologs to identify *B. oleracea* genes and examined the potential function of differentially regulated genes according to gene ontology (GO) terms from The Arabidopsis Information Resource (http://www.arabidopsis.org).

Quantitative RT-PCR

Quantitative RT-PCR was used to examine gene expression of selected genes per plot by using the RNA pools of all 18 plots separately. One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (BioRad). Gene-specific primers were designed for *B. oleracea* genes based on sequences obtained by a BLAST search in the TIGR *B. oleracea* database (*LOX2*-left 5'-CTT TGC TCA CAT ACG GTA GAA GC-3', *LOX2*-right 5'-CCT TTG CAT TGG GCT AGT TC-3'; *TPI*-left 5'-TGG TGA CAA GTA GCT GTG GTG-3', *TPI*-right 5'-TCC AAG TTA TGG GCA GTG G-3'). Primers were tested for gene specificity by performing melt curve analysis and PCR products were sequenced to confirm amplification of the gene of interest. Sequence results were checked by a BLAST search in the *B. oleracea* as well as in the *A. thaliana* TIGR database. Quantitative RT-PCR analysis was done in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR were performed in duplicate and average values were used in the analyses. The following PCR program was used for all PCR reactions: 3 min 95 ^{o}C; 40 cycles of 30 sec 95 ^{o}C and 45 sec 60 ^{o}C. Threshold cycle (Ct) values were calculated using Optical System software, version 2.0 for MyIQ (BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *glyceraldehyde-3-*

phosphate dehydrogenase (*GAPDH*-left 5'-AGA GCC GCT TCC TTC AAC ATC ATT-3', *GAPDH*-right 5'-TGG GCA CAC GGA AGG ACA TAC C-3') from the Ct value of the gene of interest. Normalized gene expression was then calculated as $2^{-\Delta Ct}$.

Herbivore biodiversity calculations

For both time points, the number of individuals per herbivore species was counted on the nine plants of a plot and herbivores were weighed on a microgram balance. These values were used to calculate per plant (1) the total herbivore abundance, (2) the species richness, and (3) the Shannon-Wiener diversity index. Total herbivore abundance represents the total number of individuals, whereas species richness represents the total number of herbivorous species. The Shannon-Wiener biodiversity index describes herbivore diversity by taking into account both the richness of species as well as the evenness of their distribution (Mendes et al., 2008).

Linear regression analysis

Herbivore abundance, species richness, total herbivore mass and biodiversity index were regressed onto plant weight and number of leaves in multiple linear regression analysis, with cultivar as grouping factor.

Results

Abundance of naturally occurring herbivores

Fourteen species of herbivorous insects were found in the field (Table 1), all of which were previously reported to be associated with *B. oleracea* (Root, 1973; Mitchell and Richards, 1979; Poelman et al., 2008c). Thirteen occurred on both cultivars and one, *Autographa gamma*, was only found on Christmas Drumhead. Early in the season, four weeks after transplanting seedlings into the field, nine herbivore species were found that were equally distributed over Rivera and Christmas Drumhead (Figure 2). At this moment in the season, *B. brassicae* was the most abundant herbivore on both cultivars with about 20 individuals per plant.

Table 1. Herbivore species found on the *Brassica oleracea* cultivars Rivera and Christmas Drumhead early and later in the season and their degree of host plant specialization.

Order	Family	Species	Feeding strategy	Specialization
Lepidoptera	Pieridae	*Pieris rapae*	Leaf chewing	Specialist
		Pieris brassicae	Leaf chewing	Specialist
	Plutellidae	*Plutella xylostella*	Leaf chewing	Specialist
	Pyralidae	*Evergestris fortificalis*	Leaf chewing	Specialist
	Noctuidae	*Mamestra brassicae*	Leaf chewing	Generalist
		Autographa gamma	Leaf chewing	Generalist
Coleoptera	Chrysomelidae	*Phyllotreta atra*	Leaf chewing	Specialist
		Phyllotreta nemorum	Leaf chewing	Specialist
Hemiptera	Aphididae	*Brevicoryne brassicae*	Phloem feeding	Specialist
		Myzus persicae	Phloem feeding	Generalist
	Aleyrodidae	*Aleyrodes proletella*	Phloem feeding	Specialist
Thysanoptera	Thripidae	*Thrips tabaci*	Cell content feeding	Generalist
Diptera	Anthomyiidae	*Delia radicum*	Root feeding	Specialist

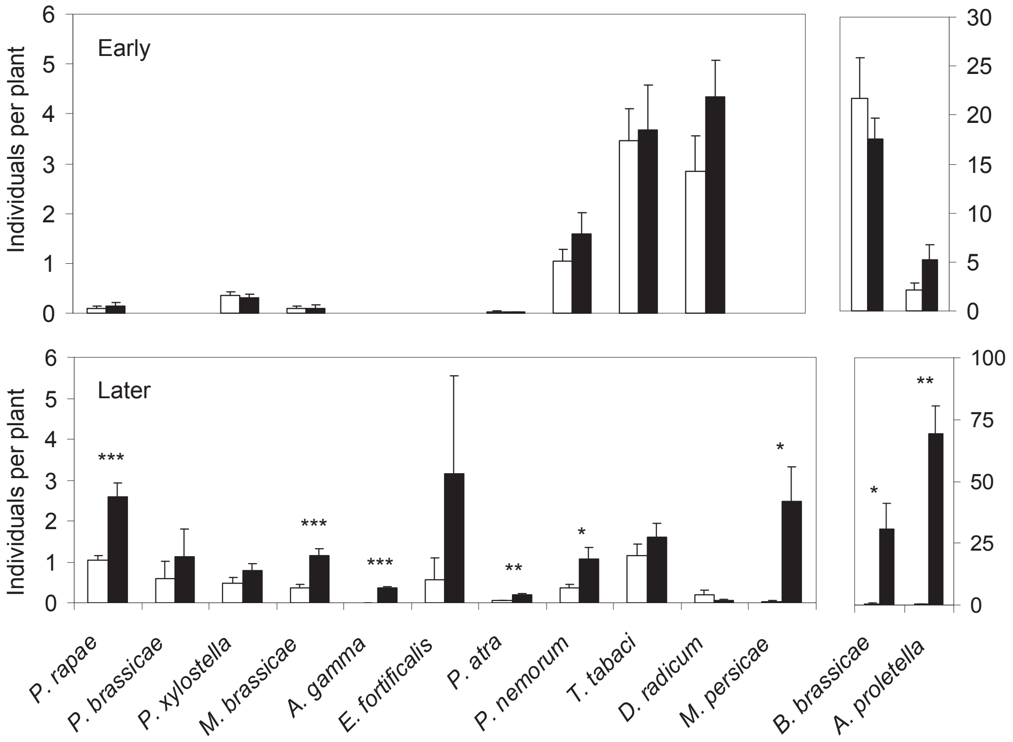

Figure 2. Numbers of naturally occurring herbivores in the field on Rivera (white bars) and Christmas Drumhead (black bars) early and later in the season. Mean numbers of individuals per plant are given with their corresponding standard error. Bars within pairs marked with one or more asterisks differ significantly (independent sample *t*-test, one asterisk: $P < 0.05$; 2 asterisks: $P < 0.01$; 3 asterisks: $P \leq 0.001$).

Nine weeks after the first time point, the abundance of herbivores on the cultivars had changed completely. At this time point later in the season, Rivera harboured significantly fewer *P. rapae* and *Mamestra brassicae* larvae than Christmas Drumhead (independent sample *t*-test, *P. rapae*: $P < 0.001$; *M. brassicae*: $P = 0.001$; Figure 2). Several larvae of *A. gamma* were found on Christmas Drumhead, whereas this species was absent on Rivera (Figure 2). The other lepidopteran larvae that were found were equally distributed over the two cultivars (*Pieris brassicae*: $P = 0.51$; *Plutella xylostella*: $P = 0.12$; *Evergestris fortificalis*: $P = 0.26$; Figure 2). Furthermore, less than half as many flea beetles were found on Rivera than on Christmas Drumhead (*Phyllotreta atra*: $P = 0.002$; *Phyllotreta nemorum*: $P = 0.01$; Figure 2). Great differences between the cultivars were found for the occurrence of cabbage aphids (*B. brassicae)* and whiteflies (*Aleyrodes proletella)* later in the season (*B. brassicae*: $P = 0.022$; *A. proletella*: $P = 0.001$). Hardly any individuals of these two species were present on Rivera,

whereas on Christmas Drumhead ca. 30 and 70 individuals per plant were found of these two species respectively (Figure 2). Remarkably, a few *A. proletella* adults were found on Rivera, but no pupae of this species were present on this cultivar. On Christmas Drumhead we found four times more *A. proletella* pupae than adults. Additionally, lower numbers of the phloem-feeding herbivore *Myzus persicae* were found on Rivera than on Christmas Drumhead (P = 0.021; Figure 2).

All together, total herbivore abundance and species richness were significantly different on the two cultivars later in the season (independent sample *t*-test, abundance: P = 0.001; richness: P < 0.001). Rivera harboured significantly lower numbers of specialist as well as generalist species than Christmas Drumhead (P < 0.001; Figure 3A). Additionally, fewer specialist and generalist species were present on Rivera than on Christmas Drumhead (P ≤ 0.001; Figure 3B). We also weighed all herbivores present on the cultivars and found that total mass of herbivores collected from Rivera was significantly lower than total mass of herbivores collected from Christmas Drumhead (P = 0.002). This difference was mostly caused by the specialist herbivores (specialists: P = 0.001; generalists: P = 0.013; Figure 3C). Due to the distribution of the herbivore species, Rivera scored significantly higher on the Shannon-Wiener biodiversity index (index value for Rivera is 1.65 ± 0.06 and for Christmas Drumhead is 1.08 ± 0.13; P = 0.001).

To assess whether plant biomass or the number of leaves could explain differences in herbivore community composition between the cultivars we weighed all plants individually and counted the number of leaves per plant. Herbivore abundance, species richness, total herbivore mass, or biodiversity were not significantly affected by plant weight or number of leaves (linear regression, abundance: weight P = 0.55, leaves P = 0.14; richness: weight P = 0.72, leaves P = 0.88; mass: weight P = 0.22, leaves P = 0.24; Shannon-Wiener index: weight P = 0.42, leaves P = 0.60).

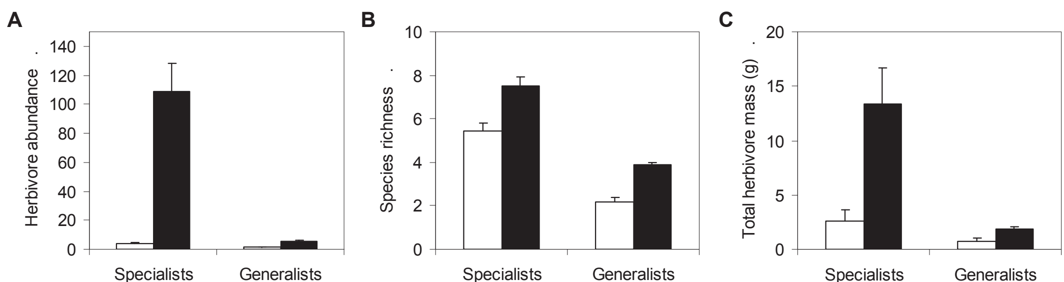

Figure 3. Herbivore community composition parameters later in the season for Rivera (white bars) and Christmas Drumhead (black bars) are given for specialist and generalist herbivores. Graphs represent: (A) total number of herbivore individuals per plant (+ SE); (B) total number of species per plant (+ SE); (C) total mass of all herbivores per plant (+ SE). Bars within pairs marked with three asterisks differ significantly (independent sample *t*-test, P ≤ 0.001).

Gene expression differences between Rivera and Christmas Drumhead in the field

From the same plants that we used to monitor naturally occurring herbivores, we had collected leaf material to examine transcriptional profiles in Rivera and Christmas Drumhead. Using *A. thaliana* full-genome microarrays, we investigated whether differences in gene expression levels exist between the two cultivars under field conditions. Early in the season only a small number of genes showed different expression levels between the two cultivars. Five and 11 genes showed higher levels of expression in Rivera or Christmas Drumhead respectively, including genes mainly involved in general metabolic processes and genes of unknown function (Table 2). Later in the season differences in expression levels between Rivera and Christmas Drumhead were more pronounced as 51 genes showed different expression levels (Table 2). The 27 genes with higher expression levels in Rivera include, among others, genes involved in defence and metabolic processes. The defence-related genes that were identified in Rivera include genes encoding lipoxygenase 2 (LOX2), two lectins (At2g39310 and At5g38540), a trypsin inhibitor, and a Bet v I allergen (Table 2). In Christmas Drumhead, 24 genes showed higher expression levels than in Rivera of which most were involved in metabolic processes and photosynthesis (Table 2). One of the genes with a higher expression level in this cultivar is involved in defence and encodes flavin-dependent monooxygenase 1 (FMO1) (Table 2). To get insight into the role of JA accumulation in the cultivars we compared the results from this study to those obtained from a glasshouse experiment in which plants had been treated with a JA solution (Chapter 2: Broekgaarden et al., 2007). We found that 37% (10/27) of the genes with higher expression levels in Rivera than in Christmas Drumhead were JA-responsive. Conversely, none of the genes with higher expression levels in Christmas Drumhead compared to Rivera were found to be JA-responsive (Table 2).

Table 2. Genes with a higher level of expression in Rivera or Christmas Drumhead under field conditions early and later in the season.

Probe identification	AGI code	Ratio	*P* value	Process category	JA-responsive
A- Early in the season					
Higher expression in Rivera					
Fucosyltransferase 12 (FUT12)	At1g49710	7.81	0.013	Metabolic processes	-
Tropinone reductase	At2g29320	7.22	0.048	Metabolic processes	-
Expressed protein	At5g38310	2.01	0.039	Unknown	-
Hypothetical protein	At3g51760	2.04	0.024	Unknown	-
Endonuclease/exonuclease/phosphatase	At1g31500	2.35	0.004	Unknown	-
Higher expression in Christmas Drumhead					
Expressed protein	At1g80245	2.44	0.012	Cell organization & biogenesis	-
Cytochrome b6f complex	At2g26500	2.30	0.017	Electron transport	-
Ribulose-phosphate 3-epimerase	At3g01850	2.05	0.041	Metabolic processes	-
MMS Zwei Homologe 4 (MMZ4)	At3g52560	2.22	0.008	Metabolic processes	-
Cardiolipin synthase (CLS)	At4g04870	2.28	0.023	Metabolic processes	-
Prenylcysteine alpha-carboxyl methyltransferase (STE14B)	At5g08335	3.11	0.002	Metabolic processes	-
Histone H4	At5g59690	2.38	0.001	Metabolic processes	-
Actin depolymerizing factor 3 (ADF3)	At5g59880	2.06	0.017	Stress response	-
33 kDa secretory protein-related	At5g48540	2.31	0.009	Unknown	-
Hypothetical protein	At1g10800	2.37	0.008	Unknown	-
Myosin heavy chain-related	At3g13190	2.04	0.025	Unknown	-

Table 2. (continued)

Probe identification	AGI code	Ratio	*P* value	Process category	JA-responsive
B- Later in the season					
Higher expression in Rivera					
Senescence-associated family protein	At5g66040	2.84	0.026	Aging	-
Giant chloroplast 1 (GC1)	At2g21280	2.29	0.023	Cell organization & biogenesis	-
Bet v I allergen	At1g24020	3.49	0.01	Defence	-
Lectin	At2g39310	5.16	0.007	Defence	+
Trypsin inhibitor	At2g43530	2.36	0.013	Defence	-
Lipoxygenase 2 (LOX2)	At3g45140	2.60	0.008	Defence	+
Lectin	At5g38540	2.24	0.038	Defence	+
Cytochrome c oxidase subunit 6b	At4g28060	2.15	0.034	Electron transport	-
60S ribosomal protein L39 (RPL39A)	At2g25210	4.44	0.005	Metabolic processes	-
Ubiquitin extension protein 2 (UBQ2)	At2g36170	2.31	0.004	Metabolic processes	-
60S ribosomal protein L41 (RPL41C)	At2g40205	2.33	0.017	Metabolic processes	-
Signal peptidase	At3g15710	3.15	0.018	Metabolic processes	-
Sugar isomerase (SIS) domain-containing protein	At3g54690	3.18	0.047	Metabolic processes	-
Rho-related protein from plants 3 (ROP3)	At2g17800	2.28	0.028	Signal transduction	-
Peroxidase 42 (PRXR1)	At4g21960	2.06	0.044	Stress response	+
Zinc finger (GATA type) family protein	At1g08010	2.26	0.044	Transcription	-
Basic helix-loop-helix (bHLH)	At4g37850	2.15	0.04	Transcription	-
Vavuolar H+-ATPase subunit E isoform 3	At1g64200	2.15	0.016	Transport	-
Protease inhibitor/seed storage/lipid transfer protein	At3g57310	3.95	0.032	Transport	+
Dehydroascorbate reductase	At1g19550	3.22	0.022	Unknown	+
Thioredoxin-dependent peroxidase 1 (TPX1)	At1g65980	2.51	0.004	Unknown	-
COP1-interacting protein-related	At1g72410	2.01	0.032	Unknown	-
Nodulin-related	At2g03440	2.31	0.049	Unknown	+
F-box family protein	At3g17530	2.41	0.048	Unknown	-
Transporter-related	At4g39390	2.57	0.047	Unknown	-
Tudor domain-containing protein	At5g07350	2.83	0.029	Unknown	-
Expressed protein	At5g26270	2.65	0.04	Unknown	-
Higher expression in Christmas Drumhead					
Flavin-dependent monooxygenase 1 (FMO1)	At1g19250	4.33	0.02	Defence	-
Kinesin-13A	At3g16630	2.23	0.012	Development	-
GDSL-motif lipase/hydrolase	At1g29660	2.41	0.012	Metabolic processes	-
J8; heat shock protein binding	At1g80920	2.66	0.05	Metabolic processes	-
Fructose-bisphosphate aldolase	At2g21330	2.04	0.05	Metabolic processes	-
Protein kinase	At3g18810	2.32	0.033	Metabolic processes	-
Prefoldin-related KE2	At3g22480	2.22	0.013	Metabolic processes	-
Tubulin beta-4 chain (TUB4)	At5g44340	2.31	0.014	Metabolic processes	-
Photosystem I subunit F (PSAF)	At1g31330	2.39	0.032	Photosynthesis	-
LHCA1	At3g54890	2.16	0.013	Photosynthesis	-
Photosynthetic electron transfer C (PETC)	At4g03280	2.07	0.027	Photosynthesis	-
DC1 domain-containing protein	At5g54050	2.77	0.018	Signal transduction	-
Iron superoxide dismutase (FSD1)	At4g25100	2.17	0.005	Stress response	-
Thiazole requiring (THI1)	At5g54770	2.55	0.048	Stress response	-
Homeobox-leucine zipper protein (HAT4)	At4g16780	2.89	0.029	Transcription	-
Calcium-binding EF hand	At1g20760	2.51	0.018	Unknown	-
Protein kinase-related	At3g03930	2.59	0.001	Unknown	-
Expressed protein	At3g12320	3.21	0.022	Unknown	-
Expressed protein	At3g44580	2.15	0.035	Unknown	-
Expressed protein	At4g20290	2.16	0.018	Unknown	-
Expressed protein	At5g25640	3.35	0.01	Unknown	-
Expressed protein	At5g55620	2.37	0.046	Unknown	-
Embryo-specific protein-related	At5g62200	2.04	0.032	Unknown	-

Relative differences in expression levels in Rivera compared to Christmas Drumhead were measured in field-grown plants early and later in the season. Mean expression ratios and *P* values (student *t*-test) were calculated from three biological replicates. The column 'JA-responsive' is based on comparisons to data obtained from (Broekgaarden et al. 2007).

Gene expression in the field compared to herbivore-induced responses in the glasshous

We compared the genes that showed different levels of expression in field-grown Rivera and Christmas Drumhead plants to previously identified *P. rapae*- and *B. brassicae*-induced genes in plants grown in the glasshouse (Broekgaarden et al., 2007; 2008). More than half (15/27) of the genes that showed a higher level of expression in Rivera compared to Christmas Drumhead in the field were previously identified as *P. rapae*-inducible in one or both cultivars in the glasshouse (Figure 4), including the defence-related genes *LOX2*, *trypsin inhibitor*, and the two genes encoding lectin. Only three of the 24 genes that showed a higher expression level in Christmas Drumhead compared to Rivera in the field were previously identified as *P. rapae*-inducible in one or both cultivars under glasshouse conditions (Figure 4) and none of them are involved in defence-related processes. None of the genes that showed a differential expression between the cultivars under field conditions were previously identified as *B. brassicae*-responsive.

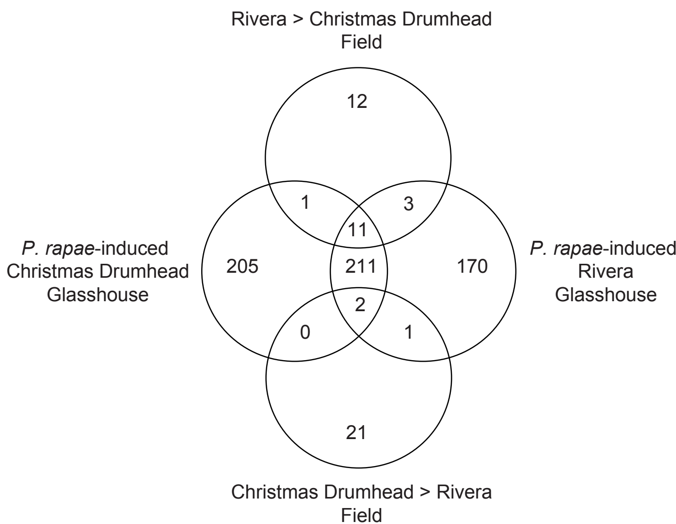

Figure 4. Venn diagram representing the distribution of genes with a higher level of expression in Rivera or Christmas Drumhead in the field, compared to genes induced by *P. rapae* after 24, 48 and/or 72 h in the glasshouse (Broekgaarden et al., 2007).

Quantitative RT-PCR analysis using *B. oleracea*-derived primers for *LOX2*, a gene known to be involved in defence, confirmed the microarray result by showing a significantly higher expression in Rivera than in Christmas Drumhead in the field (one-way ANOVA, $P < 0.001$; Figure 5). In order to compare gene expression levels between field- and glasshouse-grown plants, we differently analyzed data obtained from control plants and *P. rapae*- or *B. brassicae*-challenged plants of Rivera and Christmas Drumhead grown in the glasshouse (Broekgaarden et al., 2007; 2008). Expression levels of *LOX2* in field-grown plants were significantly higher than those in control plants grown in the glasshouse for both cultivars (one-way ANOVA, $P < 0.001$; Figure 5). Furthermore, expression levels in the field were significantly lower than the levels reached after 72 h of *P. rapae* feeding for Christmas Drumhead ($P = 0.01$; Figure 5). In glasshouse-grown plants, no *LOX2* expression could be detected after *B. brassicae* feeding in either of the two cultivars (Broekgaarden et al., 2008).

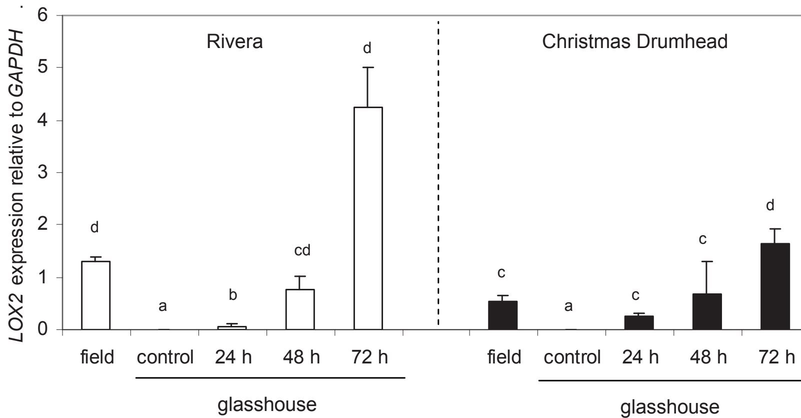

Figure 5. Expression levels of *LOX2* in field-grown plants and plants grown in the glasshouse (GH) that were either unchallenged (control) or challenged for 24, 48, or 72 h by *P. rapae*. Bars represent mean *LOX2* expression levels relative to the reference gene *GAPDH* for Rivera (white bars) and Christmas Drumhead (black bars) with standard error bars. Bars marked with different letters are significantly different (one-way ANOVA, $P < 0.05$).

We also analyzed the expression of *TPI* (*trypsin-and-protease inhibitor*), a defence-related gene whose expression negatively affects *P. rapae* and *B. brassicae* performance (Broekgaarden et al., 2008; Chapter 4). The microarray showed a tendency of differential *TPI* expression levels between the cultivars (expression ratio of 2.61, $P = 0.1$), and qRT-PCR analysis using *B. oleracea*-derived primers revealed an almost significant higher level of expression in Rivera than in Christmas Drumhead for this gene (one-way ANOVA, $P = 0.07$; Figure 6). For both cultivars, the expression levels of *TPI* were significantly higher in field-grown plants compared to control plants grown in the glasshouse for both cultivars ($P < 0.001$; Figure 6A). However, *TPI* expression levels in field-grown plants did not reach the levels of glasshouse-grown plants challenged for 48 or 72 h with *P. rapae* (Rivera 48 h: $P = 0.07$; Rivera 72 h: $P = 0.02$; Christmas Drumhead 48 h: $P = 0.001$; Christmas Drumhead 72 h: $P = 0.002$; Figure 6A). Furthermore, expression levels of *TPI* in field-grown plants were significantly higher than expression levels after *B. brassicae* feeding in the glasshouse ($P < 0.001$; Figure 6B).

Discussion

Rivera and Christmas Drumhead differentially affect herbivore communities throughout the season

In our field experiment, Rivera and Christmas Drumhead were exposed to naturally occurring populations of herbivorous insects and the abundance of these herbivores was monitored early and later in the season (Figure1). Early in the season, i.e. four weeks after seedlings were planted into the field, the two *B. oleracea* cultivars harboured similar numbers of herbivorous insects. In contrast, later in the season, when plants were present in the field for 13 weeks, clear differences in herbivore communities were found between the cultivars (Figure 2). These data show that Rivera and Christmas Drumhead differently develop their phenotype throughout the season.

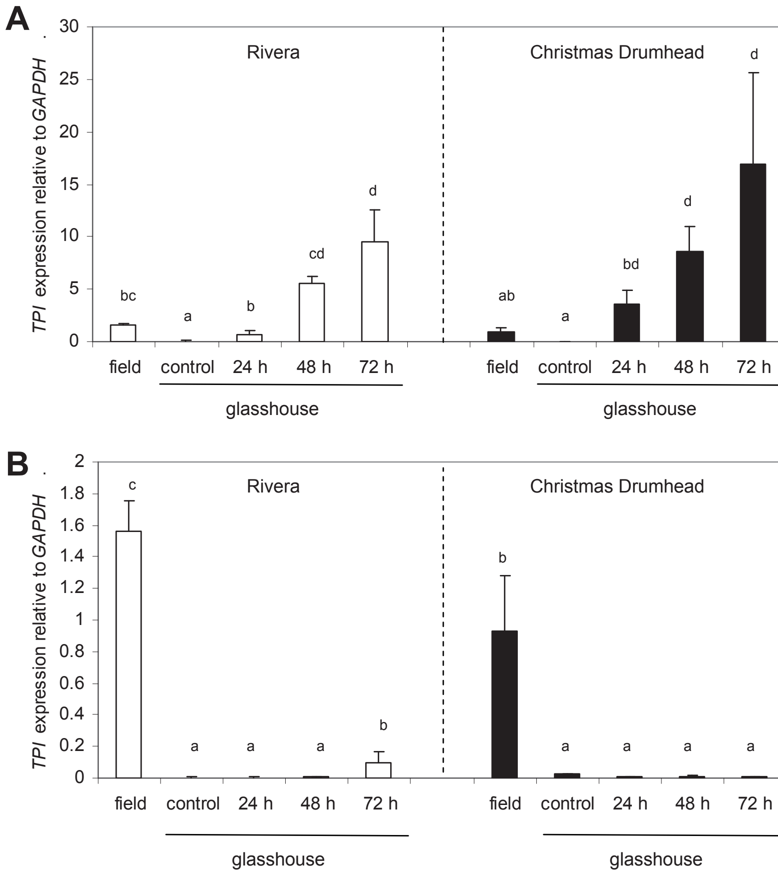

Figure 6. Expression levels of *TPI* in field-grown plants and plants grown in the glasshouse that were either unchallenged (control) or challenged for 24, 48, or 72 h of *P. rapae* (A) or *B. brassicae* (B) feeding. Bars represent mean *TPI* expression levels relative to the reference gene *GAPDH* for Rivera (white bars) and Christmas Drumhead (black bars) with their corresponding standard error. Bars marked with different letters are significantly different (one-way ANOVA, $P < 0.05$).

Genotypic differences between plants may have a stronger effect on herbivore communities than environmental factors (Johnson and Agrawal, 2005; Bangert et al., 2006). Plant morphology has been found to have a strong effect on insect community composition and may even be more important than plant defence traits (Johnson and Agrawal, 2005). Although plants of the two cultivars used here differed in fresh weight and number of leaves, neither of these parameters correlated with herbivore abundance, richness and biodiversity and therefore are not likely to explain the observed differences in herbivore communities.

Lower numbers of *P. rapae* and *M. brassicae* larvae were found on Rivera than on Christmas Drumhead later in the season, suggesting differences in larval performance and/or oviposition preference between the cultivars. Indeed, under glasshouse conditions, butterflies of *P. rapae* showed a higher preference for Christmas Drumhead than for Rivera (Poelman et al., 2008c) and *P. rapae* larvae

performed better when feeding on Christmas Drumhead compared to Rivera (Broekgaarden et al., 2007; Poelman et al., 2008c). Larvae of *M. brassicae* also performed better on Christmas Drumhead than on Rivera under glasshouse conditions (Poelman et al., 2008c). Plant phenotype changes may not only affect the performance and host plant selection behaviour of the attacking herbivore, but also that of subsequently colonizing species (Shiojiri et al., 2002; Long et al., 2007). Initial infestations with *P. rapae* on Rivera negatively affected the performance of subsequently colonizing *P. rapae* and *M. brassicae* as well as the preference of adult females from the latter species (Poelman et al., 2008a). Conversely, initial *P. rapae* infestation attracted oviposition by *P. rapae* (Agrawal and Sherriffs, 2001; Poelman et al., 2008a). The absence of *A. gamma* larvae on Rivera suggests that butterflies of this species have a strong preference for Christmas Drumhead. This species does not completely avoid Rivera as *A. gamma* caterpillars were found on this cultivar in two previous years (Poelman et al., 2008c; 2008d). Large differences in the occurrence of phloem-feeding specialists were observed between Rivera and Christmas Drumhead. No pupae of the cabbage whitefly *A. proletella* have been observed on Rivera, whereas high numbers were found on Christmas Drumhead. This suggests a strong difference in host plant selection behaviour of whitefly females. Interestingly, the number of *B. brassicae* individuals on Rivera decreased whereas population size of this aphid increased on Christmas Drumhead throughout the season. Both cultivars started with similar numbers of *B. brassicae* early in the season. In glasshouse experiments, this aphid was previously shown to be able to settle and reproduce on both cultivars (Broekgaarden et al., 2008), indicating that other factors play a role in this decrease in *B. brassicae* numbers under field conditions.

Herbivores not only differ in feeding strategy, but also in host plant range. Specialists feed on one or a few closely related plant species, whereas generalists feed on many different plants (Schoonhoven et al., 2005). Certain defence compounds may negatively affect generalist herbivores, whereas specialists may be able to detoxify these compounds (Ratzka et al., 2002; Wittstock et al., 2004; Kliebenstein et al., 2005; Després et al., 2007). In *Brassica* species glucosinolates and their breakdown products stimulate specialists and deter generalists (Renwick et al., 1992; Van Loon et al., 1992; Riggin-Bucci and Gould, 1996; Renwick et al., 2006). The abundance, species richness, and total herbivore mass of specialists differed more between the cultivars than that of generalists (Figure 3) suggesting differential induction of defence compounds between Rivera and Christmas Drumhead.

Intraspecific transcriptional variation may result in differences in herbivore communities

Early in the season, no clear differences in gene expression levels could be detected between Rivera and Christmas Drumhead. Only a small number of genes showed differences in expression levels and none of them were related to defensive processes. Conversely, clear differences in gene expression levels between the cultivars were detected later in the season, supporting the suggestion that both cultivars develop their phenotype differently throughout the season. Although a relatively small number of genes showed differences in expression levels between the two cultivars, the genes that were differently expressed are interesting in relation to insect performance.

Later in the season, seven defence-related genes showed higher levels of expression in Rivera than in Christmas Drumhead. One of these genes that probably play a central role in shaping the

herbivore community is *LOX2*. It is likely that *LOX2* in *B. oleracea* encodes a 13-LOX (Zheng et al., 2007), which is required for the first step in JA biosynthesis (Schaller et al., 2005; Wasternack et al., 2006). In *A. thaliana, LOX2* has been shown to be required for the biosynthesis of JA in leaves (Bell et al., 1995). Furthermore, RNA levels of this gene have been shown to increase in *B. oleracea* after JA treatment, wounding and herbivore feeding (Broekgaarden et al., 2007; Zheng et al., 2007). The higher expression level of *LOX2* in Rivera than in Christmas Drumhead suggests that more JA accumulates in Rivera in the field. This is supported by the observation that 37% of the genes with higher expression levels in Rivera than in Christmas Drumhead are JA-responsive (Table 2). The fact that JA mediates direct defence by inducing secondary metabolites (Van Dam et al., 2004; Bruinsma et al., 2007) suggests that the absence of JA accumulation result in higher herbivore abundance and species richness. Indeed, *Nicotiana attenuata* plants that were artificially silenced in a *13-LOX* gene harboured higher number of herbivores and were even attacked by a species that was never found on control plants in the field (Kessler et al., 2004). This indicates that altering JA accumulation can affect herbivore host selection and herbivore community composition (Kessler et al., 2004; Paschold et al., 2007; Halitschke et al., 2008).

The defence-related gene *TPI,* which encodes a trypsin-and-protease inhibitor, may also play an important role in the observed difference in herbivore community on Rivera and Christmas Drumhead. This gene is a member of the Kunitz trypsin inhibitor family that inhibits proteolytic enzymes within herbivore guts, resulting in reduced insect growth (Schuler et al., 1998; Marchetti et al., 2000). Silencing of *TPI* expression in *A. thaliana* increased *P. rapae* and *B. brassicae* performance in the glasshouse (Broekgaarden et al., 2008; Chapter 4). The higher expression level of *TPI* in Rivera compared to Christmas Drumhead is probably a result of the higher expression level of *LOX2* in Rivera as *TPI* is JA-inducible (Broekgaarden et al., 2007).

The other four defence-related genes that showed higher levels of expression in Rivera compared to Christmas Drumhead may also contribute to the difference in herbivore community composition. Lectins can function as defence proteins against herbivores (Peumans and Van Damme, 1995), Bet v 1 allergen protein is a member of the pathogenesis-related-10 family (Hoffmann-Sommergruber, 2000), and trypsin inhibitors can play a role in plant tolerance to herbivorous insects (Dunaevsky et al., 2005). However, more studies are needed to determine the role of these genes in shaping herbivore communities.

Intraspecific transcriptional variation in the context of herbivore community composition

From the moment that the plants had been transplanted into the field they have been exposed to all kinds of abiotic and biotic stresses such as temperature changes, rainfall, fungi, bacteria, and herbivorous insects that can all have an effect on the plant's phenotype and gene expression. UV-B radiation, for example, has been shown to increase expression of jasmonate-signalling genes in field-grown *Nicotiana longiflora* (Izaguirre et al., 2003). Early in the season almost no differences in gene expression between the cultivars could be detected, whereas clear differences in transcriptional profiles between Rivera and Christmas Drumhead were observed later in the season. More than 50% of the genes that showed a higher level of expression in Rivera compared to Christmas Drumhead

later in the season had previously been identified as *P. rapae*-responsive in glasshouse experiments (Broekgaarden et al., 2007). These transcriptional data recorded for field-grown plants suggests that herbivore pressure may have a strong influence on shaping a plant's phenotype in the field and thereby herbivore community composition. Indeed, initial *P. rapae* feeding on two *B. oleracea* cultivars resulted in differential regulation of gene expression upon feeding by sequential herbivores and this resulted in differential effects on performance and population development of these herbivores and on community composition (Poelman et al., 2008a).

Zheng and co-workers (2007) have shown that just a single *P. rapae* larva can induce a fast increase in *LOX2* transcript levels in *B. oleracea*. Our results show that the expression levels of the two defence-related genes *LOX2* and *TPI* were higher in field-grown plants than in glasshouse-grown control plants for both cultivars and comparable to the levels in plants that were challenged for 24 or 48 h by *P. rapae* under glasshouse conditions. However, the expression levels of *LOX2* and *TPI* were not as high as those after 72 h of feeding by *P. rapae*. This shows that genes are not necessarily expressed to a maximum level, even when more than one *P. rapae* larva is present. The lower gene expression levels in the field may be the result of crosstalk between responses to many different signals. Herbivore species differentially induce plant responses that can have different effects on subsequent herbivores or pathogens (Agrawal, 2000; Heidel and Baldwin, 2004). For example, different herbivores elicited very different transcriptional responses in *A. thaliana* (De Vos et al., 2005) and induction by *P. rapae* caterpillars affects the susceptibility to turnip crinkle virus through priming of the SA-dependent defence against this pathogen (De Vos et al., 2006b). In *N. attenuata,* prior attack by sap-feeding mirids (*Tupiocoris notatus*) resulted in reduced performance of *Manduca sexta* (Voelckel and Baldwin, 2004). Accumulation of JA, for instance by *P. rapae* feeding, may negatively affect the performance of whiteflies (Zarate et al., 2007). Thus, the induction of plant responses by herbivory affects subsequent attackers and is mediated by transcription-related changes in plant phenotype (Kessler et al., 2004; Poelman et al., 2008a). Unravelling the mechanisms underlying the dynamics of community composition is an exciting process that is now possible through a multidisciplinary approach that connects transcriptomics with metabolomics and community ecology (Kessler and Halitschke, 2007; Bruinsma and Dicke, 2008).

Conclusion

Our results show that clear differences in herbivore community composition between two *B. oleracea* cultivars develop during the season. These differences are most likely related to differences in gene expression between the cultivars. While the herbivore populations and gene expression patterns were very similar early in the season, they evolved very differently for the two cultivars. Several defence-related genes showed higher levels of expression in the cultivar that harboured the lowest numbers of herbivores. These data provide an important step in the analysis of the mechanisms that underlie the dynamics of ecological communities.

Acknowledgements

We thank André Meijaard, Stephanie Laurent, Ciska Raaijmakers, Jeroen Jansen, Greet Steenhuis, Sylvia Lenting, and Aline Boursault for assistance with collecting material; CGN and Bejo Zaden for providing seeds of the cultivars; Unifarm for maintenance of the plants and field site; André Meijaard and Stephanie Laurent for assistance in the laboratory and data analysis. This research was supported by the Dutch ministry of Agriculture, Nature, and Food quality. MD was additionally supported by a VICI grant from the Netherlands Organization for Scientific Research, NWO (865.03.002).

General discussion

Colette Broekgaarden

Introduction

Plants are at the basis of most food webs in which they interact with organisms from higher trophic levels such as herbivores. Plants have evolved several strategies to reduce or prevent damage by herbivorous insects. Mechanisms underlying these strategies can be based on direct or indirect defence, which can both be constitutively present or induced in response to herbivore attack (Kessler and Baldwin, 2002; Schoonhoven et al., 2005). Direct defence involves plant traits that interfere with herbivore feeding or oviposition. Morphological factors such as leaf surface wax layers or trichomes provide a first barrier to herbivores (Eigenbrode and Espelie, 1995; Traw and Dawson, 2002a; Picoaga et al., 2003; Schoonhoven et al., 2005). Secondly, the production of secondary metabolites, such as toxins or digestibility reducers, may have a negative effect on herbivore growth and survival (Roda and Baldwin, 2003; van Dam et al., 2004). Finally, nutritional quality can directly influence herbivore performance (Omacini et al., 2001; Schoonhoven et al., 2005; Bukovinszky et al., 2008). Direct defences can have a strong impact on insect-plant interactions. Although indirect defence mechanisms are also very important (Pichersky and Gershenzon, 2002; D'Alessandro and Turlings, 2006; Bruinsma and Dicke, 2008), I have focused on getting more insight into the molecular mechanisms underlying direct defences.

Microarrays have been used extensively to investigate transcriptional responses of plants after feeding by a single herbivore species (Korth, 2003; Reymond et al., 2004; De Vos et al., 2005; Thompson and Goggin, 2006). There is, however, limited information on intraspecific variation in global transcriptional responses of plants to herbivore feeding (Kuśnierczyk et al., 2007; Wang et al., 2008). Even much less is known about intraspecific transcriptional profiles of plants under field conditions in which plants are exposed to multiple attackers.

This project was part of a research programme which aimed to link intraspecific variation in plant defence to higher trophic level biodiversity. Several approaches, including transcriptomics, metabolomics, behavioural and community ecology were used in this programme. This integrated approach significantly advanced investigations into plant defences (Baldwin et al., 2001; Mercke et al., 2004). This thesis contributes to the programme with an integrated approach addressing plant transcriptomics, insect life history, population development and community composition related to cultivated and wild *Brassica* species and their herbivores, both under controlled and field conditions (Figure 1).

From model plant to agricultural crop: possibilities and limitations

Brassica crops suffer from many herbivores, both specialists and generalists, of which the caterpillar *Pieris rapae,* the aphid *Brevicoryne brassicae* and the whitefly *Aleyrodes proletella* are the most important specialists and *Thrips tabaci* the most important generalist. From a genetic point of view, *Brassica* crops are not the most convenient plants to work with. In strong contrast to its relative *Arabidopsis thaliana,* which has a short generation time of about 7-8 weeks (Meyerowitz, 1989; Baud et al., 2002), *Brassica oleracea* has a two-year life cycle. Most transcriptional profiling studies have focused on the model plant *A. thaliana* for which full-genome microarrays, an extensive mutant collection, and ample information on signal transduction pathways are available (Pieterse and Dicke,

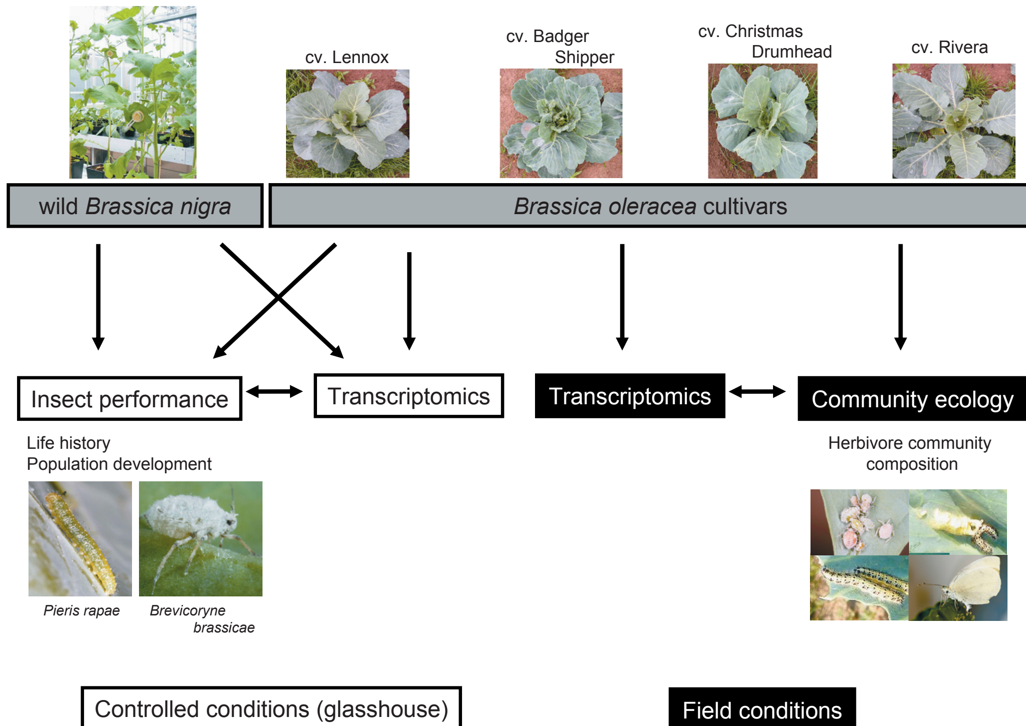

Figure 1. An integrated approach to study insect-plant interactions in *Brassica*.

2007). Gene expression studies of the interaction between *A. thaliana* and herbivorous insects have been performed, resulting in the identification of several candidate genes for direct defence (Moran et al., 2002; Reymond et al., 2004; De Vos et al., 2005; Barth and Jander, 2006). However, *A. thaliana* is less suitable to investigate the effects of gene expression on community ecology because herbivorous insects are active in a different time window than *A. thaliana* (Yano and Ohsaki, 1993). Other crucifers are more appropriate for this purpose. Many studies have examined *Brassica*-herbivore interactions from an ecological and metabolomic point of view (Snoeren et al., 2007; Zheng and Dicke, 2008). Research on plant-insect interactions now faces the challenge to translate transcriptomic information obtained from the model plant *A. thaliana* to a crop species like *B. oleracea*. This is the field of 'plant translational genomics' (Gepts et al., 2005; Stacey and VandenBosch, 2005; Salentijn et al., 2007). Information obtained from a model plant is expected to be useful for understanding the biology of crop species as well. This is based on the assumption that genes with a proven or predicted function in a model species may have a similar function in the target crop and can be translated to a target crop by using candidate gene approaches (Salentijn et al., 2007). However, not only the presence or absence of certain genes is important, but also their regulation. Within a plant species different lines of defence are present that might be activated differently in response to environmental stimuli. It is important to gain information on this before investing in translational genomics. Gene expression studies in relatives of *A. thaliana* can help to fill this gap. *Brassica* crops are not yet fully sequenced, and only very recently a microarray based on a part of the *Brassica* genome has become available (JIC/JCVI/Cogenics, 2008). *A. thaliana* and *Brassica* share about 85% sequence identity (Cavell et

al., 1998), which allows for the use of the *A. thaliana* genetic toolbox in investigating the molecular genetics of *Brassica* species (Clauss and Koch, 2006). Lee et al. (2004) had already shown that the majority of oligonucleotides present on an *A. thaliana* microarray hybridized to *B. oleracea* cDNA. In this research project, I have used a microarray containing 70-mer synthetic oligonucleotides representing the whole genome of *A. thaliana* to study transcriptional responses in *Brassica.* Taking into account all microarray analyses in this thesis, 90% of the oligonucleotides present on the microarray showed intensity signals when hybridized with *B. oleracea* amplified RNA (Chapters 2, 3, and 5). The hybridization efficiency was somewhat lower (70%) for RNA amplified from *Brassica nigra* (Chapter 4). Microarray validation with quantitative RT-PCR using *Brassica*-derived primers showed that microarray results were reliable for both *B. oleracea* and *B. nigra* (Chapters 2, 3, 4, and 5). Based on these results, I expect that transcriptional responses of all species within the Brassicaceae can be analyzed with these *A. thaliana* 70-mer oligo microarrays. However, one has to keep in mind that *Brassica*-specific genes will not be detected using these microarrays.

Studying intraspecific variation: a way to identify genes that matter

Phenotypic variation among plants of the same species is likely to be smaller than phenotypic variation between plants of different species. Plants of the same species have similar life-histories, morphological characteristics, and genetic background. For this reason, studying intra- instead of interspecific variation makes it easier to identify candidate genes for direct defence against herbivorous insects. A lot of studies have addressed intraspecific variation in insect performance (Jensen et al., 2002; Alvarez et al., 2006; Ranger et al., 2007; Wang et al., 2008) or defensive compounds (Kliebenstein et al., 2001; Kushad et al., 2004). Intraspecific variation in phenotypic traits has been shown to be a result of differences in the level to which genes are expressed (Carroll, 2000). Variation in transcription levels of particular genes among different populations of the same species has been shown to be responsible for differences in secondary metabolite production (Wu et al., 2008) or insect performance (Kuśnierczyk et al., 2007; Gao et al., 2008). In this project, I have studied intraspecific variation in transcriptional changes of *B. oleracea* cultivars in response to feeding by *P. rapae* caterpillars or *B. brassicae* aphids and combined this with insect performance studies to make a first step in identifying genes that are involved in direct defence against these herbivores.

The performance of two specialist herbivores on different cultivars of *B. oleracea* was investigated in a glasshouse experiment. Caterpillars of *P. rapae* gained less biomass and developed more slowly on Rivera than on Christmas Drumhead plants (Chapter 2). A test involving eight *B. oleracea* cultivars showed that cultivar Christmas Drumhead can be classified into a group with good larval performance (i.e. susceptible cultivars), whereas Rivera could be classified into a group with poor *P. rapae* performance, i.e. more resistant cultivars (Poelman et al., 2008b). *B. brassicae* populations developed more slowly on Rivera and Lennox than on Christmas Drumhead and Badger Shipper (Chapter 3). This shows that for both herbivores the same cultivar had the highest relative level of defence. These results led to the question which transcriptional responses underlie the intraspecific differences among the cultivars in herbivore performance. Microarray analysis revealed large differences in transcriptional responses between Rivera and Christmas Drumhead after feeding by *P. rapae* (Chapter 2). In Rivera the induction of a transcriptional response needed more time than in

Christmas Drumhead and the regulation of specific genes differed between the cultivars. Differences in *B. brassicae*-induced transcriptional responses between the two cultivars were less pronounced as a relatively small number of genes were differentially regulated after infestation by this aphid (Chapter 3). The finding that Rivera and Christmas Drumhead did not show clear differences in constitutive gene expression (Chapter 2), suggests a strong influence of inducible defence mechanisms in intraspecific variation in herbivore performance. Several defence-related genes were only induced in Rivera or showed a higher level of induction in this cultivar than in Christmas Drumhead after feeding by either *P. rapae* or *B. brassicae*. The expression of these genes may have an impact on herbivore performance, thereby underlying the direct defence mechanisms. One of these genes encodes a trypsin-and-protease inhibitor and was induced by *P. rapae* (Chapter 4) as well as *B. brassicae* (Chapter 3). To investigate whether this gene really has an effect on plant defence we went back to *A. thaliana* to study the effect of a knock-out mutation in the *TPI* gene (*trypsin-and-protease inhibitor*, At1g72290) as such experiments are difficult to perform in *B. oleracea*. The mutation showed to have an effect on the performance of both *P. rapae* (Chapter 4) and *B. brassicae* (Chapter 3), strongly suggesting that the gene is involved in the defence of *B. oleracea* as well. Overall, these data suggest that the microarray analysis, possibly in combination with a time course qRT-PCR analysis and mutant analysis in *A. thaliana* provides a good first step in identifying genes that are involved in direct plant defence.

Interspecific variation among cultivated and wild *Brassica* species

The extensive damage caused by pests in agroecosystems has promoted the study of herbivore-plant interactions in crops. However, breeders usually focus on particular yield- and quality-enhancing traits and original defence strategies may have been disrupted or lost during cultivation processes (Rosenthal and Dirzo, 1997). For example, the bitter or sharp taste of glucosinolates probably resulted in lower levels of these compounds in cultivated than in wild *B. oleracea* populations (Gols et al., 2008). This changed chemistry of cultivated plants has an effect on the performance and behaviour of certain herbivore species (Gols et al., 2008). Studying interspecific plant variation using cultivated and wild species can provide insight into plant-herbivore interactions.

B. nigra contains higher levels of glucosinolates than *B. oleracea* cultivars (Poelman et al., 2008b). High glucosinolate concentrations contribute to a higher level of direct defence against generalists and to a lesser extent to specialist herbivores (Gols et al., 2008). As shown in a study within the same research programme, *P. rapae* performed better on *B. nigra* than on any of eight *B. oleracea* cultivars tested (Poelman et al., 2008b) and *B. brassicae* populations developed faster on *B. nigra* than on four *B. oleracea* cultivars (Chapter 4). Larvae of *P. rapae* produce an enzyme, nitrile-specifying protein (NSP), in their gut that promotes the formation of nitriles instead of more toxic isothiocyanates during glucosinolate hydrolysis (Wittstock et al., 2004; Agerbirk et al., 2006). *B. brassicae* has evolved its own myrosinase and uses a bipartite glucosinolate-myrosinase system to accumulate intact glucosinolates for its own defence (Jones et al., 2002; Kazana et al., 2007; Pratt et al., 2008). Because *P. rapae* and *B. brassicae* have evolved mechanisms to deactivate the plant's glucosinolate hydrolysis system, it is unlikely that differential performance of these herbivores is due to differences in glucosinolate concentrations. The *B. oleracea* cultivars and *B. nigra* used in this thesis probably differ also in

other defence-related compounds such as proteinase inhibitors. To identify defence-related traits that may explain the differences in insect performance, transcriptional responses of *B. nigra* and the *B. oleracea* cultivars Rivera and Christmas Drumhead after feeding by either *P. rapae* or *B. brassicae* were compared (Chapter 4). Indeed, two genes encoding proteinase inhibitors showed no or lower expression in response to *P. rapae* feeding in *B. nigra* compared to the *B. oleracea* cultivars. This supports the observation that higher levels of proteinase inhibitors are present in cultivated *Brassica* plants compared to their wild relatives (Broadway, 1989).l.

Intraspecific variation in the field

The majority of gene expression studies on insect-plant interactions have been performed in the laboratory or glasshouse in which plants are exposed to a single attacker under carefully controlled conditions. In the field, plants have to deal with a great variety of stresses that can occur sequentially or simultaneously. Whether results obtained from glasshouse studies are also useful in field situations was unclear until now. Studies on plant transcriptional responses in the field will help to understand plant responses to insect attack under ecologically more relevant conditions. Until now, only few studies investigated transcriptional responses of field-grown plants. In these studies plants were artificially induced and gene expression levels compared. The investigated treatments include e.g. exposure to methyl jasmonate (Schmidt et al., 2006), induction by *Manduca sexta* herbivory (Izaguirre et al., 2003), or Japanese beetles (*Popillia japonica*) (Casteel et al., 2008). In my research, I studied intraspecific variation of two *B. oleracea* cultivars in the field and monitored naturally occurring herbivores on these cultivars. Differences in herbivore performance on *B. oleracea* cultivars as assessed in the glasshouse were consistent with performance differences as recorded in the field both for *P. rapae* (Poelman et al., 2008c) and *B. brassicae* (Chapter 3). This shows that intraspecific variation in the used cultivars, as represented by insect performance, in the glasshouse and field experiments are similar.

Intraspecific variation among four *B. oleracea* cultivars (two and all four were used in chapters 2 and 3, respectively) has also been found with regard to herbivore communities present on the cultivars, which were most likely influenced by differences in secondary metabolites (Poelman et al., 2008c). Furthermore, intraspecific variation in induced responses among *B. oleracea* cultivars has been observed and this variation may be linked to the observed differences in herbivore performance (Chapters 2 and 3). However, these studies were performed in a glasshouse and it is unclear whether the observed differences will also be recorded the field. I have investigated whether differences in herbivore community composition between two *B. oleracea* cultivars in the field can be related to intraspecific transcriptional variation (Chapter 5). Cultivars Rivera and Christmas Drumhead developed their phenotype in the field throughout the season differently. Phenotypic changes can be the result of all kinds of abiotic and biotic stresses such as temperature fluctuations, rainfall, and attack by fungi, bacteria, and herbivorous insects. In the field study described in Chapter 5, no differences in herbivore communities were found early in the season when low numbers of herbivores were present in the field. Also, no clear differences in transcriptional profiles could be detected between the cultivars at that time point. Later in the season, clear differences were found both in gene expression and in the structure of the herbivore community. In Rivera, several defence-related genes showed higher levels of expression than in Christmas Drumhead, which might, at least partly, explain the differences in herbivore communities.

Role of jasmonic acid pathway in herbivore resistance of white cabbage

Responses of plants to feeding by herbivorous insects have been shown to depend predominantly on increased levels of JA. Subsequent JA-responsive gene expression leads to the accumulation of defensive secondary metabolites, such as toxins, or digestibility reducers. These changes in plant traits result in reduced oviposition and development of herbivores (Thaler et al., 2002; Howe, 2005; Bruinsma and Dicke, 2008). For example, JA-treated *B. oleracea* plants showed reduced oviposition preference of *P. rapae* and *P. brassicae* females as well as *P. rapae* performance compared to control plants (Bruinsma et al., 2007). Blocking JA-mediated responses in *A. thaliana* increases the performance of lepidopteran herbivores (McConn et al., 1997; Stotz et al., 2002; Van Poecke and Dicke, 2002; Reymond et al., 2004).

JA biosynthesis is suggested to be regulated by positive feedback, as JA application results in the induction of all genes involved in JA biosynthesis (Wasternack, 2007). This fits well with the observed induction of *LOX2* after JA application in *B. oleracea* (Chapter 2; Zheng et al., 2007). *LOX2* in *B. oleracea* encodes a 13-LOX (Zheng et al., 2007), which is required for JA biosynthesis (Schaller et al., 2005; Wasternack et al., 2006). The expression of *LOX2* was induced after *P. rapae* feeding in *B. oleracea* (Chapter 2; Zheng et al., 2007) and *B. nigra* (Chapter 4), but not after feeding by the aphids *B. brassicae* (Chapter 3) or *M. persicae* (Zheng et al., 2007). This corresponds with the observation that JA accumulates in *A. thaliana* after *P. rapae*, but not after *M. persicae* feeding (De Vos et al., 2005).

The JA pathway also showed to play an important role in shaping the herbivore community on field-grown plants. Inhibition of the expression of a 13-LOX either by applying an inhibitor or through genetic modification has been shown to affect herbivore host selection and herbivore community composition (Kessler et al., 2004; Paschold et al., 2007; Bruinsma et al., 2008). In the field, *LOX2* expression levels were higher in *B. oleracea* cultivar Rivera than in cultivar Christmas Drumhead (Chapter 5), suggesting more JA accumulation in Rivera. Moreover, more JA-responsive genes showed higher levels of expression in Rivera than in Christmas Drumhead. The fact that JA induces secondary defence metabolites (van Dam et al., 2004; Bruinsma and Dicke, 2008) suggests that higher JA concentrations result in lower herbivore performance and species richness as observed on field-grown Rivera (Chapter 5). Accumulation of JA, for instance by *P. rapae* feeding, may negatively affect aphid and whitefly population development as these phloem-feeding herbivores have been shown to manipulate plant responses (Zhu-Salzman et al., 2004; Zhu-Salzman et al., 2005; Thompson and Goggin, 2006; De Vos et al., 2007; Zarate et al., 2007; Gao et al., 2008).

Conclusions and future perspectives

Until now, the knowledge on gene expression in plants of the Brassicaceae family has been obtained from the model plant *A. thaliana*. Furthermore, all studies on *A. thaliana*-insect interactions at the transcriptomic level have focused on plants grown under laboratory conditions and involve mostly induction with just a single herbivore. A lot of ecological and metabolomic knowledge on *Brassica*-herbivore interactions has been obtained in the past decades. However, nothing is known about global transcriptional responses of *Brassica* to herbivore feeding. Advances are therefore to be made

by (1) obtaining information on induced transcriptional responses in *Brassica* crops, (2) translating transcriptomic results from the glasshouse to the field situation, and (3) integrating transcriptomic, metabolomic, and ecological research. The results presented in this thesis provide a first step in identifying genes that play an important role in defence mechanisms against herbivores in *B. oleracea*. It was shown that intra- and/or interspecific variation in the performance of *P. rapae* or *B. brassicae* among *B. oleracea* cultivars and naturally occurring *B. nigra* can be (partly) explained by differences in transcriptional responses. As a result of this biological integration, several candidate genes were identified that may be involved in direct defence against these herbivorous insects. Furthermore, using an *A. thaliana* knock-out mutant confirmed the importance of one of these genes in direct defence against *P. rapae* and *B. brassicae.* Results of herbivore performance and transcriptional profiling obtained from glasshouse experiments could be translated to the field situation. Two field-grown *B. oleracea* cultivars showed clear differences in herbivore community composition that may be due to intraspecific transcriptional variation among certain genes.

Herbivorous insects are serious pests in agriculture, thereby hindering successful cultivation of crops. The future perspective in plant breeding to minimize herbivore damage in crops is to develop an approach that combines different plant defence traits, such as direct and indirect mechanisms. Such an approach is likely to be more effective than using a single plant trait. Chances of herbivores becoming resistant to a particular plant defence trait are most likely reduced when a plant produces a combination of resistance traits such as (1) toxins, (2) repellent compounds, and (3) volatiles to attract parasitoids or predators. In this thesis, I have shown that genes possibly involved in direct defence against *P. rapae* and/or *B. brassicae* are already present in *B. oleracea*, but their expression level may be too low or the timing of induction may not be optimal in certain cultivars to be maximally effective. Studies using mutant plants in which the candidate gene is silenced or overexpressed, through genetic modification, RNAi or virus induced gene silencing (VIGS), are needed to investigate the exact role of the genes in *B. oleracea*. To this purpose, lines in which the expression of single genes as well as in multiple genes is manipulated will be useful. The use of *A. thaliana* manipulated lines, in which homologues of certain *Brassica* genes are silenced or overexpressed, may be useful to get a first impression about their role. However, *B. oleracea* lines silencing or overexpressing genes of interest are needed to determine the exact role of candidate genes in this crop. When eventually defence genes have been identified, breeders can implement the results of this thesis study. Direct implementation might be possible using GM approaches such as cisgenesis. However, at present the public opinion in Europe is not in favour of GM food. A more likely implementation is via traditional breeding. Using molecular marker technologies it should be possible to select the best alleles involved in defence into one cultivar.

Acknowledgements

I thank Roeland Voorrips, Ben Vosman and Marcel Dicke for valuable suggestions that helped to improve this chapter.

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Summary

In their natural environment, plants are under constant pressure from all kinds of herbivorous insects. Plants have evolved several defence strategies to prevent or reduce attack by herbivorous insects. These strategies are classified as direct and indirect defence, which can both be constitutively present or induced upon herbivore feeding. Direct defence affects the performance and behaviour of the herbivore through, for example, morphological characteristics or the production of defensive compounds. Indirect defence on the other hand enhances the effectiveness of the natural enemies of the herbivore. Transcriptional profiling after herbivore feeding reveals, at the molecular level, how plants respond to this type of stress. Microarrays have been used extensively to investigate transcriptional responses of plants after feeding by a single herbivore species. In the Brassicaceae family most of these studies have focused on the model plant *Arabidopsis thaliana*. However, this plant is less suitable to investigate the effects of gene expression on community ecology because herbivorous insects and *A. thaliana* are active in different time windows. Other crucifers, such as white cabbage (*Brassica oleracea* var. *capitata*), are more appropriate for this purpose. White cabbage and *A. thaliana* are closely related and share a high sequence identity, which makes it possible to use genetic tools from the model plant to study the molecular basis of plant defence mechanisms in *Brassica* species. Because full genome *Brassica* microarrays are not available, 70-mer oligonucleotide microarrays based on the whole genome of *A. thaliana* were used to study responses of *Brassica* species to herbivore feeding.

Variation in plant defence traits may result in differences in herbivore feeding behaviour. Phenotypic variation for defence-related traits among plants from the same species therefore plays an important role in plant-herbivore interactions and may be used for identifying defence mechanisms. Consequently, intraspecific variation in plant traits may influence the composition and diversity of herbivore communities on plants grown under natural conditions. Differences in the expression of particular genes often underlie intraspecific variation in susceptibility to herbivorous insects. However, few studies link herbivore performance data with a full transcriptomic analysis. Even much less is known about intraspecific variation in transcriptional profiles of plants under field conditions in which plants are exposed to multiple attackers and all kinds of other stress factors.

This study was part of a research programme which aimed to link intraspecific variation in plant defence to higher trophic level biodiversity via an integrated approach in which transcriptomics, metabolomics, behavioural and community ecology are combined. This thesis contributes to the programme by combining plant transcriptomics, insect life history, population development and community composition to identify genes that are important in defence mechanisms against herbivores in cultivated and wild *Brassica* species, both under controlled and field conditions.

Intraspecific variation among white cabbage cultivars in respoknse to herbivore feeding

Larvae of the cabbage white butterfly (*Pieris rapae*) cause extensive damage to white cabbage plants by removing whole leaf areas. Conversely, feeding by cabbage aphids (*Brevicoryne brassicae*) results in chlorosis and curling of cabbage leaves. Both herbivore species negatively influence plant growth

thereby negatively affecting crop yield. To investigate the responses of white cabbage cultivars to feeding by these chewing and phloem-feeding herbivores, I monitored plant susceptibility and the induced transcriptional responses.

Cabbage white caterpillars gained more biomass during 6 days of feeding and needed longer time to develop into pupae on cultivar Rivera than on cultivar Christmas Drumhead, indicating a lower susceptibility of Rivera. Intraspecific variation in susceptibility against cabbage aphids among four white cabbage cultivars was also observed. Cultivars Rivera and Lennox clearly supported slower population increase of aphids than cultivars Christmas Drumhead and Badger Shipper. This shows that for both herbivores the same cultivar displayed the highest relative defence level.

To investigate which transcriptional responses underlie the observed intraspecific variation in herbivore performance, I performed microarray analysis to compare non-challenged and herbivore-induced plants of the cultivars Rivera and Christmas Drumhead. Caterpillar feeding for 24, 48, and 72 h resulted in transcriptional responses of the cultivars that differed in timing as well as in the regulation of individual genes. Jasmonic acid is an important plant hormone involved in the induction of defence responses against herbivore damage. Analyses of transcriptional responses after applying jasmonic acid to the cultivars revealed that the difference in timing did not hold for this type of treatment. Application of this hormone to a plant induces a reaction that is similar (although not identical) to that induced by insect herbivores. Indeed, the majority of caterpillar-induced genes in the two white cabbage cultivars were also jasmonic-acid responsive.

Aphid feeding, in contrast to caterpillar-induced responses, resulted in the differential regulation of only a small number of genes in cultivars Rivera and Christmas Drumhead. The transcriptional responses induced after caterpillar or aphid feeding were highly cultivar-specific and several defence-related genes were only induced in Rivera or showed a higher level of induction in this cultivar than in Christmas Drumhead. The expression of these genes may account for the observed differences in herbivore performance. Targeted studies employing an *A. thaliana* silenced mutant showed that the expression of a trypsin-and-protease inhibitor negatively influences caterpillar as well as aphid performance.

The results on this interaction between white cabbage cultivars and cabbage white caterpillars or cabbage aphids clearly show that there is intraspecific variation in plant susceptibility and whole-genome transcriptional responses. Several genes that were only induced in cultivar Rivera may underlie direct defence mechanisms against these herbivores in this cultivar.

Interspecific variation among white cabbage cultivars and wild black mustard in response to herbivore feeding

Investigating interspecific variation, i.e. differences among plants from different species, may contribute to understanding plant-herbivore interactions by comparing responses of wild and cultivated plants. Therefore, I studied interspecific variation in susceptibility and transcriptional responses to cabbage white caterpillars as well as cabbage aphids among white cabbage cultivars and plants from a wild

black mustard (*Brassica nigra*) population. Transcriptional responses in cabbage as well as black mustard after feeding by these two herbivores were highly insect-specific.

Comparing the results from black mustard to those from white cabbage after caterpillar feeding suggests that certain mechanisms of defence that are present in black mustard are lacking in the cultivated material and vice versa. This suggests that both *Brassica* species use different defence strategies to survive cabbage white caterpillar attack. The observed lower numbers of larvae on black mustard than white cabbage in the field and the better performance of caterpillars on the wild than cultivated *Brassica* in the glasshouse support this hypothesis. The expression of a gene that influences herbivore host plant selection was only induced in black mustard, whereas several direct defence-related genes were induced only in the white cabbage cultivars Rivera and Christmas Drumhead.

Performance of cabbage aphids on black mustard was also monitored and showed that black mustard is highly susceptible to this phloem-feeding herbivore in comparison to white cabbage cultivars. Microarray analysis revealed that black mustard also regulated a small number of genes after cabbage aphid feeding. The genes that were differentially expressed were different from the ones regulated in white cabbage cultivars after aphid feeding. The absence of induced expression of certain defence-related genes in black mustard can at least partly explain the differences in aphid performance between black mustard and white cabbage cultivars.

Intraspecific variation among white cabbage cultivars in the field

Herbivore communities on plants grown in the field are influenced by intraspecific plant variation. However, all previously mentioned experiments as well as most other transcriptomic studies have been carried out under carefully controlled conditions in a glasshouse in which plants were exposed to a single herbivore. In the field, plants are exposed to a whole range of biotic and abiotic stresses and results obtained from glasshouse experiments do not necessarily represent the field situation. Examining intraspecific variation in herbivore communities and transcriptional profiles between white cabbage cultivars in the field are therefore needed to investigate if results obtained from glasshouse studies are also useful under field conditions.

Recording cabbage aphid numbers on four cultivars in the field showed that the performance of this herbivore was similar under glasshouse and field conditions and thus relative cultivar susceptibility was largely independent of the environmental conditions. Monitoring all naturally occurring herbivores on field-grown Rivera and Christmas Drumhead revealed that herbivore community composition was similar on both cultivars early in the season. Conversely, clear differences in herbivore abundance, species richness, and biodiversity were observed later in the season. This suggests that the cultivars developed clearly different phenotypes during the growing season. Microarray analysis revealed significant differences in the expression levels of 51 genes between the cultivars later in the season, but only few differences earlier on. Several defence-related genes showed higher levels of expression in the white cabbage cultivar that harboured the lowest numbers of herbivores. These results obtained from the field show that intraspecific variation in plant phenotype between white cabbage cultivars develops during the season, resulting in differential composition of herbivore communities. The

observed differences in herbivore communities on the two white cabbage cultivars can be, at least partly, explained intraspecific variation in the expression of particular defence-related genes.

Conclusion

The results obtained in this thesis show that intra- and interspecific variation between *Brassica* plants has a strong impact on susceptibility and transcriptional responses of these plants in response to herbivore feeding both under glasshouse and field conditions. The integrated approach of the research programme, i.e. combining transcriptomics, metabolomics, behavioural and community ecology, contribute to a better understanding of *Brassica*-insect interactions. This thesis forms the basis for further unravelling direct defence mechanisms of white cabbage.

Samenvatting

In hun natuurlijke omgeving worden planten continu belaagd door allerlei plantenetende insecten. Om te kunnen overleven hebben ze verschillende strategieën ontwikkeld om deze aanval te verhinderen of de gevolgen te verminderen. Deze verdedigingsmechanismen worden gedefinieerd als directe of indirecte verdediging en kunnen altijd aanwezig zijn of geactiveerd worden in de aanwezigheid van plantenetende insecten. Directe verdediging beïnvloedt de groei en ontwikkeling van de insecten in de vorm van bijvoorbeeld morfologische barrières (zoals bladharen of een waslaag) of de productie van, voor het insect, schadelijke stoffen. Indirecte verdediging bevordert de aantrekking van natuurlijke vijanden van de insecten door bijvoorbeeld het produceren van lokstoffen. Het transcriptie profiel van een plant, d.w.z. de groep genen die wordt afgeschreven, bepaalt welke verdedigingsmechanismen worden gebruikt tegen de aanvallende plantenetende insecten. Microarrays worden veel gebruikt om transcriptie profielen of reacties van planten te onderzoeken. Een microarray is een glasplaatje met daarop een grote hoeveelheid “spots” met in elke spot een DNA fragment van een ander gen. Met microarrays is het mogelijk om de transcriptie profielen van twee verschillende behandelingen of rassen te vergelijken. Materiaal van monster A wordt gelabeld met fluorescerend groen terwijl materiaal van monster B wordt gelabeld met fluorescerend rood. Beide monsters worden vervolgens samen op de microarray gegoten zodat het aanwezige RNA, het afgeschreven DNA van genen, in de monsters kunnen binden aan de passende genen op de microarray. Wanneer de microarray onder de fluorescentiemicroscoop bekeken wordt, zijn er drie mogelijkheden: een spot is groen (het gen is alleen actief in monster A), rood (het gen is alleen actief in monster B), of geel (het gen is actief in beide monsters).

In de Kruisbloemigenfamilie (*Brassicaceae*) zijn de meeste microarray studies uitgevoerd met de modelplant *Arabidopsis thaliana* (zandraket). Hierbij is voornamelijk gekeken naar de transcriptie reactie van deze plant op insectenvraat. In zijn natuurlijke omgeving komt de zandraket echter niet of nauwelijks in aanraking met plantenetende insecten waardoor deze plant minder geschikt is om de effecten van transcriptie reacties op insecten ontwikkeling te onderzoeken. Andere kruisbloemigen, zoals witte kool (*Brassica oleracea* var. *capitata*), zijn meer geschikt voor dit doel. Het totale DNA van witte kool en zandraket komt voor 85% overeen waardoor het mogelijk is om genetische technieken van de modelplant te gebruiken voor het bestuderen van de verdedigingsmechanismen in *Brassica* soorten. In dit onderzoek werden daarom microarrays gebruikt die alle genen van de zandraket vertegenwoordigen om transcriptie reacties van *Brassica* soorten op insectenvraat te bestuderen.

Verschillende verdedigingsstrategieën van planten hebben een verschillend effect op de groei en ontwikkeling van planteneters. Variatie in verdediging tussen planten van dezelfde soort, ook wel intraspecifieke variatie genoemd, speelt daarom een belangrijke rol in plant-insect interacties en kan worden gebruikt voor het identificeren van verdedigingsmechanismen. Deze variatie kan ook het voorkomen van insecten beïnvloeden op planten die groeien onder natuurlijke omstandigheden. Verschillen in transcriptie profielen of reacties zijn vaak verantwoordelijk voor intraspecifieke variatie in groei en ontwikkeling van insecten. Er zijn maar weinig onderzoeken die resultaten van insectengroei en -ontwikkeling koppelen aan microarray analyses. Nog minder is bekend over intraspecifieke variatie

in transcriptie profielen van planten onder veldomstandigheden waarin ze worden blootgesteld aan allerlei belagers en ook aan andere vormen van stress.

Dit onderzoek maakte deel uit van een onderzoeksprogramma dat probeerde intraspecifieke variatie in plantverdediging te koppelen aan biodiversiteit van plantenetende insecten en hun natuurlijke vijanden. Dit proefschrift combineert transcriptie analyses van planten met groei, ontwikkeling en voorkomen van insecten om genen te identificeren die belangrijk zijn in de verdediging tegen insecten in gecultiveerde en wilde *Brassica* soorten, zowel in de kas als in het veld.

Intraspecifieke variatie tussen witte koolrassen in reactie op insectenvraat

Rupsen van het kleine koolwitje (*Pieris rapae*) eten van bladeren waarbij ze enorme schade aanrichten aan de plant. De melige koolluis (*Brevicoryne brassicae*) zuigt sap uit het vaatweefsel van de plant wat leidt tot het krullen of verbleken van de bladeren. Beide insecten eten van witte kool en vormen een serieus probleem tijdens de teelt ervan. Om meer te weten te komen over de interactie tussen witte koolrassen en deze insecten heb ik gekeken naar groei en ontwikkeling van de insecten en naar de transcriptie reacties van de plant.

De resultaten laten zien dat rupsen langzamer groeiden en zich sneller ontwikkelden tot een pop op ras Rivera dan op ras Christmas Drumhead. Rivera is dus beter bestand tegen rupsenvraat dan Christmas Drumhead. We vonden ook verschillen in groei en ontwikkeling van de melige koolluis op vier witte koolrassen. Bladluizen die uitgezet waren ontwikkelden een kleinere populatie op de rassen Rivera en Lennox dan op de rassen Christmas Drumhead en Badger Shipper. Voor beide insecten laten dezelfde rassen dus de sterkste relatieve verdediging zien.

Om te onderzoeken waardoor de gevonden verschillen in insectengroei en -ontwikkeling kunnen worden verklaart, heb ik gekeken naar de transcriptie reacties van de rassen Rivera en Christmas Drumhead. Met behulp van de microarray werden controle planten vergeleken met planten die waren aangevallen door insecten om te zien welke genen werden geactiveerd door de aanwezigheid van de insecten. De transcriptie reacties van de rassen na rupsenvraat verschilde niet alleen in timing, maar er werden ook verschillende genen aangeschakeld. Jasmonzuur is een belangrijk plantenhormoon dat betrokken is bij de activatie van verdedigingsmechanismen tegen planteneters. Na het aanbrengen van een oplossing met jasmonzuur op de bladeren verschilden de transcriptie reacties van de rassen niet in timing. De transcriptie reactie na deze behandeling was gelijk, maar niet identiek, aan die na rupsenvraat. De meerderheid van de door rupsen aangeschakelde genen in de twee witte koolrassen werden ook aangeschakeld door jasmonzuur. Wanneer de rassen werden aangevallen door melige koolluizen, werden er minder genen geactiveerd in de rassen (Rivera en Christmas Drumhead) dan na rupsenvraat.

De transcriptie reacties op een aanval door rupsen of bladluizen waren zeer specifiek per ras en een aantal genen die iets te maken zouden kunnen hebben met plantenverdediging waren alleen of sterker aangeschakeld in Rivera. De eiwitten die ontstaan na het activeren van deze genen kunnen verantwoordelijk zijn voor de gevonden verschillen in groei en ontwikkeling van de insecten. Door een

van deze genen uit te schakelen in een plant is het mogelijk om de functie van dit gen te onderzoeken. Helaas is het nog niet mogelijk om dit te doen in *Brassica* planten. Om toch een idee te krijgen over de functie van bepaalde genen heb ik knock-out mutanten, planten waarin één bepaald gen is uitgeschakeld, van de zandraket gebruikt. Onderzoek naar de groei en ontwikkeling van de insecten op deze knock-out planten liet zien dat een “trypsine-en-protease inhibitor” gen een negatief effect had op zowel rupsen als bladluizen.

De resultaten van de interactie tussen witte koolrassen en rupsen van het kleine koolwitje of melige koolluizen laten duidelijk zien dat er intraspecifieke variatie is voor insectengroei en transcriptie reacties van planten. Een aantal genen die alleen aangeschakeld werden in Rivera kunnen de basis zijn voor directe verdedigingsmechanismen tegen deze plantenetende insecten.

Interspecifieke variatie tussen witte koolrassen en wilde zwarte mosterd op plantenetende insecten

Het bestuderen van interspecifieke variatie, d.w.z. verschillen tussen planten van verschillende soorten, kan meer inzicht geven in de verdedigingmechanismen van planten in reactie op de aanwezigheid van insecten door de reacties van wilde planten en rassen te vergelijken. Daarom heb ik ook gekeken naar de groei van rupsen van het kleine koolwitje en melige koolluizen op wilde zwarte mosterdplanten (*Brassica nigra)* en naar de transcriptie reactie van deze plant op een aanval door deze insecten. De resultaten werden vervolgens vergeleken met de resultaten verkregen van de witte kool rassen. De transcriptie reacties van zwarte mosterd waren, net als bij de witte kool rassen, afhankelijk van het aanwezige insect.

De transcriptie reacties suggereren dat bepaalde verdedigingsmechanismen die geactiveerd worden in zwarte mosterd niet actief zijn in witte koolrassen en andersom. Dit wil zeggen dat beide *Brassica* soorten waarschijnlijk verschillende verdedigingsstrategieën gebruiken om een aanval van rupsen van het kleine koolwitje te overleven. Het lagere aantal rupsen dat werd gevonden op zwarte mosterdplanten in het veld en de betere groei van rupsen op de wilde *Brassica* dan op de *Brassica* rassen draagt bij aan deze suggestie. Zwarte mosterd beïnvloedt waarschijnlijk de aantrekking van vlinders omdat een gen betrokken bij dit proces alleen actief was in deze plant. Een aantal genen die betrokken zijn bij directe verdediging waren juist alleen geactiveerd in de witte koolrassen Rivera en Christmas Drumhead.

De groei en ontwikkeling van de melige koolluis op zwarte mosterd is ook onderzocht en liet zien dat er, na een bepaalde tijd, meer luizen aanwezig waren op zwarte mosterd dan op de witte kool rassen. Microarray experimenten lieten zien dat zwarte mosterd een lager aantal, en ook andere genen activeert in reactie op een aanval door luis dan de witte koolrassen. Het verschil in bladluisgroei en -ontwikkeling tussen zwarte mosterd en de witte kool rassen zou veroorzaakt kunnen worden doordat een aantal verdedigingsgenen niet in zwarte mosterd worden geactiveerd en wel in witte kool.

Intraspecifieke variatie tussen witte kool rassen in het veld
Populaties van insecten op planten in het veld worden beïnvloed door intraspecifieke planten variatie. Alle bovengenoemde experimenten zijn uitgevoerd onder zorgvuldig gecontroleerde omstandigheden in een kas waarbij planten werden aangevallen door één enkele insectensoort. In het veld hebben planten echter te maken met een heel scala aan stress factoren, zoals wind, regen, ziektes of allerlei insecten. Resultaten verkregen uit kasexperimenten zijn niet noodzakelijkerwijs een afspiegeling van de veldsituatie. Veldexperimenten zijn daarom nodig om te bepalen of de resultaten uit kasexperimenten kunnen worden gebruikt in het veld.

Het tellen van melige koolluis op vier witte koolrassen in het veld liet zien dat de groei en ontwikkeling van dit insect relatief ongeveer gelijk was in kas en veld. De verdediging van de rassen is daarom waarschijnlijk onafhankelijk van de omgevingsfactoren. Vroeg in het seizoen was het aantal natuurlijk voorkomende plantenetende insecten in het veld gelijk op Rivera en Christmas Drumhead. Op dit tijdstip waren de transcriptie profielen van de beide rassen nagenoeg gelijk. Later in het seizoen waren er duidelijke verschillen te zien in de verdeling, het voorkomen en de biodiversiteit van plantenetende insecten op beide rassen. De rassen ontwikkelen zich waarschijnlijk verschillend tijdens het groeiseizoen. Microarray experimenten lieten zien dat de transcriptie profielen van de rassen later in het seizoen verschilden in 51 genen. Een aantal verdedigingsgenen waren sterker geactiveerd in het ras met de kleinste aantallen insecten. Deze resultaten laten zien dat intraspecifieke plantenvariatie tussen witte koolrassen zich ontwikkelt door het seizoen heen wat verschillen in insecten populaties veroorzaakt. Deze verschillen in insecten populaties kunnen, in ieder geval gedeeltelijk, worden gekoppeld aan de verschillen in activatie van bepaalde verdedigingsgenen.

Conclusies
De resultaten in dit proefschrift laten zien dat intra- en interspecifieke variatie tussen *Brassica* planten een sterk effect hebben op de groei van plantenetende insecten en op de transcriptie reacties van de plant na aanval door insecten, zowel in de kas als in het veld. Het combineren van onderzoek naar transcriptie profielen van de plant en de groei van insecten, draagt bij aan een het beter begrijpen van de interactie tussen *Brassica* planten en plantenetende insecten. Dit proefschrift vormt de basis voor het verder onderzoeken van directe verdedigingsmechanismen van witte kool.

Dankwoord

Vier jaar geleden leek dit moment nog heel ver weg, maar de tijd is voorbij gevlogen en dat is volgens mij een duidelijk teken dat ik een erg leuke periode ga afsluiten. Ik wil deze pagina's dan ook gebruiken om iedereen te bedanken die direct of indirect een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift.

Als eerste wil ik graag mijn begeleiders bedanken. Ben, jouw positieve insteek en enthousiasme hebben zeker bijgedragen aan het hooghouden van mijn motivatie. Soms kwam je vlak voor het submitten van een manuscript nog even binnen wandelen met een gevatte opmerking waardoor ik even door de bomen het bos niet meer zag. Gelukkig zag ik, na het even te laten bezinken, dat je meestal toch wel een goed punt had waardoor het verhaal net weer even beter op papier kwam. Roeland, jij hebt ook een belangrijke rol gespeeld in het tot stand komen van dit proefschrift. Je was altijd erg precies en jouw statistische inzichten hebben mij beter wegwijs gemaakt in deze verwarrende tak van wetenschap. Marcel, dankzij jou heb ik op een andere manier naar insecten leren kijken. Jouw positieve kritiek en enthousiasme hebben zeker bijgedragen aan het afronden van dit proefschrift. Ook vond je altijd wel een gaatje in je drukke schema om mijn manuscripten razend snel te corrigeren. Ik ben erg blij dat ik jullie als begeleiders heb gehad. Bedankt!

Erik, tijdens de afgelopen vier jaar heb ik met veel plezier met je samengewerkt. Ik heb veel van je geleerd over de ecologische kant van ons project en vond het erg motiverend om experimenten samen met jou te bedenken, uit te voeren en te publiceren. Ook het telllen van insecten op eindeloos veel planten (waarom hadden we ook al weer zoveel herhalingen ingebouwd?!) was een stuk aangenamer in jouw gezelschap. Ron, jammer dat je besloot om je plaats als derde AIO in het triplet op te geven. Maar, ik ben blij dat je een baan hebt gevonden die helemaal bij je past. Jeroen, bedankt voor je statistische adviezen en ideeën als plaatsvervanger. Voor alle adviezen en commentaren tijdens menig triplet vergadering die zeker hebben bijgedragen aan dit onderzoek ben ik ook nog dank verschuldigd aan Nicole, Joop, Wim en Louise.

Ook wil ik graag alle mensen bedanken die mij geholpen hebben met de praktische zaken. Een aantal mensen wil ik daarbij in het bijzonder even noemen. Greet, jouw kennis en ervaring hebben het opzetten van experimenten en het verzamelen van materiaal een stuk makkelijker gemaakt. Ook jouw gezelligheid en humor hebben daar natuurlijk aan bijgedragen. Doret, ik vond het leuk om samen met jou de wondere wereld van de microarrays en real-time PCR te ontdekken. Onze gesprekken over shoppen, vakanties en andere alledaagse dingen hebben het labwerk nog leuker gemaakt dan het al was. Si-jun, bedankt voor al je moleculaire adviezen en materialen. Ronny, bedankt voor je hulp bij het transformeren van Arabidopsis plantjes. Helaas is het me niet gelukt om met deze plantjes nog data te genereren voor dit proefschrift.

I have been lucky to involve some enthousiastic students in my project and I am grateful for their help with lab- and/or fieldwork. Ana, Merlijn, Stephanie and André, it was a pleasure to work with you.

Het uitvoeren van alle experimenten was niet gelukt zonder de goede verzorging van planten en insecten door Leo, André G, Frans, Bert, Maarten en vele anderen van Unifarm. André M en Bertus kregen het gelukkig ook altijd voor elkaar om een goede ruimte voor mij te regelen. Bedankt daarvoor! Noortje, jou wil ik bedanken voor het snelle afhandelen van de koolzaadaanvragen. Leuk dat je ze vaak persoonlijk kwam afgeven. Ook wil ik Mariame, Annie, Lettie, Sabine en Angelique bedanken voor de nodige administratieve ondersteuning.

Ook heb ik nog tijd kunnen vinden om mee te werken aan andere projecten. Nina, ik vond het erg leuk dat je me vroeg voor de versterking van je paper en ik heb deze dan ook vol trots aan mijn publicatie lijst toegevoegd. Tjeerd, ik vond het erg leuk om je kennis te laten maken met de moleculaire aspecten van plant-insect interacties. Bedankt voor al je inspirerende opmerkingen en by the way: als ik evil ben, ben jij het zeker! Maaike, bedankt dat je mij hebt gevraagd als mede organisator voor de AIO excursie naar London. Ik vond het een erg leuke ervaring en een project op zich.

De afgelopen vier jaar waren zeker weten een stuk minder leuk geweest zonder mijn “roomies” Adriana, Marleen, Mirjam, Eveline, Brigitte en Hulya. Bedankt voor al jullie steun, interesse, meedenken, opbeurende gesprekken, gezellige uitstapjes en etentjes. Ik heb een super tijd gehad in ons kippenhok! Ook de lunch pauzes zijn vaak een belangrijke adempauze geweest, vooral tijdens stressvolle dagen. Rene, Paul en Martijn, bedankt voor alle peptalks en gezellige afleiding tijdens de lunch.

Aangezien het onmogelijk is om iedereen bij naam te noemen, wil ik bij deze al mijn collega’s van Ento en Plant Breeding bedanken voor de prettige werksfeer, gezelschap tijdens congressen, adviezen, interesse en belangstelling. Bij deze wil ik ook graag alle collega AIO’s bedanken voor alle gezelligheid tijdens congressen, excursies en PhD dagen.

Gelukkig is er na het werk ook nog wat tijd overgebleven voor wat ontspanning. Waterpolo is een goede sport voor het afreageren van frustraties en ik wil daarom mijn teamgenootjes bedanken voor het in stand houden van deze mogelijkheid. Ook wil ik “de boys” bedanken voor de gezellige eet- en drinkavonden in Utrecht. Ik vind het erg leuk dat we elkaar na de studie nog steeds regelmatig spreken. Verder wil ik al mijn vrienden bedanken voor hun interesse, steun, afleiding en gezelligheid.

Tenslotte wil ik natuurlijk ook mijn familie bedanken. Pap en mam, jullie zijn echt super ouders. Bedankt voor jullie onvoorwaardelijke steun, interesse en hulp op wat voor manier dan ook. Kristel en Mans, ik ben erg blij met zo’n leuke zus en broer. Carel, Gaby, Bert en Marijke, jullie horen natuurlijk ook in dit rijtje thuis. Allerliefste John, zonder jou zou mijn leven een stuk minder leuk zijn! Bedankt voor al je interesse, geduld, liefde en steun de afgelopen jaren.

Curriculum Vitae

Colette Broekgaarden was born on 18th of August 1980 in Ede, the Netherlands. After obtaining het HAVO diploma at the Johannes Fontanus College in Barneveld in 1997, she started her study Medical Biotechnology at the Hogeschool van Utrecht. As part of her bachelor she carried out a research project at the Hubrecht Institute in Utrecht. In this project, she studied the promotor region of a gene involved in pipid frog development. She obtained her degree in 2001 and started to study Biology at the Free University in Amsterdam in the same year. There she became fascinated with plant research and went for a master thesis project to the department of Biochemistry and Plant Molecular Physiology in Montpellier, France. During this period she studied the role of an Arabidopsis potassium channel that controls the plant hydric and ionic states . After her graduation in 2004 she started her PhD at the Laboratory of Entomology and Plant Research International, Wageningen University. The research focussed on idenitfiying and characterizing new genes that are involved in insect resistance in Brassica species.The results of this research project are described in this thesis. After defending her PhD thesis, she will continue working on plant-insect interactions as a postdoc at Plant Breeding, Wageningen University.

List of publications

Peer-reviewed publications

Broekgaarden C, Poelman EH, Steenhuis G, Voorrips RE, Dicke M, Vosman B (2008) Responses of *Brassica oleracea* cultivars to infestation by the aphid *Brevicoryne brassicae*: an ecological and molecular approach. Plant, Cell and Environment DOI: 10.1111/j.1365-3040.2008.01871.x

Fatouros NE, **Broekgaarden C**, Bukovinszkine'Kiss G, van Loon JJA, Mumm R, Huigens ME, Dicke M, Hilker M (2008) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. Proceedings of the National Academy of Sciences of the USA 105: 10033-10038

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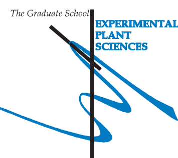
Submitted

Broekgaarden C, Poelman EH, Voorrips RE, Dicke M, Vosman B. Intraspecific variation in herbivore community composition and transcriptional profiles in field-grown *Brassica oleracea* cultivars.

Poelman EH, Oduor AMO **Broekgaarden C**, Hordijk CA, Janssen JJ, van Loon JJA, van Dam NM, Vet LEM, Dicke M. Field parasitism rates of caterpillars on *Brassica oleracea* plants are reliably predicted by differential attraction of *Cotesia* parasitoids.

Broekgaarden C, Voorrips RE, Dicke M, Vosman B. Transcriptional responses of wild and cultivated *Brassica* to two specialist insect herbivore species.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Colette Broekgaarden
Date: 21 October 2008
Group: Laboratory of Entomology & Plant Research International (PRI)
Wageningen University and Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Identification and expression of genes related to herbivory	Dec 20, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Radboud University Nijmegen	Jun 02, 2005
EPS PhD student day, Wageningen University	Sep 19, 2006
EPS PhD student day, Wageningen University	Sep 13, 2007
► **EPS theme symposia**	
Theme 2 symposium 'Interactions between plants and biotic agents', Utrecht University	Sep 17, 2004
Theme 2 symposium 'Interactions between plants and biotic agents', Leiden University	Jun 23, 2005
Theme 2 symposium 'Interactions between plants and biotic agents', Amsterdam University	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Plant Sciences meeting, Lunteren	Apr 04-05, 2005
17e Meeting Netherlands Entomological Society, Ede	Dec 16, 2005
NWO-ALW Plant Sciences meeting, Lunteren	Apr 05-06, 2006
NERN annual meeting, Lunteren	Feb 12-13, 2008
NWO-ALW Plant Sciences meeting, Lunteren	Apr 07-08, 2008
► **Seminars (series), workshops and symposia**	
Symposia YELREM, 'Biodiversity and Convergence of Sciences' de Bosbeek, Renkum	Jun 29, 2005
Symposia YELREM, 'Molecular Ecology and Insect learning' de Bosbeek, Renkum	Jun 20, 2006
Symposia YELREM, 'Metabolomics data processing and Insect behaviour' de Bosbeek, Renkum	Jun 18, 2007
CBSG Micro Array meeting	Oct 15, 2004
Workshop Plant-Insect Interactions: 'From Molecular Biology to Ecology, Wageningen University	Apr 25, 2006
EPS symposium 'Ecology and Experimental Plant Sciences: From Molecules to Multitrophic Interactions', WU	Mar 23, 2007
Seminar series Entomology	2004-2008
Microarray workshop, Leiden	Sep 21, 2007
Seminar series PRI	2004-2008
► **Seminar plus**	
► **International symposia and congresses**	
IOBC Workshop: 'Methods in Induced Resistance' Delémont, Switserland	Nov 02-04, 2004
Symposia 'Regulatory Oxylipins' Lausanne, Switserland	Sep 15-16, 2005
Plant GEMs, Amsterdam, NL	Sep 20-23, 2005
IOBC Workshop: 'Breeding for inducible resistance against pests and diseases' Heraklion-Crete, Greece	Apr 27-29, 2006
Intl Joint Workshop on "PR proteins" and "Induced Resistance Against Pathogens and Insects" Doorn, NL	May 10-14, 2007
► **Presentations**	
Poster, NWO-ALW Plant Sciences meeting, Lunteren	Apr 04-05, 2005
Poster, Symposia Regulatory Oxylipins/Plant GEMs	Sep 15-16, 2005/Sep 20-23, 2005
Oral presentation, 17e Meeting Netherlands Entomological Society	Dec 16, 2005
Oral presentation, NWO-ALW Plant Sciences meeting, Lunteren	Apr 05-06, 2006
Oral presentation, Workshop Plant-Insect Interactions/IOBC Workshop	Apr 25, 2006/Apr 27-29, 2006
Oral presentation, Joint Workshop PR Proteins and Induced Resistance	May 10-14, 2007
Oral presentation, NERN annual meeting, Lunteren	Feb 12-13, 2008
► **IAB interview**	Sep 14, 2007
► **Excursions**	
PhD Excursion UK	Mar 04-08, 2007
Subtotal Scientific Exposure	*19.4 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Course 'Bioinformation Technology - 1', VLAG, Wageningen	Nov 08-16, 2004
Springschool 'Chemical Communication' WICC, Wageningen	Mar 19-23, 2005
► **Journal club**	
Plant-Insect Interactions, Laboratory of Entomology, WU	2004-2007
Theme group 'Abiotic and Biotic stress', Plant Research International	2004-2007
Weekly meetings Plant Breeding, PRI	2004-2007
► **Individual research training**	
Long Oligonucleotide Microarray Workshop, Arizona, USA	Dec 12-17, 2004
Subtotal In-Depth Studies	*8.4 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD Assessment	May 04, 2005
English Scientific Writing, CENTA, WU	Mar 15-May 10, 2006
► **Organisation of PhD students day, course or conference**	
Organisation of PhD Excursion to United Kingdom	Mar 04-08, 2007
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

Supplemental Tables Chapter 4

Supplemental Table 1. Mean log_2 expression ratio after challenge with *Pieris rapae* for 24 h in *Brassica nigra* and *Brassicae oleracea* cultivar Christmas Drumhead

Brassica nigra	*P* value	Christmas Drumhead	*P* value	AGI code	Name
-0.12	0.218	-1.00	0.009	At1g04480	60S ribosomal protein L23 (RPL23A)
-0.67	0.096	-1.19	0.035	At1g05190	ribosomal protein L6 family protein
1.00	0.061	1.23	0.013	At1g07230	phosphoesterase family protein
0.46	0.052	1.14	0.001	At1g08860	copine; putative
1.13	0.019	0.54	0.490	At1g10360	glutathione S-transferase; putative
1.33	0.173	2.03	0.021	At1g11840	lactoylglutathione lyase; putative / glyoxalase I; putative
1.43	0.002	0.25	0.243	At1g12110	nitrate/chlorate transporter (NRT1.1) (CHL1)
-1.06	0.029	-0.22	0.388	At1g12200	flavin-containing monooxygenase family protein / FMO family protein
0.61	0.114	1.85	0.001	At1g19550	dehydroascorbate reductase; putative
-2.72	0.247	-2.90	0.027	At1g20360	F-box protein-related
0.98	0.051	1.16	0.020	At1g20620	catalase 3 (SEN2)
-1.46	0.031	-0.90	0.004	At1g22990	heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related
0.06	0.293	-1.02	0.030	At1g23690	expressed protein
-0.71	0.196	-1.14	0.018	At1g24020	Bet v I allergen family protein
-0.03	0.318	1.30	0.046	At1g24070	glycosyl transferase family 2 protein
-0.42	0.251	-1.16	0.049	At1g24240	ribosomal protein L19 family protein
*		-1.21	0.050	At1g27140	glutathione S-transferase; putative
0.81	0.115	1.84	0.021	At1g29390	stress-responsive protein; putative
-0.53	0.131	-1.43	0.000	At1g29660	GDSL-motif lipase/hydrolase family protein
-1.69	0.123	-1.56	0.041	At1g29910	chlorophyll A-B binding protein 2; chloroplast / LHCII type I CAB-2 / CAB-140 (CAB2A)
-0.39	0.310	1.48	0.016	At1g35720	annexin 1 (ANN1)
1.15	0.005	1.04	0.171	At1g47350	F-box family protein-related
0.27	0.040	1.24	0.044	At1g47540	trypsin inhibitor; putative
*		-2.10	0.026	At1g49240	actin 8 (ACT8)
1.22	0.035	2.40	0.019	At1g52070	jacalin lectin family protein
0.03	0.764	3.82	0.019	At1g54020	myrosinase-associated protein; putative
0.98	0.047	1.83	0.007	At1g54030	GDSL-motif lipase; putative
1.10	0.041	0.70	0.067	At1g54040	kelch repeat-containing protein
-1.19	0.053	-1.01	0.009	At1g55915	expressed protein
-1.51	0.049	-1.13	0.041	At1g56420	expressed protein
-0.55	0.120	1.69	0.001	At1g60080	3' exoribonuclease family domain 1-containing protein
1.07	0.037	1.75	0.031	At1g61120	terpene synthase/cyclase family protein similar to S-linalool synthase
0.15	0.063	1.18	0.006	At1g63410	expressed protein
1.39	0.003	1.57	0.009	At1g70230	expressed protein
1.28	0.122	3.72	0.001	At1g72290	trypsin and protease inhibitor family protein / Kunitz family protein
1.35	0.098	3.04	0.007	At1g73325	trypsin and protease inhibitor family protein / Kunitz family protein
0.93	0.082	1.37	0.024	At1g74950	expressed protein
-1.02	0.011	-0.93	0.001	At1g79510	expressed protein
1.48	0.021	2.53	0.020	At1g79920	heat shock protein 70; putative / HSP70; putative
*		-1.09	0.025	At1g80180	expressed protein
0.42	0.012	1.05	0.003	At1g80420	DNA repair protein; putative (XRCC1)
0.13	0.469	1.63	0.018	At1g80920	DNAJ heat shock N-terminal domain-containing protein
1.14	0.004	0.83	0.259	At2g03140	CAAX amino terminal protease family protein
-0.89	0.077	-1.44	0.017	At2g10940	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
-0.30	0.205	1.47	0.003	At2g11490	hypothetical protein
1.00	0.028	0.81	0.298	At2g15910	CSL zinc finger domain-containing protein
1.42	0.041	0.01	0.874	At2g17785	zinc-binding protein-related
0.19	0.087	1.22	0.023	At2g19540	transducin family protein / WD-40 repeat family protein
-0.17	0.584	-1.11	0.009	At2g21920	hypothetical protein
-1.49	0.032	*		At2g24340	hypothetical protein
1.04	0.038	0.85	0.260	At2g28610	homeobox-leucine zipper transcription factor (PRESSED FLOWER)
-1.70	0.065	-1.72	0.003	At2g29320	tropinone reductase; putative / tropine dehydrogenase; putative
-0.18	0.697	1.21	0.034	At2g29450	glutathione S-transferase (103-1A)
1.70	0.004	0.45	0.510	At2g32150	haloacid dehalogenase-like hydrolase family protein
0.45	0.541	1.33	0.011	At2g33340	transducin family protein / WD-40 repeat family protein
-0.47	0.060	-1.29	0.006	At2g36170	ubiquitin extension protein 2 (UBQ2) / 60S ribosomal protein L40 (RPL40A)
-0.09	0.713	-1.03	0.003	At2g36830	major intrinsic family protein / MIP family protein
-0.61	0.005	-1.44	0.023	At2g37220	29 kDa ribonucleoprotein; chloroplast; putative / RNA-binding protein cp29; putative
-0.03	0.665	1.65	0.041	At2g38150	alpha 1;4-glycosyltransferase family protein
0.37	0.124	3.20	0.024	At2g39310	jacalin lectin family protein similar to myrosinase-binding protein
0.61	0.020	3.38	0.006	At2g39330	jacalin lectin family protein similar to myrosinase-binding protein
-1.12	0.002	-1.24	0.001	At2g40100	chlorophyll A-B binding protein (LHCB4.3)
0.38	0.164	1.23	0.046	At2g44830	protein kinase; putative
1.18	0.034	0.45	0.169	At2g47890	zinc finger (B-box type) family protein
-0.25	0.204	1.49	0.016	At3g01420	pathogen-responsive alpha-dioxygenase; putative
-1.05	0.040	-1.03	0.006	At3g01500	carbonic anhydrase 1; chloroplast / carbonate dehydratase 1 (CA1)
-1.32	0.052	-1.09	0.003	At3g03110	exportin 1; putative
1.26	0.047	1.85	0.042	At3g06547	expressed protein
1.09	0.002	0.75	0.372	At3g06870	proline-rich family protein
1.06	0.022	-0.34	0.341	At3g10470	zinc finger (C2H2 type) family protein
-0.13	0.181	-1.30	0.014	At3g11990	expressed protein
0.02	0.673	1.60	0.004	At3g12720	myb family transcription factor
1.03	0.026	0.86	0.294	At3g12870	expressed protein
1.64	0.031	1.96	0.048	At3g16160	tesmin/TSO1-like CXC domain-containing protein
-0.14	0.206	1.16	0.025	At3g16220	expressed protein
1.19	0.042	-0.08	0.614	At3g16680	expressed protein

Supplemental Table 1. (continued)

Brassica nigra	*P* value	Christmas Drumhead	*P* value	AGI code	Name
1.06	0.016	0.55	0.178	At3g19170	peptidase M16 family protein / insulinase family protein
-0.21	0.243	1.50	0.003	At3g20240	mitochondrial substrate carrier family protein
-1.29	0.023	-1.06	0.012	At3g21960	receptor-like protein kinase-related
1.04	0.027	0.86	0.210	At3g24495	DNA mismatch repair protein MSH6-2 (MSH7)
-1.31	0.006	-0.98	0.011	At3g26060	peroxiredoxin Q; putative
0.28	0.051	1.61	0.031	At3g27270	expressed protein
2.05	0.079	3.54	0.002	At3g45140	lipoxygenase (LOX2)
1.65	0.073	3.07	0.001	At3g45410	lectin protein kinase family protein
1.51	0.019	1.72	0.024	At3g46970	starch phosphorylase; putative
-1.01	0.010	-0.44	0.050	At3g51760	hypothetical protein
-0.16	0.337	-1.25	0.010	At3g52590	ubiquitin extension protein 1 (UBQ1) / 60S ribosomal protein L40 (RPL40B)
-1.13	0.002	0.01	0.926	At3g53420	plasma membrane intrinsic protein 2A (PIP2A) / aquaporin PIP2.1 (PIP2.1)
-0.50	0.116	-1.41	0.001	At3g53740	60S ribosomal protein L36 (RPL36B)
-0.30	0.156	-1.36	0.017	At3g53890	40S ribosomal protein S21 (RPS21B)
-0.79	0.030	-1.13	0.030	At3g56910	expressed protein
0.23	0.098	3.37	0.007	At3g57310	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
-1.23	0.001	-1.04	0.040	At3g62030	peptidyl-prolyl cis-trans isomerase
1.40	0.038	0.03	**	At3g62810	complex 1 family protein / LVR family protein
-0.56	0.022	-1.18	0.038	At4g00810	60S acidic ribosomal protein P1 (RPP1B)
1.24	0.029	1.77	0.016	At4g01080	expressed protein
1.04	0.026	-1.41	**	At4g05010	F-box family protein
0.08	0.762	1.29	0.009	At4g09150	T-complex protein 11
1.01	0.071	1.73	0.009	At4g15415	serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B' (B'gamma)
1.19	0.040	0.25	0.527	At4g15417	ribonuclease III family protein
-1.15	0.012	-0.08	0.737	At4g15740	C2 domain-containing protein
-1.08	0.047	-0.88	0.048	At4g16980	arabinogalactan-protein family
1.12	0.019	0.81	0.143	At4g18150	hypothetical protein
1.22	0.019	-0.07	**	At4g23090	hypothetical protein
1.19	0.147	3.27	0.011	At4g23600	coronatine-responsive tyrosine aminotransferase / tyrosine transaminase
-1.23	0.020	0.00	0.995	At4g25100	superoxide dismutase [Fe]; chloroplast (SODB) / iron superoxide dismutase (FSD1)
0.31	0.291	1.69	0.035	At4g31500	cytochrome P450 83B1 (CYP83B1)
1.39	0.086	3.08	0.011	At4g32110	expressed protein
-0.08	0.207	1.02	0.035	At4g32860	expressed protein
1.21	0.072	2.64	0.045	At4g33140	expressed protein
0.84	0.010	1.59	0.016	At4g37990	mannitol dehydrogenase; putative (ELI3-2)
0.27	0.333	1.05	0.025	At4g38260	expressed protein
-1.20	0.044	-0.39	0.403	At4g38880	amidophosphoribosyltransferase; putative
-1.11	0.027	-1.08	0.045	At5g01300	phosphatidylethanolamine-binding family protein
-0.36	0.287	1.18	0.007	At5g03170	fasciclin-like arabinogalactan-protein (FLA11)
0.03	0.278	1.67	0.023	At5g07580	ethylene-responsive element-binding family protein
1.14	0.008	0.61	0.297	At5g11010	pre-mRNA cleavage complex-related
0.01	0.468	1.70	0.006	At5g16120	hydrolase; alpha/beta fold family protein
-0.45	0.005	-1.10	0.019	At5g16130	40S ribosomal protein S7 (RPS7C)
1.02	0.029	0.80	0.261	At5g25475	expressed protein
0.84	0.050	1.36	0.040	At5g26270	expressed protein
0.84	0.080	1.79	0.002	At5g30520	hypothetical protein
-1.09	0.036	-0.98	0.022	At5g38410	ribulose bisphosphate carboxylase small chain 3B
1.30	0.032	2.71	0.013	At5g38540	jacalin lectin family protein similar to myrosinase-binding protein
-0.26	0.284	-1.29	0.021	At5g39160	germin-like protein (GLP2a) (GLP5a)
-1.23	0.033	-0.58	0.011	At5g41260	protein kinase family protein
-1.04	0.009	-0.47	0.278	At5g43730	disease resistance protein (CC-NBS-LRR class); putative
1.01	0.005	0.99	0.067	At5g45660	expressed protein
-1.46	0.040	-0.80	0.037	At5g49770	leucine-rich repeat transmembrane protein kinase; putative
-0.06	0.690	1.19	0.010	At5g52980	expressed protein
0.60	0.062	1.38	0.047	At5g53420	expressed protein
-0.55	0.019	-1.20	0.013	At5g54600	50S ribosomal protein L24; chloroplast (CL24)
1.06	0.022	0.81	0.250	At5g55970	zinc finger (C3HC4-type RING finger) family protein
0.75	0.154	2.22	0.042	At5g58310	hydrolase; alpha/beta fold family protein
0.07	0.582	1.32	0.044	At5g58790	expressed protein
0.64	0.129	2.14	0.046	At5g62290	nucleotide-sensitive chloride conductance regulator (ICln) family protein
-1.34	0.042	-1.61	0.042	At5g62780	DNAJ heat shock N-terminal domain-containing protein
-0.49	0.403	-1.08	0.039	At5g64580	AAA-type ATPase family protein similar to zinc dependent protease
0.76	0.094	1.02	0.016	At5g67030	zeaxanthin epoxidase (ZEP) (ABA1)

Mean log_2 expression ratios are calculated from three biologically independent replicates.
*70-mer-oligonucleotide did not hybridize in any of the three replicates;
**70-mer oligonucleotide only hybridized in one of the three replicates.
AGI, Arabidopsis Genome Initiative.

Supplemental Table 2. Mean log_2 expression ratio after 48 h of *Brevicoryne brassicae* feeding in *Brassica nigra* and *Brassicae oleracea* cultivars Rivera and Christmas Drumhead

Brassica nigra	*P* value	Rivera	*P* value	Christmas Drumhead	*P* value	AGI code	Name
-0.24	0.743	0.07	**	-1.48	0.037	At1g01070	nodulin MtN21 family protein
-0.53	0.483	0.08	0.867	-0.71	0.042	At1g02180	ferredoxin-related
-0.51	0.342	0.17	0.348	-0.62	0.028	At1g02580	maternal embryogenesis control protein / MEDEA (MEA)
1.04	0.029	0.36	0.500	0.38	0.609	At1g02820	late embryogenesis abundant 3 family protein / LEA3 family protein
-0.02	0.904	-0.15	0.787	1.13	0.048	At1g04030	expressed protein
-0.54	0.122	-0.91	0.038	-0.52	0.132	At1g05190	ribosomal protein L6 family protein
0.85	0.040	0.82	0.017	1.07	0.026	At1g06040	zinc finger (B-box type) family protein / salt-tolerance protein (STO)
0.15	0.536	0.17	0.147	1.18	0.034	At1g06140	pentatricopeptide (PPR) repeat-containing protein
-0.07	0.859	0.68	0.263	1.00	0.047	At1g06430	FtsH protease; putative
-0.33	0.371	-0.29	0.228	-0.67	0.019	At1g06680	photosystem II oxygen-evolving complex 23 (OEC23)
-0.13	0.417	-0.31	0.331	1.11	0.017	At1g07020	expressed protein
0.02	0.893	-0.06	0.842	0.84	0.035	At1g07570	protein kinase (APK1a)
0.20	0.414	1.11	**	-0.71	0.023	At1g09250	expressed protein
-0.96	0.028	-0.68	0.065	-0.96	0.073	At1g09570	phytochrome A (PHYA)
-0.60	0.030	-0.27	0.361	-0.36	0.100	At1g09795	ATP phosphoribosyl transferase 2 (ATP-PRT2)
0.24	0.369	0.02	0.770	0.84	0.004	At1g09950	transcription factor-related
-0.19	0.422	-0.04	0.959	1.26	0.044	At1g10095	protein prenyltransferase alpha subunit-related
0.26	0.048	0.71	0.045	0.33	0.181	At1g10770	invertase/pectin methylesterase inhibitor family protein
-0.54	0.360	-0.70	0.026	-0.13	0.407	At1g10950	endomembrane protein 70; putative
-1.08	0.024	-0.21	0.610	-0.86	0.084	At1g11850	expressed protein
-1.08	0.023	-0.27	**	0.17	0.064	At1g13370	histone H3; putative
-1.77	0.516	0.33	0.302	-0.67	0.049	At1g13460	serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B
0.58	0.019	0.19	0.477	0.43	0.076	At1g14530	tobamovirus multiplication protein 3; putative / TOM3; putative (THH1)
-0.09	0.520	0.17	0.710	-0.68	0.011	At1g14870	expressed protein
-0.65	0.206	-0.30	0.571	-1.23	0.012	At1g15920	CCR4-NOT transcription complex protein; putative
1.53	0.018	0.70	0.500	-0.02	0.964	At1g16410	cytochrome P450; putative
-0.28	0.283	0.07	0.789	-0.76	0.002	At1g16900	curculin-like (mannose-binding) lectin family protein
-1.27	0.335	-0.31	0.330	-0.87	0.025	At1g18540	60S ribosomal protein L6 (RPL6A)
0.41	0.347	0.12	0.561	-0.81	0.003	At1g20060	kinesin motor protein-related
-0.75	0.483	1.12	0.024	0.66	0.152	At1g20575	dolichyl-phosphate beta-D-mannosyltransferase; putative
0.64	0.020	0.06	0.283	0.29	0.223	At1g21760	F-box family protein
1.25	0.131	0.91	0.010	1.23	0.115	At1g23310	glutamate:glyoxylate aminotransferase 1 (GGT1)
-1.57	0.090	-0.66	0.021	-1.01	0.069	At1g25275	expressed protein
1.20	0.026	0.02	**	-0.15	0.356	At1g27730	zinc finger (C2H2 type) family protein (ZAT10)
-2.93	0.042	-1.93	0.092	-0.94	0.216	At1g28330	dormancy-associated protein; putative (DRM1)
-1.22	0.235	-0.16	0.559	-0.90	0.033	At1g28400	expressed protein
0.94	0.079	0.98	0.021	0.31	0.126	At1g29700	expressed protein
-0.92	0.059	-0.72	0.010	-0.99	0.014	At1g29910	chlorophyll A-B binding protein 2
-0.24	0.220	-0.10	0.813	0.79	0.044	At1g30850	hypothetical protein
-0.40	0.061	-0.20	0.526	-0.62	0.037	At1g31850	dehydration-responsive protein; putative
-0.14	0.421	0.05	0.936	1.14	0.012	At1g32380	ribose-phosphate pyrophosphokinase 2
-0.51	0.081	-0.76	0.211	-0.69	0.014	At1g32570	hypothetical protein
-0.45	0.142	-0.39	0.419	-0.80	0.038	At1g32700	zinc-binding family protein
-0.30	0.037	-0.61	0.027	-0.09	0.697	At1g32990	ribosomal protein L11 family protein
-1.22	0.022	-0.31	0.347	0.59	0.466	At1g33030	O-methyltransferase family 2 protein
-0.98	0.289	-0.87	0.029	0.32	0.514	At1g33850	40S ribosomal protein S15; putative
-0.70	0.364	-0.83	0.031	-0.35	0.275	At1g36230	hypothetical protein
-0.03	0.950	0.08	**	1.12	0.035	At1g47350	F-box family protein-related
0.18	0.092	0.26	0.640	-0.62	0.029	At1g48870	WD-40 repeat family protein
-0.60	0.005	0.11	0.177	0.04	0.607	At1g49100	leucine-rich repeat protein kinase; putative
0.59	0.039	0.62	0.176	-0.04	0.871	At1g49500	expressed protein
0.19	0.452	0.20	0.384	-0.69	0.022	At1g50000	hypothetical protein
-0.99	0.255	-0.78	0.005	-0.87	0.062	At1g50010	tubulin alpha-2/alpha-4 chain (TUA2)
-0.10	0.712	-0.11	0.607	-0.65	0.041	At1g51650	ATP synthase epsilon chain; mitochondrial
0.83	0.018	0.66	0.068	0.03	0.927	At1g52590	expressed protein
0.29	0.324	0.38	0.003	0.83	0.001	At1g53035	expressed protein
-1.05	0.035	0.14	0.302	0.43	0.636	At1g54920	expressed protein
0.09	0.661	0.02	0.952	-0.60	0.045	At1g55150	DEAD box RNA helicase; putative (RH20)
-0.79	0.005	-0.46	0.523	0.02	0.839	At1g55260	protease inhibitor/seed storage/lipid transfer protein (LTP) family
0.22	0.569	0.87	0.025	0.66	0.025	At1g55650	high mobility group (HMG1/2) family protein
-0.21	0.489	-0.95	0.017	-0.16	0.459	At1g56070	elongation factor 2; putative / EF-2; putative
-0.36	0.658	0.02	0.743	-2.01	0.016	At1g56690	pentatricopeptide (PPR) repeat-containing protein
0.72	0.039	0.39	0.034	-0.21	0.356	At1g57990	purine permease-related
0.68	0.036	0.83	0.202	-0.23	0.048	At1g58230	WD-40 repeat family protein / beige-related
*	**	0.87	0.206	0.97	0.013	At1g58460	expressed protein
0.29	0.333	0.23	0.236	1.11	0.023	At1g61120	terpene synthase/cyclase family protein similar to S-linalool synthase
-0.18	0.527	-0.78	**	0.92	0.042	At1g61140	SNF2 domain-containing protein
0.02	**	*		2.40	0.015	At1g61820	glycosyl hydrolase family 1 protein
0.39	0.194	1.20	0.009	0.19	0.292	At1g64310	pentatricopeptide (PPR) repeat-containing protein
0.07	0.892	0.13	0.300	0.82	0.001	At1g65180	DC1 domain-containing protein
-0.42	0.418	-0.25	0.844	3.31	0.040	At1g65270	expressed protein
-0.90	0.019	-0.26	0.057	-0.56	0.035	At1g65740	F-box family protein
0.81	**	-0.07	**	0.94	0.038	At1g66145	CLE18; putative CLAVATA3/ESR-Related 18 (CLE18)
-0.60	0.410	0.36	0.517	-1.14	0.002	At1g66470	basic helix-loop-helix (bHLH) family protein
-0.32	0.547	-0.37	0.661	3.27	0.028	At1g66820	glycine-rich protein
-0.09	0.781	0.28	0.428	-0.71	0.024	At1g67420	24 kDa vacuolar protein; putative
-1.04	0.254	-0.85	0.010	-0.12	0.262	At1g67430	60S ribosomal protein L17 (RPL17B)
0.11	0.573	0.68	0.031	0.18	0.522	At1g68220	expressed protein
-0.35	0.184	-0.07	0.915	-0.80	0.006	At1g68380	expressed protein
0.88	0.001	0.89	0.081	0.40	0.328	At1g70680	caleosin-related family protein
-0.63	0.039	0.09	0.801	-0.71	0.117	At1g70710	endo-1;4-beta-glucanase (EGASE) / cellulase
-0.08	0.757	-0.11	0.577	-0.61	0.042	At1g71300	Vps52/Sac2 family protein
-0.32	0.513	-0.70	0.028	0.07	0.651	At1g72730	eukaryotic translation initiation factor 4A; putative / eIF-4A; putative
0.75	0.037	0.51	0.086	0.19	0.185	At1g73700	MATE efflux family protein
-1.99	0.367	-0.71	0.022	-0.18	0.071	At1g74270	60S ribosomal protein L35a (RPL35aC)

Supplemental Table 2. (continued)

Brassica nigra	*P* value	Rivera	*P* value	Christmas Drumhead	*P* value	AGI code	Name
-1.20	0.013	*		-0.08	0.105	At1g74830	expressed protein
-0.59	0.060	-0.19	0.270	-0.76	0.012	At1g76990	ACT domain containing protein
-0.26	0.060	0.03	0.894	-0.58	0.023	At1g77210	sugar transporter; putative
-0.62	0.021	-0.63	0.492	0.32	0.455	At1g77740	1-phosphatidylinositol-4-phosphate 5-kinase; putative
-0.63	0.330	-0.47	0.106	-0.82	0.021	At1g77810	galactosyltransferase family protein
0.06	0.334	0.06	0.866	-0.67	0.029	At1g79870	oxidoreductase family protein
0.52	0.082	0.67	0.010	0.79	0.009	At1g80245	expressed protein
-0.08	0.474	0.12	0.744	0.61	0.027	At1g80380	phosphoribulokinase/uridine kinase-related
-1.08	0.179	-0.60	0.019	-1.14	0.040	At2g01250	60S ribosomal protein L7 (RPL7B)
-0.37	0.420	0.93	0.009	0.27	0.141	At2g02730	expressed protein
0.02	0.879	0.49	0.033	0.61	0.001	At2g03840	senescence-associated family protein
0.98	0.046	0.85	0.102	0.95	0.306	At2g05620	expressed protein
0.38	0.344	-0.02	**	-0.74	0.009	At2g05752	hypothetical protein
0.27	0.204	0.32	0.417	-0.66	0.036	At2g05830	eukaryotic translation initiation factor 2B family protein
-0.15	0.582	0.08	**	0.73	0.025	At2g07280	hypothetical protein
-0.80	0.028	-0.29	0.165	-0.14	0.493	At2g07739	expressed protein
0.81	0.377	-0.10	0.621	-0.58	0.039	At2g10340	hypothetical protein
-0.85	0.472	0.82	0.050	1.19	0.095	At2g11490	hypothetical protein
1.57	**	-0.01	0.879	0.59	0.042	At2g14750	adenylylsulfate kinase 1 (AKN1)
-0.51	0.360	-0.21	0.713	-0.85	0.029	At2g15830	expressed protein
-2.53	0.101	-3.50	0.037	-1.94	0.080	At2g15890	expressed protein
0.38	0.094	0.67	0.026	0.18	0.379	At2g16070	expressed protein
-0.62	0.580	0.89	0.017	0.30	0.190	At2g17975	zinc finger (Ran-binding) family protein
0.92	0.007	0.09	0.532	0.36	0.258	At2g18030	peptide methionine sulfoxide reductase family protein
-1.00	0.208	-1.08	0.035	-0.04	0.861	At2g18050	histone H1-3 (HIS1-3)
-0.11	0.626	0.01	0.819	0.79	0.005	At2g20750	beta-expansin; putative (EXPB1)
-0.31	0.683	0.77	0.007	0.88	0.192	At2g21030	expressed protein
-0.14	0.385	0.00	0.991	1.30	0.019	At2g21140	hydroxyproline-rich glycoprotein family protein
-0.16	0.549	0.22	0.805	0.80	0.050	At2g22250	aminotransferase class I and II family protein
-0.35	0.004	-0.31	0.417	-0.79	0.010	At2g22430	homeobox-leucine zipper protein 6 (HB-6)
0.53	0.190	0.21	0.354	-0.72	0.012	At2g23130	arabinogalactan-protein (AGP17)
-0.09	0.803	0.07	0.815	-0.64	0.017	At2g23690	expressed protein
-0.13	0.324	-0.02	0.903	-0.65	0.050	At2g23820	metal-dependent phosphohydrolase HD domain-containing protein
0.61	0.047	0.07	0.557	0.56	0.146	At2g24210	myrcene/ocimene synthase (TPS10)
-0.37	0.456	*		-0.98	0.019	At2g24600	ankyrin repeat family protein
0.48	0.198	0.07	0.766	1.93	0.022	At2g25080	phospholipid hydroperoxide glutathione peroxidase / PHGPx (GPX1)
0.68	0.045	0.38	0.094	0.12	0.476	At2g25490	F-box family protein (FBL6)
-0.13	0.488	0.10	0.853	-0.72	0.004	At2g25720	expressed protein
-1.21	0.150	0.14	0.900	-1.19	0.005	At2g25990	hypothetical protein
0.10	0.477	0.07	0.783	-0.60	0.009	At2g26160	F-box family protein
0.82	0.032	0.91	0.200	0.59	0.073	At2g26800	hydroxymethylglutaryl-CoA lyase; putative
-0.39	0.666	-0.81	0.161	-1.41	0.006	At2g27450	carbon-nitrogen hydrolase family protein
-0.52	0.184	-0.46	0.369	-0.67	0.047	At2g27530	60S ribosomal protein L10A (RPL10aB)
-0.10	0.416	-0.44	0.001	-0.60	0.039	At2g27720	60S acidic ribosomal protein P2 (RPP2A)
0.30	0.178	0.50	0.380	0.73	0.022	At2g29340	short-chain dehydrogenase/reductase (SDR) family protein
1.77	0.103	1.29	0.019	1.09	0.106	At2g29650	inorganic phosphate transporter; putative
-0.14	0.762	0.70	0.000	0.20	0.403	At2g30440	chloroplast thylakoidal processing peptidase
0.47	0.355	0.68	0.455	-0.66	0.041	At2g30620	histone H1.2
0.04	0.876	0.28	0.463	-0.69	0.028	At2g30940	protein kinase family protein
-0.17	0.254	-0.06	0.827	-0.60	0.039	At2g31160	expressed protein
-1.58	0.441	-2.31	0.097	-1.49	0.048	At2g31900	myosin family protein
0.25	0.466	0.65	0.043	-0.44	0.330	At2g32270	zinc transporter (ZIP3)
-0.45	0.294	2.05	0.543	-0.92	0.002	At2g33310	auxin-responsive protein / indoleacetic acid-induced protein 13 (IAA13)
-2.69	0.025	-2.09	0.073	-1.31	0.191	At2g33830	dormancy/auxin associated family protein
-0.81	0.031	-0.18	0.715	-1.21	0.019	At2g34420	chlorophyll A-B binding protein / LHCII type I (LHB1B2)
0.08	0.919	0.03	0.916	-0.64	0.010	At2g34430	chlorophyll A-B binding protein / LHCII type I (LHB1B1)
-1.45	0.007	-0.81	0.070	0.19	0.165	At2g34680	leucine-rich repeat family protein
-0.28	0.119	-0.65	0.047	0.29	0.233	At2g34860	chaperone protein dnaJ-related
0.83	0.050	0.11	0.806	1.26	0.193	At2g35260	expressed protein
-0.71	0.301	-0.79	0.034	-0.50	0.052	At2g36530	enolase
-0.60	0.135	0.18	0.137	0.60	0.046	At2g36590	proline transporter; putative
-0.47	0.173	-0.85	0.017	-0.66	0.002	At2g36620	60S ribosomal protein L24 (RPL24A)
1.08	0.118	1.67	0.026	0.95	0.188	At2g37170	aquaporin PIP2.2 (PIP2.2)
0.77	0.342	1.46	0.010	1.20	0.081	At2g37180	aquaporin PIP2.3 (PIP2.3)
-1.34	0.034	-1.82	0.103	-0.66	0.151	At2g37220	29 kDa ribonucleoprotein; chloroplast; putative
-0.82	0.064	-0.49	0.137	-0.73	0.031	At2g37270	40S ribosomal protein S5 (RPS5A)
0.31	0.354	-0.01	0.984	-0.72	0.039	At2g37600	60S ribosomal protein L36 (RPL36A)
0.58	0.037	*		-0.21	0.287	At2g39980	transferase family protein
0.53	0.262	-0.63	0.010	-0.83	0.240	At2g40750	WRKY family transcription factor
-1.77	0.003	-0.88	0.034	-0.92	0.044	At2g41430	dehydration-induced protein (ERD15)
-0.68	0.387	-0.67	0.033	0.45	0.201	At2g41530	esterase; putative
-0.59	0.032	-0.62	0.463	0.57	0.356	At2g42220	rhodanese-like domain-containing protein
-0.19	0.362	-0.58	0.037	-0.58	0.037	At2g42870	expressed protein
0.20	0.421	0.67	0.045	0.38	0.402	At2g43100	aconitase C-terminal domain-containing protein
0.73	0.016	0.99	0.094	0.25	0.274	At2g43340	expressed protein
-0.10	0.446	*		0.99	0.018	At2g44330	zinc finger (C3HC4-type RING finger) family protein
0.06	0.865	0.21	0.622	-0.97	0.018	At2g44910	homeobox-leucine zipper protein 4 (HB-4) / HD-ZIP protein 4
0.03	0.941	0.18	0.618	-0.59	0.029	At2g45050	zinc finger (GATA type) family protein
0.50	0.045	0.66	0.045	0.16	0.529	At2g45660	MADS-box protein (AGL20)
0.87	0.019	0.55	0.312	0.49	0.184	At2g46270	G-box binding factor 3 (GBF3)
1.07	0.037	0.37	0.306	-0.16	0.455	At2g46600	calcium-binding protein; putative
-0.80	0.005	-0.53	0.044	-0.94	0.128	At2g46870	DNA-binding protein; putative
-1.19	0.231	-0.91	0.019	0.36	0.419	At2g47110	ubiquitin extension protein 6 (UBQ6)
-0.12	0.112	-0.11	0.785	0.68	0.035	At3g01410	RNase H domain-containing protein
0.64	0.028	0.88	0.199	-0.20	0.525	At3g01930	nodulin family protein

Supplemental Table 2. (continued)

Brassica nigra	*P* value	Rivera	*P* value	Christmas Drumhead	*P* value	AGI code	Name
-0.14	0.313	-1.10	0.019	-0.40	0.334	At3g02200	proteasome family protein
-0.70	0.031	0.00	**	0.28	**	At3g02450	cell division protein ftsH; putative
-0.92	0.267	-0.62	0.013	0.07	0.354	At3g02560	40S ribosomal protein S7 (RPS7B)
-0.96	0.001	-0.06	0.845	-0.03	0.918	At3g03150	expressed protein
-0.80	0.247	0.35	0.452	-0.61	0.007	At3g03660	homeobox-leucine zipper transcription factor family protein
-0.77	0.016	-0.78	0.043	-0.91	0.109	At3g06250	far-red impaired responsive protein; putative
-0.59	0.289	0.02	0.954	-0.83	0.020	At3g07565	expressed protein
-1.94	0.213	-0.87	0.045	-1.07	0.041	At3g08030	expressed protein
0.62	0.058	0.75	0.022	0.21	0.495	At3g09880	serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B
-0.65	0.032	-1.56	**	0.32	0.301	At3g10470	zinc finger (C2H2 type) family protein
-0.19	0.231	0.09	0.727	-0.65	0.036	At3g10640	SNF7 family protein
-0.12	0.073	0.44	0.158	1.16	0.007	At3g11000	expressed protein
0.01	0.993	-0.42	0.391	-1.21	0.043	At3g11200	PHD finger family protein
-0.60	0.414	0.01	0.988	-0.75	0.036	At3g11890	expressed protein
1.02	0.018	0.30	0.135	-0.38	0.361	At3g11910	ubiquitin-specific protease; putative
0.93	0.182	0.26	0.609	-0.72	0.039	At3g11940	40S ribosomal protein S5 (RPS5B)
0.18	0.321	0.38	0.237	-0.79	0.024	At3g12030	expressed protein
0.73	0.027	0.55	0.285	0.52	0.208	At3g12890	expressed protein
0.68	0.004	-0.04	0.500	-0.06	0.533	At3g12940	expressed protein
0.99	0.004	0.92	0.165	1.07	0.152	At3g13110	serine O-acetyltransferase (SAT-1)
-0.58	0.002	-0.75	0.027	-0.74	0.008	At3g15630	expressed protein
*		0.84	0.368	0.90	0.029	At3g16150	L-asparaginase; putative / L-asparagine amidohydrolase; putative
-0.52	0.258	-0.42	0.085	-0.86	0.010	At3g16370	GDSL-motif lipase/hydrolase family protein
-0.61	0.535	0.82	0.022	-0.03	0.876	At3g16590	F-box family protein
0.64	0.434	0.71	0.001	0.05	0.885	At3g18350	expressed protein
-0.33	0.263	-0.43	0.013	-0.60	0.042	At3g18820	Ras-related GTP-binding protein; putative
0.43	0.039	0.07	**	0.88	0.047	At3g20555	hypothetical protein
-0.53	0.283	-0.20	0.544	-1.02	0.045	At3g20850	proline-rich family protein
-1.32	0.025	-0.56	0.506	1.95	0.015	At3g21055	photosystem II 5 kD protein; putative
0.20	0.546	0.14	0.691	-0.59	0.006	At3g21440	myb family transcription factor
-1.97	0.218	-1.07	0.007	-0.45	0.203	At3g22200	4-aminobutyrate aminotransferase
-0.05	0.930	-0.01	0.972	-1.05	0.026	At3g22460	cysteine synthase; putative
-0.21	0.329	-0.04	0.901	-0.61	0.007	At3g22630	20S proteasome beta subunit D (PBD1) (PRGB)
0.63	0.314	1.59	0.110	0.62	0.046	At3g22840	chlorophyll A-B binding family protein
-0.20	0.565	0.00	0.989	-0.65	0.020	At3g23050	auxin-responsive protein / indoleacetic acid-induced protein 7 (IAA7)
-0.06	0.819	-0.08	0.527	0.61	0.040	At3g24060	self-incompatibility protein-related
0.76	0.045	0.15	0.490	-0.56	0.052	At3g24506	expressed protein
-0.88	0.039	-1.12	0.044	-0.91	0.202	At3g25890	AP2 domain-containing transcription factor; putative
-0.76	0.008	-0.44	0.558	0.89	0.316	At3g26640	transducin family protein / WD-40 repeat family protein
0.59	0.016	0.57	0.096	-0.10	0.736	At3g26850	expressed protein
0.93	0.182	-0.26	0.484	0.88	0.010	At3g28300	integrin-related protein 14a
-2.05	0.034	-0.93	0.314	0.46	0.589	At3g29320	glucan phosphorylase; putative
0.07	0.741	-0.06	0.721	-0.66	0.003	At3g31320	hypothetical protein
0.45	0.596	0.41	0.295	-0.59	0.044	At3g43190	sucrose synthase; putative / sucrose-UDP glucosyltransferase; putative
-0.88	0.176	-0.71	0.008	-0.35	0.032	At3g43810	calmodulin-7 (CAM7)
0.12	0.745	-0.48	0.532	1.37	0.004	At3g44080	F-box family protein
-0.74	0.009	-0.42	0.056	-0.78	0.144	At3g44950	glycine-rich protein
-0.45	0.474	-1.20	0.194	-0.87	0.021	At3g45050	expressed protein
-0.70	0.430	-1.18	0.007	-0.64	0.086	At3g45320	hypothetical protein
-0.52	0.394	-0.68	0.050	-0.55	0.044	At3g47370	40S ribosomal protein S20 (RPS20B)
-0.44	0.006	-1.09	0.047	-0.84	0.124	At3g47650	bundle-sheath defective protein 2 family / bsd2 family
-1.47	0.210	-1.63	0.028	0.69	0.391	At3g48960	60S ribosomal protein L13 (RPL13C)
-1.17	0.619	0.76	0.026	0.37	0.021	At3g49210	expressed protein
-0.39	0.048	-0.10	0.666	0.91	0.029	At3g51230	hypothetical protein
0.92	0.166	0.85	0.006	1.05	0.039	At3g51510	expressed protein
-1.68	0.220	-0.92	0.028	0.49	0.208	At3g52590	ubiquitin extension protein 1 (UBQ1)
-0.63	0.011	-0.58	0.041	-0.57	0.170	At3g53020	60S ribosomal protein L24 (RPL24B)
-1.21	0.000	-1.68	0.033	-1.26	0.028	At3g53460	29 kDa ribonucleoprotein; chloroplast / RNA-binding protein cp 29
-1.47	0.255	-0.95	0.031	0.46	0.074	At3g53740	60S ribosomal protein L36 (RPL36B)
-0.92	0.022	-0.40	0.472	0.05	0.466	At3g53990	universal stress protein (USP) family protein
-0.93	0.071	-0.64	0.008	-0.68	0.025	At3g54400	aspartyl protease family protein
-0.94	0.001	-0.59	0.101	1.15	0.181	At3g55280	60S ribosomal protein L23A (RPL23aB)
-0.61	0.041	-0.24	0.755	1.78	0.140	At3g55740	proline transporter 2 (ProT2)
0.08	0.541	0.50	0.338	-0.60	0.002	At3g55940	phosphoinositide-specific phospholipase C; putative
-0.69	0.002	-0.18	0.702	-0.35	0.173	At3g56910	expressed protein
1.05	0.003	1.09	0.060	0.44	0.043	At3g56940	dicarboxylate diiron protein; putative (Crd1)
1.13	0.036	1.01	0.099	0.62	0.082	At3g57120	protein kinase family protein
0.13	0.709	0.00	**	0.91	0.026	At3g57620	glyoxal oxidase-related
-0.79	0.004	-1.01	**	-0.03	**	At3g58750	citrate synthase; glyoxysomal; putative
0.58	0.009	*		0.15	0.100	At3g59845	NADP-dependent oxidoreductase; putative
0.85	**	-0.10	**	-0.91	0.033	At3g61270	expressed protein
-0.58	0.214	-0.64	0.048	-0.71	0.033	At3g62550	universal stress protein (USP) family protein
-0.11	0.742	-0.31	0.438	-0.80	0.020	At3g63260	protein kinase; putative (MRK1)
-0.73	0.044	-0.70	0.045	-0.48	0.069	At4g00810	60S acidic ribosomal protein P1 (RPP1B)
-0.45	0.413	-0.54	0.446	0.60	0.041	At4g01610	cathepsin B-like cysteine protease; putative
-0.56	**	*		0.93	0.011	At4g04220	disease resistance family protein
0.63	0.015	0.57	0.181	0.15	0.089	At4g06746	AP2 domain-containing transcription factor family protein
-0.19	0.269	1.05	0.050	0.37	0.294	At4g09150	T-complex protein 11
0.73	0.044	0.28	0.081	0.12	0.160	At4g10120	sucrose-phosphate synthase; putative
0.65	0.009	0.34	0.109	0.02	0.747	At4g10170	synaptobrevin-related family protein
0.77	0.013	0.12	0.340	0.15	0.164	At4g10570	ubiquitin carboxyl-terminal hydrolase family protein
1.99	0.022	0.71	0.008	0.73	0.036	At4g12510	protease inhibitor/seed storage/lipid transfer protein (LTP) family
0.58	0.216	0.80	0.033	0.16	0.614	At4g13770	cytochrome P450 family protein
-1.21	0.040	-1.18	0.045	-1.04	0.253	At4g13850	glycine-rich RNA-binding protein (GRP2)
-1.06	0.043	-1.10	0.087	-1.24	0.035	At4g14270	expressed protein

Supplemental Table 2. (continued)

Brassica nigra	*P* value	Rivera	*P* value	Christmas Drumhead	*P* value	AGI code	Name
-0.24	0.382	-0.08	0.708	-0.75	0.004	At4g14455	Bet1-like SNARE 1-2 / Bet1 / Sft1-like SNARE 14b / BS14b (BET12)
0.73	0.044	0.12	0.546	-0.29	0.264	At4g14805	protease inhibitor/seed storage/lipid transfer protein (LTP)-related
-1.06	0.197	-0.69	0.016	-0.85	0.009	At4g14960	tubulin alpha-6 chain (TUA6)
0.86	0.023	*		0.39	0.439	At4g15350	cytochrome P450 family protein
-0.41	0.297	-0.19	0.492	-0.85	0.044	At4g15460	glycine-rich protein
-0.90	0.040	-0.24	0.496	0.21	0.216	At4g15930	dynein light chain; putative
0.95	0.047	0.20	0.359	0.56	0.075	At4g17050	expressed protein
-0.66	0.029	-0.28	0.436	0.43	0.311	At4g17170	Rab2-like GTP-binding protein (RAB2)
0.22	0.225	-0.42	0.380	0.89	0.008	At4g17190	farnesyl pyrophosphate synthetase 2 (FPS2)
0.27	0.554	-0.12	0.800	-1.07	0.007	At4g17615	calcineurin B-like protein 1 (CBL1)
0.38	0.339	0.65	0.034	0.22	0.595	At4g17840	expressed protein
-0.96	0.228	-0.90	0.024	-0.65	0.032	At4g18100	60S ribosomal protein L32 (RPL32A)
0.82	0.069	1.13	0.043	0.12	0.761	At4g18335	hypothetical protein
0.32	0.443	-0.07	0.691	0.66	0.022	At4g18780	cellulose synthase; catalytic subunit (IRX1)
-0.58	0.374	0.20	0.557	-0.60	0.005	At4g19020	chromomethylase 2 (CMT2)
0.22	0.111	0.26	0.161	0.76	0.019	At4g19420	pectinacetylesterase family protein
0.00	0.997	0.00	0.991	-0.66	0.041	At4g19670	zinc finger (C3HC4-type RING finger) family protein
0.35	0.462	0.04	0.592	-0.66	0.044	At4g20250	hypothetical protein
-0.69	0.002	-0.45	0.574	0.33	0.213	At4g21450	vesicle-associated membrane family protein / VAMP family protein
-0.80	0.012	-0.87	0.512	0.41	0.431	At4g22380	ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
0.44	0.080	0.25	0.451	0.87	0.024	At4g22890	expressed protein
-1.11	0.158	-1.50	0.032	-0.85	0.018	At4g25000	alpha-amylase; putative / 1;4-alpha-D-glucan glucanohydrolase; putative
-0.31	0.137	-1.02	0.045	0.84	0.012	At4g25080	magnesium-protoporphyrin O-methyltransferase; putative
0.50	0.638	0.75	0.014	0.66	0.034	At4g25100	superoxide dismutase [Fe]; chloroplast (SODB)
-0.04	0.906	0.22	0.424	-0.67	0.024	At4g25550	expressed protein
0.73	0.049	0.06	0.445	0.35	0.040	At4g26710	ATP synthase subunit H family protein contains
1.15	0.023	1.10	0.084	1.26	0.066	At4g26850	expressed protein
0.08	0.649	0.69	0.012	-0.06	0.687	At4g26870	aspartyl-tRNA synthetase; putative / aspartate--tRNA ligase; putative
0.70	0.020	0.16	0.479	-0.32	0.130	At4g26940	galactosyltransferase family protein
-1.47	**	-0.86	0.048	-0.09	0.632	At4g27090	60S ribosomal protein L14 (RPL14B)
*		0.59	0.039	0.11	0.243	At4g28680	tyrosine decarboxylase; putative
-0.27	0.721	0.13	0.779	-0.66	0.041	At4g29650	cytidine deaminase 4 (CDA4) (desH) / cytidine aminohydrolase
-0.60	0.074	0.06	0.867	-0.66	0.021	At4g29660	expressed protein
-0.20	0.051	0.03	0.921	-0.59	0.028	At4g30370	zinc finger (C3HC4-type RING finger) family protein
-2.07	0.031	-2.37	0.152	0.06	0.968	At4g30650	hydrophobic protein; putative
0.59	0.025	-0.08	**	0.08	0.083	At4g31390	ABC1 family protein
-1.08	0.197	-0.82	0.011	-0.45	0.041	At4g31985	60S ribosomal protein L39 (RPL39C)
-1.00	0.041	-0.20	0.473	-0.17	0.500	At4g32470	ubiquinol-cytochrome C reductase complex 14 kDa protein; putative
-0.10	0.549	1.06	0.028	0.83	0.023	At4g32860	expressed protein
0.59	0.029	-0.01	0.860	0.04	0.841	At4g33500	protein phosphatase 2C-related / PP2C-related
-0.90	0.223	-0.47	0.345	-0.71	0.049	At4g33640	expressed protein
0.84	0.007	0.63	0.407	0.25	**	At4g34240	aldehyde dehydrogenase (ALDH3)
-0.76	0.095	-0.85	0.009	-0.53	0.054	At4g34670	40S ribosomal protein S3A (RPS3aB)
2.08	0.024	2.06	0.026	1.03	0.156	At4g35090	catalase 2
1.07	0.020	*		0.09	0.448	At4g36050	endonuclease/exonuclease/phosphatase family protein
0.22	0.122	0.13	0.657	-0.67	0.049	At4g36060	basic helix-loop-helix (bHLH) family protein
-0.10	0.335	0.01	0.922	0.79	0.023	At4g36105	expressed protein
-0.22	0.268	-0.31	0.288	-0.75	0.047	At4g36130	60S ribosomal protein L8 (RPL8C)
0.62	0.009	0.20	0.237	0.10	0.232	At4g38810	calcium-binding EF hand family protein
-0.04	0.896	0.46	0.436	-0.69	0.010	At4g39540	shikimate kinase family protein
0.26	0.309	1.21	0.030	0.71	0.127	At5g01410	stress-responsive protein; putative
-0.28	0.375	-0.09	0.827	0.58	0.043	At5g01580	gamma interferon responsive lysosomal thiol reductase family protein
-0.81	0.241	-1.17	0.030	-0.51	0.340	At5g02160	expressed protein
0.39	0.189	0.93	0.043	0.17	0.773	At5g02490	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)
-0.60	0.032	-0.58	0.172	-0.50	0.106	At5g02550	expressed protein
-0.13	0.599	-0.08	0.724	-1.02	0.011	At5g02560	histone H2A; putative
0.78	0.037	0.14	0.353	-0.14	0.599	At5g03490	UDP-glucoronosyl/UDP-glucosyl transferase family protein
-0.06	0.784	0.42	0.413	-0.82	0.046	At5g04820	ovate family protein 62%
0.40	0.222	0.07	0.423	0.68	0.009	At5g06920	hypothetical protein
0.01	0.979	0.90	0.114	1.00	0.034	At5g08335	isoprenylcysteine carboxyl methyltransferase family protein
-0.43	0.184	-0.30	0.168	-0.66	0.047	At5g09500	40S ribosomal protein S15 (RPS15C)
-0.96	0.218	-0.79	0.028	0.62	0.423	At5g10390	histone H3
-0.30	0.400	-0.17	0.569	-0.98	0.017	At5g10570	basic helix-loop-helix (bHLH) family protein
-0.88	0.038	-0.99	0.149	0.74	0.198	At5g10980	histone H3
-0.51	0.103	-0.18	0.403	-0.89	0.043	At5g12280	hypothetical protein
-0.28	**	-0.21	**	0.64	0.003	At5g12330	lateral root primordium 1 (LRP1)
-0.01	0.960	0.49	0.260	0.73	0.016	At5g13630	magnesium-chelatase subunit chlH; chloroplast; putative
0.73	0.019	0.61	0.174	-0.58	0.226	At5g14570	transporter; putative
-0.86	0.112	-0.45	0.015	-0.72	0.034	At5g15200	40S ribosomal protein S9 (RPS9B)
1.90	0.007	0.03	0.876	0.11	0.182	At5g15860	expressed protein
-1.17	0.146	-0.60	0.345	-1.30	0.006	At5g15970	stress-responsive protein (KIN2)
-1.15	0.031	-1.06	0.014	-0.73	0.074	At5g16130	40S ribosomal protein S7 (RPS7C)
0.87	0.185	0.91	0.048	0.14	0.625	At5g17170	rubredoxin family protein
0.59	0.038	0.19	**	0.08	0.355	At5g17400	ADP; ATP carrier protein; mitochondrial; putative
0.81	0.039	-1.06	0.084	0.48	0.234	At5g18020	auxin-responsive protein; putative
-0.32	0.207	0.33	0.188	1.23	0.045	At5g19440	cinnamyl-alcohol dehydrogenase; putative (CAD)
-1.34	0.389	-0.94	0.067	-0.72	0.045	At5g20290	40S ribosomal protein S8 (RPS8A)
-2.75	0.040	-2.12	0.154	-1.40	0.273	At5g20630	germin-like protein (GER3)
-0.96	0.274	1.10	0.024	0.61	0.282	At5g20935	expressed protein
-0.54	0.175	-0.55	0.136	-0.79	0.046	At5g21920	YGGT family protein
-0.93	0.261	-0.65	0.001	-0.07	0.005	At5g22440	60S ribosomal protein L10A (RPL10aC)
-0.08	0.833	0.68	0.086	0.72	0.025	At5g23080	SWAP (Suppressor-of-White-APricot)/surp domain-containing protein
-1.01	0.335	-1.60	0.001	-0.83	0.199	At5g23240	DNAJ heat shock N-terminal domain-containing protein
1.21	0.191	1.20	0.096	1.05	0.001	At5g24150	squalene monooxygenase 1;1 / squalene epoxidase 1;1 (SQP1;1)
-0.45	0.547	0.01	0.933	0.96	0.039	At5g24390	RabGAP/TBC domain-containing protein

Supplemental Table 2. (continued)

Brassica nigra	***P* value**	**Rivera**	***P* value**	**Christmas Drumhead**	***P* value**	**AGI code**	**Name**
0.60	0.020	0.45	0.203	0.01	0.940	At5g24460	expressed protein
0.69	0.030	0.53	0.523	-0.45	0.138	At5g25090	plastocyanin-like domain-containing protein
-1.27	0.187	-1.38	0.012	-1.15	0.123	At5g25460	expressed protein
-0.43	0.398	-0.36	0.252	1.36	0.036	At5g25980	glycosyl hydrolase family 1 protein
0.48	0.176	0.46	0.427	0.82	0.016	At5g26280	meprin and TRAF homology domain-containing protein
1.00	0.009	0.62	0.514	0.19	0.461	At5g27000	kinesin motor protein-related
-0.26	0.658	-0.16	0.718	-0.73	0.023	At5g37520	hypothetical protein
0.67	0.020	0.57	0.329	-0.07	0.766	At5g37680	ADP-ribosylation factor; putative
0.20	0.556	0.00	**	-0.85	0.041	At5g37820	major intrinsic family protein / MIP family protein
0.55	0.066	0.76	0.043	0.37	0.199	At5g38360	esterase/lipase/thioesterase family protein
0.73	0.022	0.33	0.053	0.51	0.134	At5g40030	protein kinase; putative
0.13	0.591	0.08	0.802	1.02	0.022	At5g40940	hypothetical protein
0.09	0.762	0.76	0.031	-0.40	0.197	At5g41700	ubiquitin-conjugating enzyme 8 (UBC8)
0.09	0.494	0.45	0.230	-0.74	0.037	At5g42570	expressed protein
1.02	0.010	0.61	0.197	0.00	0.976	At5g43260	chaperone protein dnaJ-related
-0.72	0.118	-0.80	0.030	-0.21	0.482	At5g43830	expressed protein
-1.10	0.022	-0.47	0.286	0.10	0.087	At5g44020	acid phosphatase class B family protein
0.03	0.804	-0.37	0.650	0.85	0.012	At5g45130	Ras-related protein (RHA1) / small GTP-binding protein
-0.32	0.213	-0.75	0.033	-0.92	0.040	At5g45280	pectinacetylesterase; putative
0.83	0.046	0.22	0.265	-0.05	0.889	At5g45410	expressed protein
-1.26	0.010	-0.14	0.522	-0.24	0.540	At5g45650	subtilase family protein
0.74	0.015	0.74	0.087	1.68	0.195	At5g46880	homeobox-leucine zipper family protein
0.17	0.556	0.72	0.030	0.07	0.069	At5g48540	33 kDa secretory protein-related
-0.22	0.129	-0.07	0.669	0.73	0.046	At5g49310	importin alpha-1 subunit; putative
0.87	0.003	0.20	0.618	0.19	0.400	At5g49740	ferric reductase-like transmembrane component family protein
0.11	0.650	-0.02	0.929	-0.74	0.022	At5g49840	ATP-dependent Clp protease ATP-binding subunit ClpX; putative
0.82	0.023	0.36	0.156	0.21	0.000	At5g51970	sorbitol dehydrogenase; putative / L-iditol 2-dehydrogenase; putative
0.11	0.458	0.79	0.029	0.16	0.355	At5g52370	expressed protein
-0.72	0.055	-0.02	0.975	-0.73	0.012	At5g52840	NADH-ubiquinone oxidoreductase-related
-0.35	0.332	0.99	0.026	0.57	0.101	At5g52980	expressed protein
0.22	0.244	0.24	0.465	0.60	0.033	At5g53230	hypothetical protein
-0.12	0.470	0.08	0.826	1.72	0.002	At5g54170	expressed protein
-1.34	0.012	-0.91	0.062	-0.71	0.322	At5g54190	protochlorophyllide reductase A; chloroplast
-0.01	0.970	0.02	0.915	0.69	0.043	At5g54200	WD-40 repeat family protein
0.21	0.275	0.02	**	0.87	0.015	At5g55020	myb family transcription factor (MYB120)
0.31	0.299	-0.08	0.892	0.63	0.050	At5g55460	protease inhibitor/seed storage/lipid transfer protein (LTP) family
-0.02	0.904	-0.29	0.415	1.10	0.004	At5g55560	protein kinase family protein
1.56	0.048	0.90	0.067	0.87	0.015	At5g55620	expressed protein
-0.29	0.000	-0.32	0.694	0.73	0.016	At5g55710	expressed protein
-0.79	**	0.04	0.612	1.15	0.011	At5g55880	hypothetical protein
0.10	0.729	0.51	0.290	0.71	0.014	At5g55980	serine-rich protein-related
0.60	0.009	-0.11	0.162	0.35	0.255	At5g57170	macrophage migration inhibitory factor family protein
0.70	0.178	0.77	0.048	-0.01	0.921	At5g57345	expressed protein
-1.53	0.001	*		-0.20	**	At5g57760	expressed protein
0.11	0.701	0.18	0.716	-0.67	0.032	At5g57790	expressed protein
0.30	0.426	-0.16	0.759	-0.61	0.001	At5g59030	copper transporter 1 (COPT1)
-0.77	0.384	-0.08	0.904	-1.16	0.039	At5g59950	RNA and export factor-binding protein; putative
-0.96	0.124	-0.88	0.021	-0.24	0.056	At5g60390	elongation factor 1-alpha / EF-1-alpha
0.22	0.509	0.35	0.522	-0.71	0.002	At5g62300	40S ribosomal protein S20 (RPS20C)
0.15	0.292	0.14	0.749	-0.65	0.019	At5g62575	expressed protein
-1.28	0.020	-1.14	0.178	-1.17	0.136	At5g62670	ATPase; plasma membrane-type; putative / proton pump; putative
-0.17	0.367	*		0.72	0.015	At5g64630	transducin family protein / WD-40 repeat family protein
-0.66	0.037	-0.68	0.028	-0.25	0.262	At5g65430	14-3-3 protein GF14 kappa (GRF8)
1.12	0.030	1.89	0.045	1.71	0.021	At5g65730	xyloglucan:xyloglucosyl transferase; putative
0.81	0.018	0.38	0.271	-0.18	0.217	At5g65840	expressed protein
-0.08	0.668	0.11	0.838	-1.18	0.028	At5g66320	zinc finger (GATA type) family protein
1.64	0.050	-0.14	0.537	1.05	0.527	At5g66400	dehydrin (RAB18)
-0.04	0.865	0.02	0.796	0.68	0.026	At5g66600	expressed protein
1.10	0.031	0.42	0.589	0.70	0.310	At5g67030	zeaxanthin epoxidase (ZEP) (ABA1)
-0.97	0.018	-0.92	0.035	-0.64	0.164	At5g67250	SKP1 interacting partner 2 (SKIP2)
-0.63	0.289	-0.08	0.890	0.69	0.031	At5g67590	NADH-ubiquinone oxidoreductase-related

Mean log_2 expression ratios are calculated from three biologically independent replicates.
*70-mer-oligonucleotide did not hybridize in any of the three replicates;
**70-mer oligonucleotide only hybridized in one of the three replicates.
AGI, Arabidopsis Genome Initiative.

This project was funded by the Dutch Ministry of Agriculture, Nature, and Food quality.

Cover design and thesis layout by Jovanka van Otterloo and Colette Broekgaarden.
Cover pictures by Colette Broekgaarden and Tibor Bukovinszky.

Printed at Wöhrmann Print Service in Zutphen, the Netherlands.