Ecogenomics of plant resistance to biotic and abiotic stresses

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To my family,
Para mi familia,

Contents

Chapter 1

General Introduction

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Introduction

In nature, plants are exposed to diverse stresses (Buchanan *et al.*, 2000). Stresses can be classified into abiotic stresses such as drought, flooding, heat, cold, nutrient deficiency, and ozone stress (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Roy *et al.*, 2011; Fahad *et al.*, 2015; Mickelbart *et al.*, 2015) or biotic stresses, when imposed by other living organisms such as bacteria, fungi, viruses, insects, or other plants (Jones & Dangl, 2006; Howe & Jander, 2008; Dicke & Baldwin, 2010; Mithofer & Boland, 2012; Pieterse *et al.*, 2012; Dangl *et al.*, 2013; Pierik *et al.*, 2013). Under natural conditions, these stresses do not occur in isolation but commonly occur simultaneously (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014). To survive under suboptimal conditions, plants have developed sophisticated mechanisms for resisting or tolerating stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Mickelbart *et al.*, 2015).

This chapter discusses how plants respond to environmental perturbations either by defending themselves against pathogens and herbivores, or acclimating to abiotic conditions. Furthermore, I compiled evidence for how plant defences are altered when multiple stresses co-occur. Finally, I discuss current technological advancements that may accelerate the discovery of genes involved in resistance or susceptibility to single abiotic or biotic stresses as well as their combinations.

Plant defences against pathogens

Plant pathogens are divided according to their life styles in biotrophs, necrotrophs and hemi-biotrophs (Glazebrook, 2005; Jones & Dangl, 2006; Pieterse *et al.*, 2012). Biotrophs use living host tissue as source of nutrients, whereas necrotrophs first kill the host tissue and then use the dead tissue as source of nutrients (Glazebrook, 2005). Hemi-biotrophs make use of living or dead host tissue depending on their life stage (Koeck *et al.*, 2011). Pathogens of different classes display also diverse life strategies. For instance, bacteria grow in intercellular spaces (the apoplast) after entering through stomata, hydathodes or wounds. Nematodes feed by inserting a stylet directly into a plant cell. Fungi are able to penetrate plant epidermal cells, or extend hyphae between and through plant cells. Some fungi form feeding structures called haustoria that are in direct contact with the host cell plasma membrane (Dangl & Jones, 2001; Jones & Dangl, 2006; Dangl *et al.*, 2013). Plant immunity against viruses depends on RNA-silencing mechanisms that have not been demonstrated in response to bacteria, fungi and nematodes; for an overview of plant defences against

viruses see Mandadi & Scholthof (2013). Plant immune responses differ depending on the type of attacker but I will focus on the common aspects rather than differences (Figure 1). Once a pathogen is in the interior of the plant, the first barrier that it encounters is the plant cell wall which is a rigid 3D network of polysaccharides (mostly cellulose), lignin and proteins that surrounds every plant cell (Gilbert *et al.*, 2013).

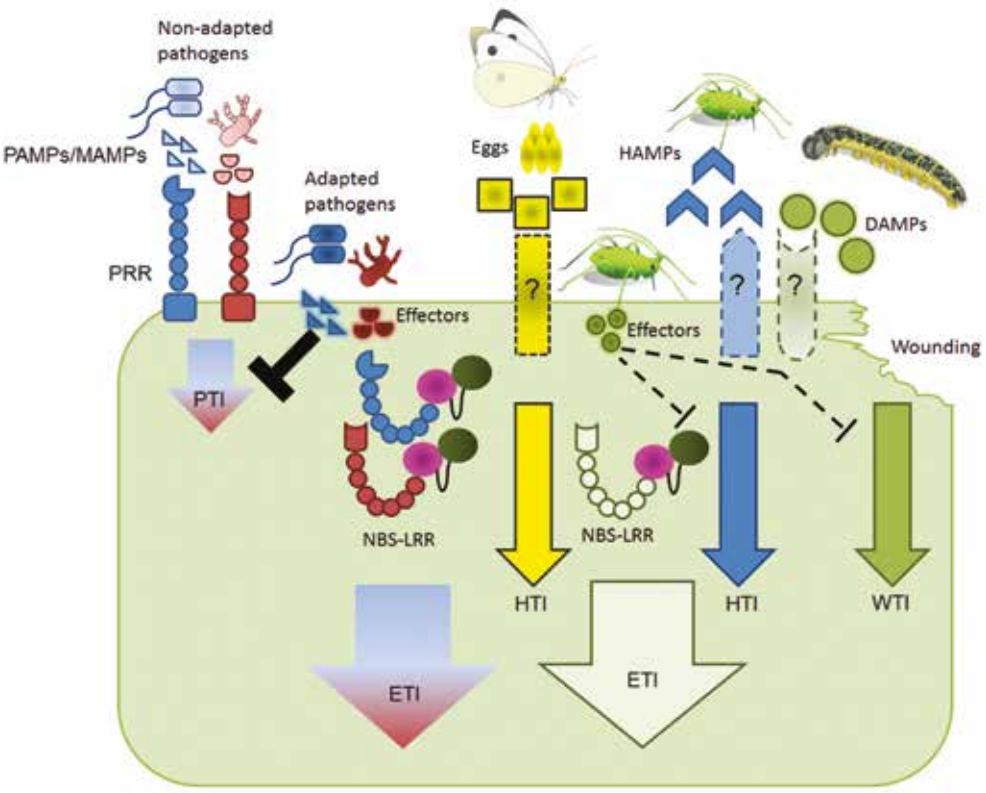

Figure 1. Plant immune system. Modified from Erb *et al.* (2012). In the diagram a plant cell is represented. Plant cells are able to identify cues that alert them from danger such as PAMPs, DAMPs, HAMPs or wounding. Recognition is perceived at plasma membrane by receptors (PRRs) and intracellularly by the products of R genes (NBS-LRR). Upon danger perception plants elicit defence responses that are attacker specific. PRRs = Pattern Recognition Receptors, PAMPs = Pathogen Associated Molecular Patterns, DAMPs = Damage Associated Molecular Patterns, HAMPs = Herbivore Associated Molecular Patterns, PTI= PAMP-Triggered Immunity, ETI = Effector-Triggered Immunity, HTI = Herbivore-Triggered Immunity and WIR = Wound Induced Resistance, NBS-LRR = Nucleotide-Binding Site – Leucine Rich Repeat. Broken lines indicate uncharacterized elements.

Some bacteria, fungi and insects are equipped with enzymes that degrade cell wall polysaccharides such as polygalacturonases (Zhang *et al.*, 2014). Plants on the other hand are equipped with extracellular surface receptors, pattern recognition receptors (PRRs), that recognize proteinaceous molecules from the pathogen, pathogen-

associated molecular patterns (PAMPs) or breakdown products from the cell-wall, damage-associated molecular patterns (DAMPs). Upon pathogen perception, plants activate the first line of defence, PAMP-Triggered Immunity (PTI) (Jones & Dangl, 2006; Dodds & Rathjen, 2010; Dangl *et al.*, 2013). PTI renders effective defence against non-adapted attackers, so-called non-host resistance (Dodds & Rathjen, 2010). Adapted attackers of diverse classes interfere with PTI by avoiding recognition at the plasma membrane or by delivering effector molecules (secreted proteins) into the plant cell. Plants that are resistant to adapted pathogens are able to recognize pathogen effectors through intracellular receptors which activate the second line of defence, Effector-Triggered Immunity (ETI) (Dangl & Jones, 2001; Jones & Dangl, 2006; Dodds & Rathjen, 2010; Dangl *et al.*, 2013). Effectors and their intracellular receptors are thought to be engaged in a co-evolutionary arms race (Ellis *et al.*, 2000).

Plant defences against insects herbivores

Plants have been interacting with insects for over 350 million years (Gatehouse, 2002; Schoonhoven *et al.*, 2005). In fact, a quarter of all described eukaryotic species are insects that feed on plants (Futuyma & Agrawal, 2009). Insects exert selection pressure on plants and as a result plants have developed defensive traits against insects; many traits evolved from defensive strategies against pathogens (Gatehouse, 2002; Mithofer & Boland, 2012). Plant traits that limit the damage imposed by insects can be divided in resistance, traits that limit the damage by the attacker, and tolerance, traits that help plants to compensate damage (e.g. increase growth, faster reproduction, repair of damage) (Strauss & Agrawal, 1999; Stout, 2013). Resistance and tolerance are based on distinct genetic mechanisms (Strauss & Agrawal, 1999; Carmona *et al.*, 2011; Karinho-Betancourt & Nunez-Farfan, 2015). Resistance can be further divided in constitutive (always present) or induced (expressed upon insect attack) resistance and can be direct (acting directly against the attacker) or indirect (promoting the effectiveness of a natural enemy of the attacker) (Mithofer & Boland, 2012; Stout, 2013). At the plant-insect interface, plants are able to perceive herbivores through damage-associated molecular patterns (DAMPs) such as cell wall fragments, or herbivore-associated molecular patterns (HAMPs) (Figure 1). HAMPs can be related to the mode of insect feeding (e.g. piercing-sucking insects such as aphids cause very little tissue damage compared to chewing insects) and molecules from insect origin or plant molecules modified by the insect (Felton *et al.*, 2014). Analogous to plant immunity against pathogens, upon perception of insect damage through DAMPs and HAMPs the plant is able to mount a wound-induced resistance (WIR) or herbivore-induced resistance (HIR) (Erb *et al.*, 2012). Insects are also known to introduce effectors that interfere with induced plant defences (Hogenhout & Bos,

2011; Felton *et al.*, 2014). Analogous to adapted and non-adapted pathogens, insects can be classified in specialist and generalist insects. In general, specialists are less affected by their host-plant defences than generalist insects (Barrett & Heil, 2012; Mithofer & Boland, 2012).

Plant tolerance to abiotic stresses

Beside stresses imposed by other living organisms, plants also experience stress imposed by abiotic conditions such as extreme temperatures, water availability and ion toxicity (Roy *et al.*, 2011; Mickelbart *et al.*, 2015). Abiotic stress is defined as sub-optimal climatic or edaphic conditions that affect the physiological homeostasis of the plant resulting in impairment of growth and fitness (Mickelbart *et al.*, 2015). Abiotic stresses can account for up to 80% of yield losses in major crops (Buchanan *et al.*, 2000). Model-based predictions indicate that climate change will increase severity of abiotic stresses (Zhao & Running, 2010; Sheffield *et al.*, 2012; Wheeler & von Braun, 2013). Abiotic stresses can be classified as transient stress (occurring in episodes) or chronic stress (always present) (Mickelbart *et al.*, 2015).

Phytohormones as mediators of induced plant responses to environmental stimuli

Induced plant responses to biotic and abiotic stresses can be divided in three conceptual phases: perception, signalling and response (Heidel-Fischer *et al.*, 2014). After stress perception, a plant needs to reprogram its phenotype in order to mount an appropriate response that allows survival under stress conditions (Erb *et al.*, 2012). Plant hormones have emerged as major players in controlling the signal-transduction pathways that regulate stress responses (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Erb *et al.*, 2012; Pieterse *et al.*, 2012). Jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) have emerged as important signalling molecules in plant defences against pathogens and insects, whereas abscisic acid (ABA) is important for acclimation to abiotic stresses (Shinozaki & Yamaguchi-Shinozaki, 2007; Verhage *et al.*, 2010; Erb *et al.*, 2012; Pieterse *et al.*, 2012). JA activates signalling pathways that mediate responses against chewing herbivores, thrips and necrotrophic fungi (Reymond *et al.*, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2005). For example, several mutants impaired in JA signalling have been shown to be more susceptible to chewing herbivores (Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Vos *et al.*, 2013a), a necrotrophic fungus (De Vos *et al.*, 2005) and thrips (Abe *et al.*, 2009). Reymond *et al.* (2004) estimated that *A. thaliana*

transcriptional responses to caterpillars of *Pieris rapae*, were mediated for 67- 84% by JA. Although the transcriptional responses to chewing herbivores, necrotrophs, and thrips are mediated by the JA-signalling pathway, the transcriptional responses are attacker- specific (De Vos *et al.*, 2005). Two branches of the JA-signalling pathway have been identified, the ERF branch that renders effective defence against necrotrophic pathogens and the MYC branch that provides effective defence against herbivorous caterpillars (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). The ERF branch is activated by JA/ET through the transcription factors ERF1 and ORA59 and results in the expression of genes such as *PDF1.2* (Lorenzo *et al.*, 2003; Pre *et al.*, 2008; Verhage *et al.*, 2010). The MYC branch is activated by JA/ABA through the transcription factor MYC2 and results in the expression of genes such as *VSP2* (Verhage *et al.*, 2011; Vos *et al.*, 2013b). The ERF and MYC2 transcription factors antagonistically regulate the two branches of the JA signalling pathway (Verhage *et al.*, 2011). The defence responses elicited through the JA signalling pathway can be direct and involve secondary metabolites of different chemical structures such as terpenoids, alkaloids, glucosinolates, anthocyanins, phytoalexins, flavonoids, and phenylpropanoids (Howe & Jander, 2008; De Geyter *et al.*, 2012) or indirect such as terpenes and methyl salicylate (Snoeren *et al.*, 2010). The SA-signalling pathway activates effective defences against biotrophic and hemi-biotrophic pathogens, nematodes and phloem feeders such as aphids (Glazebrook, 2005; Vlot *et al.*, 2009; Vos *et al.*, 2013a). Activation of the SA signalling pathway results in the expression of defence-related genes such as *PR1* and genes encoding WRKY transcription factors (van Verk *et al.*, 2011; Vos *et al.*, 2013a). The ABA signalling pathway is required for effective acclimation to several abiotic stresses imposed by drought, osmotic stress, cold and heat (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Fahad *et al.*, 2015). For instance, Huang *et al.* (2008) estimated that 67% of the transcriptional response to drought is ABA-dependent. Activation of the ABA signalling pathway results in the expression of genes related to tolerance (e.g. late embryogenesis abundant proteins (LEA), anti-freeze proteins, osmolyte biosynthesis, proline and sugar transport) or genes related to regulatory mechanisms such as transcription factors (e.g. *RD26*, *RD29B*, *RD20A*), protein phosphatases (e.g. *ABI1*, *ABI5*) (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007). Phytohormonal signalling pathways are not independent from each other, rather they interact through a phenomenon coined “crosstalk”, that has been hypothesized to allow plants to respond in a fast and cost-effective manner to stresses (Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Vos *et al.*, 2013a). Interactions between phytohormonal signalling pathways can be antagonistic or synergistic. For example, SA- and JA-mediated defences are known to exert negative effects on each other (Verhage *et al.*, 2010; Sendon *et al.*, 2011; Van der Does *et al.*, 2013; Caarls *et al.*,

2015), whereas ET and ABA have emerged as positive modulators of JA-mediated responses (Van der Ent *et al.*, 2008; Verhage *et al.*, 2010; Verhage *et al.*, 2011; Vos *et al.*, 2013b). In addition, antagonistic interactions between SA and ABA have also been reported (Mauch-Mani & Mauch, 2005; Vlot *et al.*, 2009; Pieterse *et al.*, 2012). Other hormones such as cytokinins, brassinosteroids, strigolactones, gibberellins and auxin have been implicated in modulating the equilibrium of the crosstalk described above (Vlot *et al.*, 2009; Erb *et al.*, 2012; Pieterse *et al.*, 2012; Song *et al.*, 2014).

Plant responses to multiple stresses

In natural and agricultural ecosystems plants experience stresses that commonly occur simultaneously, rather than sequentially or in isolation. Therefore, a central question is how plants mount an effective defence response to multiple stresses. Antagonistic or synergistic phythormonal signalling pathways can be activated when plants are challenged by attackers with different strategies and lifestyles (Pieterse *et al.*, 2009). For SA-JA crosstalk, it has been shown that plants prioritize one pathway over the other and this depends on the relative concentration of each hormone, the sequence of attack and the attackers involved (Pieterse *et al.*, 2012; Vos *et al.*, 2013a). Several studies have shown that when plants experience a certain stress, this compromises the plant's ability to respond to subsequent stresses. For example, multiple studies have reported that abiotic stresses tend to have a negative impact on plant defence against pathogens (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). For instance, tomato plants exposed to simultaneous stress imposed by salinity and *Oidium neolycopersici*, a biotrophic fungus that causes powdery mildew, were more susceptible to the pathogen than when exposed to the pathogen alone (Kissoudis *et al.*, 2015). Moreover, *A. thaliana* plants that were exposed to water deficit were more susceptible to infection by the biotrophic bacterial pathogen *Pseudomonas syringae* (Goel *et al.*, 2008). Furthermore, in *A. thaliana*, drought promoted population growth of generalist but not specialist aphids (Mewis *et al.*, 2012). Interactions between attackers of biotic nature have also been observed. For example, herbivory by *P. rapae*, a specialist chewing insect, in *A. thaliana* resulted in enhanced resistance to the biotroph *Turnip crinkle virus* (TCV) (De Vos *et al.*, 2006). These results were contrary to expectations, because *Arabidopsis* responds to viral and caterpillar attack through antagonistic signalling pathways. This suggested that unique interactions between the signalling pathways may occur when plants are exposed to combination of stresses. Indeed, ethylene induced by caterpillar feeding appeared to prime the SA-signalling pathway induced by TCV (De Vos *et al.*, 2006). Novel interactions have also been observed at the molecular level. In one of the first studies addressing multiple stress responses at the transcriptional level, Voelckel & Baldwin

(2004) found that transcriptional changes in *Nicotiana attenuata* plants exposed to sequential or simultaneous attack by the sap-feeding hemipteran *Tupiocoris notatus* and the chewing lepidopteran *Manduca sexta* were very similar. Furthermore, these transcriptional changes were different from the changes in response to the single stress situation. In tomato, simultaneous attack by the phloem-feeder *Macrosiphum euphorbiae* and the chewing herbivore *Spodoptera exigua* did not induce a different set of genes compared to the single stress situation; however, the expression patterns were different (Rodriguez-Saona *et al*., 2010). Several recent studies that conducted whole transcriptome profiling using microarrays (Prasch & Sonnewald, 2013; Rasmussen *et al*., 2013; Sewelam *et al*., 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al*., 2015) and QTL analysis (Makumburage & Stapleton, 2011; Cairns *et al*., 2013; Kissoudis *et al*., 2015) on plants exposed to multiple abiotic and biotic stresses have come to the conclusion that responses to combined stresses could not be predicted from the responses to individual stresses. These reports underline the complex events that take place when plants are challenged by multiple stresses and highlight the importance of studying sequential or simultaneous attack as different kinds of stress.

New approaches for identification of genes involved in resistance to plant responses to stress

With the completion of genome sequencing for several model organisms and important crops, the emphasis in genomic research has shifted to understand gene function (Feng & Mundy, 2006). The most robust method to determine gene function is to identify mutations in a gene of interest and then to compare the mutants harbouring the mutation to wild type plants (Feng & Mundy, 2006). Strategies used for gene discovery can be broadly classified into forward and reverse genetics (Azpiroz-Leehan & Feldmann, 1997; Wilson, 2000; Feng & Mundy, 2006). In forward genetic strategies one starts with plants displaying diverse phenotypes and works towards the identification of the genotypes responsible for the observed phenotypes. In reverse genetics, one starts with a gene of interest and experimentally evaluates the effect of its disruption (Azpiroz-Leehan & Feldmann, 1997; Feng & Mundy, 2006). Forward genetics is the classical way of gene identification by disrupting gene function through mutagenic agents (EMS, UV, X-ray, T-DNA) and subsequent cloning and sequencing of the gene. Natural populations of plants constitute a pool of natural mutants that harbours a rich reservoir of alleles. These natural populations offer an advantage to traditional disruption of genes by mutagenic agents where the potential number of alleles is limited to the genetic background of the wild type. Furthermore, they offer the opportunity to study mutations that are relevant to adaptation to stresses

in natural environments (Alonso-Blanco & Koornneef, 2000; Mitchell-Olds, 2001; Koornneef *et al.*, 2004; Shindo *et al.*, 2007; Nordborg & Weigel, 2008). Finding the causative mutation responsible for natural variation to a certain trait can be done through linkage mapping (QTL mapping) or association mapping (Doerge, 2002; Koornneef *et al.*, 2004; Kloth *et al.*, 2012; Weigel, 2012). QTL mapping makes use of genetic mapping and statistical tools to find an association between a region in the genome and a phenotype. QTL mapping usually takes place in synthetic populations generated by crosses. QTL mapping has been successfully applied for dissecting key components of several traits involved in plant development, flowering time, and resistance to biotic and abiotic stresses (Kowalski *et al.*, 1994; Jander *et al.*, 2001; Pfalz *et al.*, 2007; McKay *et al.*, 2008; Alonso-Blanco *et al.*, 2009; Lacaze *et al.*, 2009; Jimenez-Gomez *et al.*, 2010; Perez-Perez *et al.*, 2010; Trontin *et al.*, 2011; Wu *et al.*, 2013). Association mapping makes use of linkage disequilibrium (LD), when two loci in the genome are statistically more or less often inherited together due to recombination history, to associate genotypes with phenotypes. Association mapping can be based on an hypothesis on specific candidate genes which are suspected to be associated with a phenotype "candidate gene association mapping" or without a prior hypothesis using markers spread over the whole genome of an organism through a method called Genome Wide Association (GWA) analysis (Yu & Buckler, 2006; Zhu *et al.*, 2008). Association mapping overcomes several of the pitfalls of QTL mapping; (1) it offers higher resolution, (2) it is less time consuming and requires fewer resources, (3) it considers more or less all allelic diversity present in nature. On the other hand, association mapping has some limitations; (1) it requires large population sizes, (2) it can generate a large number of false positives due to population structure, (3) it has low statistical power to identify rare alleles and (4) it has difficulties dissecting complex traits (many rare variants of large effect or many common variants of small effect) (Nordborg & Weigel, 2008; Zhu *et al.*, 2008; Korte *et al.*, 2012). In fact, both strategies complement each other leading to a higher power of finding causal genetic variation (Zhu *et al.*, 2008; Myles *et al.*, 2009; Brachi *et al.*, 2010; Kloth *et al.*, 2012). Despite some limitations, association mapping has been successfully implemented in model plants such *Arabidopsis* (Atwell *et al.*, 2010; Baxter *et al.*, 2010; Chan *et al.*, 2010; Chan *et al.*, 2011; Chao *et al.*, 2012) and also in economically important crops such as rice (Huang *et al.*, 2010; Huang *et al.*, 2012), tomato (Ranc *et al.*, 2012), barley (Wang *et al.*, 2012), and wheat (Joukhadar *et al.*, 2013). Traits successfully dissected through association mapping include e.g. glucosinolate content (Chan *et al.*, 2011), shade avoidance (Filiault & Maloof, 2012), heavy metal accumulation (Chao *et al.*, 2012), salt stress resistance (Baxter *et al.*, 2010), flowering time (Li *et al.*, 2010), and heat tolerance (Bac-Molenaar *et al.*, 2015). Contrasting phenotypes are often due to allelic differences at several loci, and each

locus contributes to a small extent to the phenotype. Thus, it is difficult to obtain definitive proof that a QTL identified by linkage or association mapping is responsible for contrasting phenotypes. Once the association of a locus to a trait has been proven, several approaches can be used to provide evidence that an allele variant is linked to a phenotype, such as allelic complementation or mutational analysis of candidate genes (Weigel & Nordborg, 2005; Nordborg & Weigel, 2008; Weigel, 2012).

An alternative to traditional genetics for studying gene function is whole genome expression analysis (Schena *et al.*, 1995). The expression of a gene under a physiological condition and not another may indicate that the gene plays a biological role contributing to the biological difference between conditions. Whole genome profiling has been traditionally carried out using microarrays. However, the reduction in costs of Next Generation Sequencing (NGS) has opened up the possibility to carry out transcriptome analysis using RNA sequencing (RNA-seq) (Wang *et al.*, 2009; Van Verk *et al.*, 2013). For instance, a simple search in the Scopus database using as query “RNA-seq” and “expression” showed an increase in articles from 5 in 2008 to 1235 in 2014. RNA-seq offers several advantages over microarrays. (1) There is no restriction to known genes, (2) unlimited dynamic range in quantitation, (3) more accurate expression level assessment, (4) less sophisticated normalization procedures, (5) no problems with cross-hybridization of similar transcripts (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). In addition, RNA-seq can extend the studies of transcriptomes to the analysis of splice variants. One important consideration in this kind of studies is that gene expression under a certain physiological condition and not another is not proof that the gene contributed to the biological differences between conditions. Reverse genetic strategies such as mutant analysis will be required to validate the gene function.

Study system

This thesis addresses the study of the genetic architecture of and transcriptome changes underlying plant responses to biotic and abiotic stress, either in isolation or in combination. In this section I will introduce my study system.

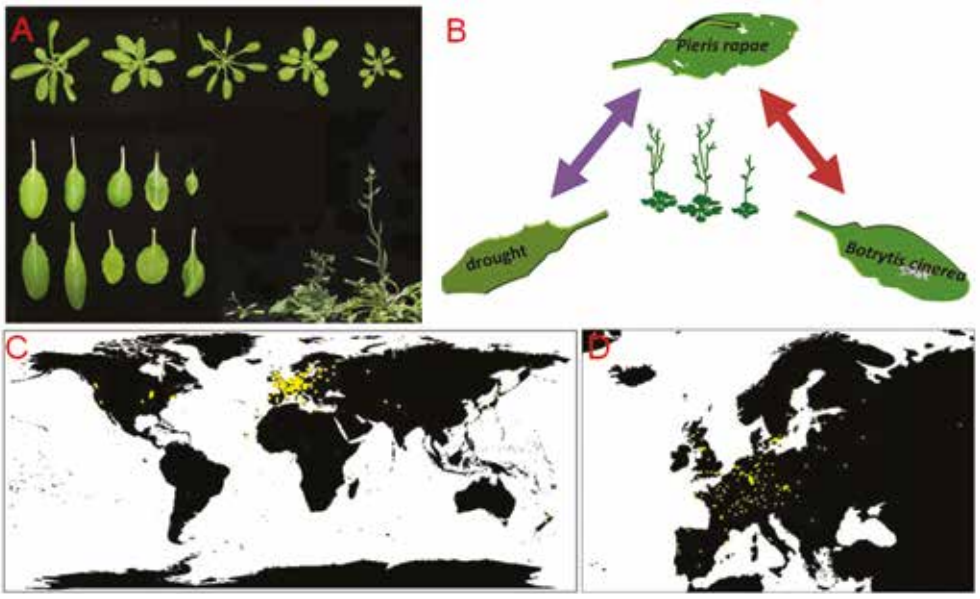

Figure 2. Natural variation in *Arabidopsis thaliana*. (A) Variation in developmental and morphological traits. Modified from Weigel (2012). (B) Study system of this thesis. (C) Geographic distribution of accessions from the Hapmap collection. D. Magnification of origin of European accessions.

1. Plant species

Arabidopsis thaliana has been the model of choice for many genetic studies in plant biology because of its suitability for laboratory studies. It offers several advantages such a small size, short life cycle, amenability for genetic studies, relatively small genome, vast collection of mutants, and publicly available resources (Alonso-Blanco & Koornneef, 2000; Kaul *et al.*, 2000; Koornneef *et al.*, 2004; Koornneef & Meinke, 2010). *Arabidopsis thaliana* has also become an interesting model system for ecologists and evolutionary biologists due to the large collections of natural accessions that have been re-sequenced (Mitchell-Olds, 2001; Shindo *et al.*, 2007; Gan *et al.*, 2011; Hancock *et al.*, 2011; Horton *et al.*, 2012; Lasky *et al.*, 2012; Weigel, 2012). *Arabidopsis thaliana* is native to Eurasia but has been introduced world-wide, and is widely distributed in the northern hemisphere (Al-Shehbaz & O'Kane, 2002). *Arabidopsis thaliana* accessions can be found in diverse and contrasting habitats such as open or disturbed land, sandy or loamy soils, at sea level or high altitude

(Hoffmann, 2002; Hoffmann, 2005). Thus, it is also a good model for studying local adaptation and responses to stresses (Hoffmann, 2002; Hoffmann, 2005; Shindo *et al*., 2007). *Arabidopsis thaliana* accessions display substantial natural variation in developmental and physiological traits, and in resistance to abiotic and biotic stresses (Figure 2A) (Alonso-Blanco & Koornneef, 2000; Koornneef *et al*., 2004; Alonso-Blanco *et al*., 2009; Weigel, 2012). In this thesis we used a world-wide collection of 350 accessions from the Hapmap population (http://naturalvariation.org/hapmap) (Figure 2C & D). This population was developed to minimize redundancy and close family relatedness, a common problem in GWA studies (Baxter et al. 2010; Platt et al. 2010a; Chao et al. 2012).

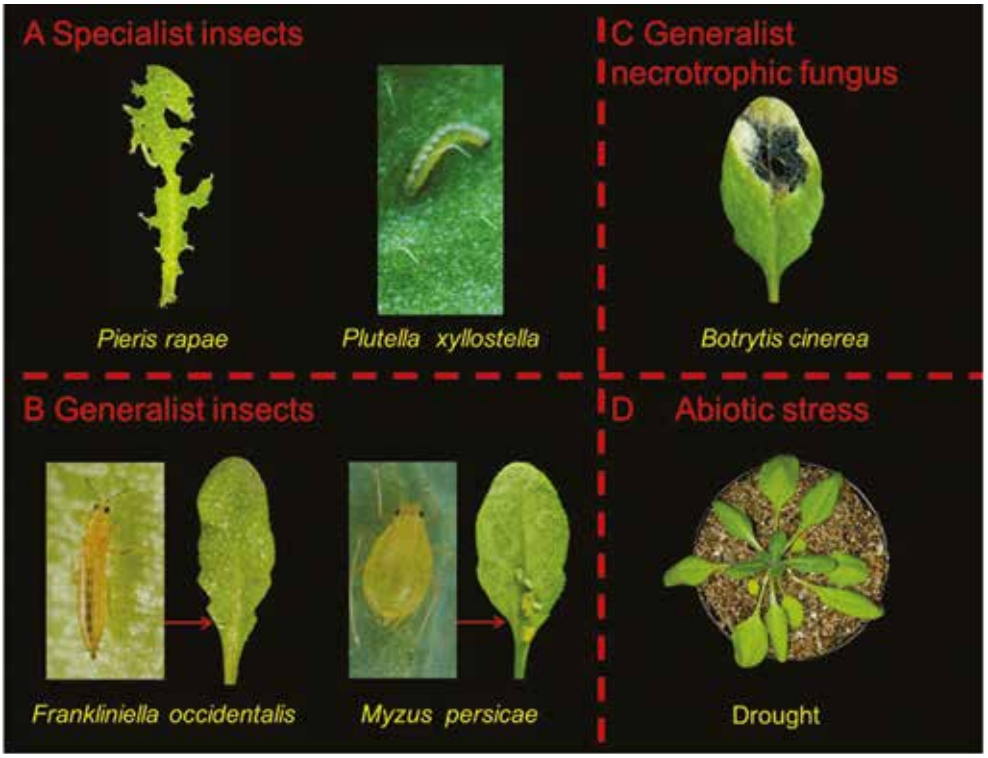

Figure 3. *Arabidopsis thaliana* interaction with biotic and abiotic stresses. (A) Specialist insects that feed on plants from the Brassicaceae family, including *A. thaliana*. (B) Generalist insects that have a broad range of host plants. (C) Plant pathogen known to infect more than 200 plant species. Pictures of *A. thaliana* (A-D): courtesy of Plant-Microbe Interactions group at Utrecht University (Hans van Pelt). *P. xyllostella* in (A): courtesy of Anneke Kroes. *Frankliniella occidentallis* and *Myzus persicae* in (B) : courtesy www.bugsinthepicture.com (Tibor Bukovinszky).

2. Specialist insect species

Caterpillars of *Pieris rapae* (Lepidoptera: Pieridae), the Small Cabbage White butterfly, are largely restricted to feeding from plants of the Brassicaceae family, including *A. thaliana,* by removing strips of leaf tissue (Figure 3A) (Schoonhoven *et al.*, 2005). Most members of the Brassicaceae family contain distinct chemical defence compounds, glucosinolates (Hopkins *et al.*, 2009; Mithofer & Boland, 2012). Glucosinolates are biosynthetically derived from amino acids and can be hydrolysed by myrosinase (thioglucosidase) enzymes upon insect herbivory (Fahey *et al.*, 2001; Kliebenstein *et al.*, 2005; Brachi *et al.*, 2015). Specialist insects such as *P. rapae*, have developed detoxification mechanisms (Brachi et al., 2015; Fahey et al., 2001)and are not affected by glucosinolates, and proteinase inhibitors produced by brassicaceous plants (Schoonhoven *et al.*, 2005; De Vos *et al.*, 2008; Muller *et al.*, 2010). On the other hand, *P. rapae* utilizes glucosinolates as oviposition and feeding stimulants (Ratzka *et al.*, 2002; De Vos *et al.*, 2008; Muller *et al.*, 2010). Herbivory by *P. rapae* in *A. thaliana* activates the MYC branch of the JA signalling pathways through the transcription factor MYC2 and results in the expression of genes such as *VSP2* (Verhage et al., 2011; Vos et al., 2013b). *Arabidopsis thaliana* mutants defective in the JA signalling pathway are more susceptible to herbivory by *P. rapae* than the wildtype, suggesting that the JA signalling pathway is a component in defences elicited against this insect herbivore (Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Vos *et al.*, 2013b) Recently, the flavonol glycoside kaempferol-3,7-dirhamnoside was shown to be involved in resistance of *A. thaliana* against the closely related caterpillar *Pieris brassicae* (Onkokesung *et al.*, 2014).

Another specialist insect used in Chapters 2 and 5 of this thesis is the Diamondback moth (Figure 3A) (*Plutella xylostella;* Lepidoptera: Yponomeutidae). The Diamondback moth originates in Europe but is now found throughout the Americas, Southeast Asia, Australia and also New Zealand (Talekar & Shelton, 1993). Annual control costs of *P. xylostella* are estimated to be nearly US$ 4-5 billion (Zalucki *et al.*, 2012). Similar to *P. rapae*, JA signalling pathways seem to play a role in mediating responses in *A. thaliana* to *P. xyllostella* (Kroes *et al.*, 2015). However, transcriptional and phenotypic responses to both specialist herbivores in *A. thaliana* are different (Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011).

3. Generalist insects

The generalist insects Western Flower Thrips (*Frankliniella occidentalis* (Pergande); Thysanoptera: Thripidae) and Green Peach Aphid (*Myzus persicae* (Sulzer); Hemiptera: Aphididae) were used in Chapters 2 and 5 of this thesis (Figure 3B). Thrips are tiny piercing-sucking insects that feed from the contents of mesophyll cells, producing visible damage in the plant tissue known as silver damage (Schoonhoven

et al., 2005). In *A. thaliana* they are known to elicit JA-mediated responses (De Vos *et al.*, 2005). *Arabidopsis thaliana* mutants impaired in JA signalling display more susceptibility to thrips feeding than the wild type (Abe *et al.*, 2009). Contrary to thrips that feed mostly from mesophyll cells, aphids need a more intimate relationship with their host plant, because they feed mostly from phloem sap and can do so during long periods of time (Schoonhoven *et al.*, 2005; Bos *et al.*, 2010). Aphids use their stylets to penetrate the epidermis and move them between several cell layers until they reach a phloem sieve element where they feed for hours to days (Tjallingii, 1995). Aphids elicit plant defences such as phloem clogging and callose deposition that are mediated by salicylic acid signalling pathways (De Vos *et al.*, 2005; Hogenhout & Bos, 2011). However, some aphids are able to supress these defence mechanism by depletion of effectors (Hogenhout & Bos, 2011).

4. Necrotrophic fungus

Botrytis cinerea (hereafter: *Botrytis*), known as gray mould, is considered the second most important fungal plant pathogen from both scientific and economic perspectives (van Kan, 2005; Williamson *et al.*, 2007; Dean *et al.*, 2012). It is known to attack approximately 1000 plant species, including 200 crop species and *Arabidopsis* (Dean *et al.*, 2012; Zhang *et al.*, 2014). Upon infection in *Arabidopsis*, *Botrytis* activates the ERF branch of the JA signalling pathway through the transcription factors ERF1 and ORA59 and results in the expression of genes such as *PDF1.2* (Lorenzo *et al.*, 2003; Pre *et al.*, 2008; Verhage *et al.*, 2011)(Figure 3C).

5. Drought

Drought stress is one of the most, if not the most, limiting abiotic stresses for crop productivity and plant fitness (Figure 3D) (Bartels & Sunkar, 2005; Riera *et al.*, 2005; Juenger, 2013). Models indicate that climate change will increase the incidence of drought along with increasing temperatures (Zhao & Running, 2010; Sheffield *et al.*, 2012; Wheeler & von Braun, 2013). Thus, drought tolerance has become a trait of interest for breeding firms and scientists from diverse fields including geneticists and evolutionary biologists (Mitchell-Olds, 2001; Yang *et al.*, 2010; Skirycz *et al.*, 2011; Juenger, 2013). Plants are able to survive drought through phenological and physiological traits by deployment of three strategies named tolerance, avoidance and escape (McKay *et al.*, 2003; Des Marais *et al.*, 2012; Juenger, 2013; Easlon *et al.*, 2014). Tolerance refers to mechanisms that protect cells and tissues under limited water conditions (e.g. LEA proteins). Avoidance refers to mechanisms that maintain the internal water status under limited water conditions (e.g. stomatal closure). Escape refers to a shift in phenology that allows plants to grow and reproduce by avoiding activity during periods of water scarcity (e.g. short life cycle in annuals) (McKay *et al.*, 2003; Juenger, 2013). Upon

drought perception, two signalling pathways are involved in mediating plant responses to drought: ABA-dependent and ABA-independent pathways (Riera *et al.*, 2005; Shinozaki & Yamaguchi-Shinozaki, 2007). ABA-dependent and ABA-independent gene expression are regulated by different sets of transcription factors: AREB/ABFs and DREBs, respectively (Shinozaki & Yamaguchi-Shinozaki, 2007; Yoshida *et al.*, 2014). For instance, one of the best studied phenotypic changes mediated by the ABA-dependent signalling pathway that allows plants to acclimate to limited water availability is stomatal closure (Shinozaki & Yamaguchi-Shinozaki, 2007; Yang *et al.*, 2010). It has been estimated that the ABA-dependent pathway mediates approximately 67 % of *A. thaliana*'s transcriptional response to drought (Huang *et al.*, 2008). On the other hand, overexpression of *DREB* genes in several plant species enhanced tolerance to several abiotic stresses such as drought, heat and salinity. The physiological and biochemical mechanisms are subject of investigation (Lata & Prasad, 2011). Furthermore, crosstalk between ABA-dependent and ABA-independent pathways has been observed (Yoshida *et al.*, 2014).

Scope and thesis outline

Plants are exposed to a broad diversity of biotic and abiotic stresses. In nature these stresses commonly occur simultaneously. Although considerable progress has been made towards the identification of genes that code for resistance to a single biotic or abiotic stress, little is known on how plants cope with multiple stresses. In a coordinated effort, we aimed to explore responses of *A. thaliana* to a range of abiotic and biotic stresses and combinations at the phenotypic, genetic and transcriptional level. We implemented two strategies for this purpose, (1) genome wide associations, that allowed us to explore the genetic architecture of plant responses to stress and to identify candidate genes having a role in resistance to more than one stress; (2) RNA-seq that allowed us to get insights into the transcriptome changes that occur in plants under stress. Thus, the approach of this thesis was an integrated ecogenomics approach that connects phenotypical analysis and genome-wide association for a large number of plant accessions with an in-depth transcriptional analysis.

The focus of this thesis was mainly on drought, herbivory by *Pieris rapae* caterpillars, and infection by the necrotrophic fungal pathogen *Botrytis cinerea* (Figure 2B, Figure 3 A,E&F). These stresses were chosen because the responses of *Arabidopsis* to these three stresses are highly divergent but at the same time regulated by the plant hormones JA and/or ABA. In a coordinated effort, we aimed (1) to explore the extent of natural variation in *A. thaliana* to abiotic, biotic and combined stresses, and (2) to understand the commonalities and differences between individual and combined stresses at the phenotypic, genetic and transcriptional level.

Chapter 2 focusses on natural variation in European accessions of *A. thaliana* in response to one abiotic stress (drought), four biotic stresses (*Pieris rapae* caterpillars*, Plutella xyllostella* caterpillars*, Frankliniella occidentallis* thrips*, Myzus persicae* aphids) and two combined stresses (drought plus *P. rapae,* and *Botrytis cinerea* fungus plus *P. rapae*). This chapter focusses on the effect of life-history strategy (i.e. summer or winter annual) of *A. thaliana* on the response to the different stresses addressed. Furthermore, gradients in response to stresses along geographical gradients were investigated.

Chapter 3 and **Chapter 4** address *A. thaliana's* response to single versus multiple stresses. Differences between single and multiple stresses, both at the phenotypic and transcriptional level, are presented.

Chapter 3 presents the results of an RNA-seq study for *A. thaliana* responses to the necrotrophic plant pathogen *B. cinerea*, the abiotic stress drought and herbivory by *P. rapae* caterpillars as single stresses. Furthermore, mathematical modelling was used to identify groups of genes that displayed altered expression patterns under combined stress compared to the single stress situations.

Chapter 4 presents an in-depth analysis of the data presented in Chapter 3 with a focus on herbivory by *P. rapae*. The chapter focuses on the temporal transcriptional changes that occur in *A. thaliana* in response to *P. rapae* herbivory. The RNA-seq analysis was contrasted to microarray studies reported in the literature. Multivariate analysis was used to dissect how transcriptional changes in *A. thaliana* to *P. rapae* feeding as single stress were altered by a preceding drought stress or infection by *B. cinerea*.

Chapter 5 presents a univariate GWA analysis that allowed the identification of regions associated with resistance to two specialist insect herbivores (*P. rapae* and *P. xyllostella),* one abiotic stress (drought) and two combined stresses (*B. cinerea* plus *P. rapae* and drought plus *P. rapae*). Candidate genes are presented and discussed.

Chapter 6 presents the results of a multi-trait GWA analysis for *A. thaliana* resistance to 11 single stresses and several of their combinations with a focus on candidate genes for contributing to resistance to several stresses. Candidate genes identified are discussed based on the literature. Mutant analysis for some of the candidate genes under a subset of stresses is also presented**.**

Finally, **Chapter 7** discusses the most important finding of this thesis and places them in the context of the current knowledge of how plants respond to multiple environmental perturbations**.**

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Chapter 2

Natural variation in life-history strategy of *Arabidopsis thaliana* determines stress responses to drought and insects of different feeding guilds

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Abstract

Plants are sessile organisms and, consequently, are exposed to a plethora of stresses in their local habitat. As a result, different populations of a species are subject to different selection pressures leading to adaptation to local conditions and intraspecific divergence. The annual brassicaceous plant *Arabidopsis thaliana* is an attractive model for ecologists and evolutionary biologists due to the availability of a large collection of re-sequenced natural accessions. Accessions of *A. thaliana* display one of two different life-cycle strategies: summer and winter annuals. We exposed a collection of 308 European *Arabidopsis* accessions, that have been genotyped for 250K SNPs, to a range of stresses: one abiotic stress (drought), four biotic stresses (*Pieris rapae* caterpillars*, Plutella xylostella* caterpillars*, Frankliniella occidentallis* thrips*, Myzus persicae* aphids) and two combined stresses (drought plus *P. rapae,* and *Botrytis cinerea* fungus plus *P. rapae*). We identified heritable genetic variation for responses to the different stresses, estimated by narrow-sense heritability. We found that accessions displaying different life-cycle strategies differ in their response to stresses: winter annuals are more resistant to drought, aphids and thrips and summer annuals are more resistant to *P. rapae* and *P. xylostella* caterpillars and to the combined stresses of drought plus *P. rapae* and infection by the fungus *Botryris cinerea* plus herbivory by *P. rapae*. Adaptation to drought displayed a longitudinal gradient. Finally, trade-offs were recorded between the response to drought and responses to herbivory by caterpillars of the specialist herbivore *P. rapae.*

Keywords: biotic stress, drought, herbivory, fungal pathogen, specialist, generalist, summer annual, winter annual.

Introduction

Biotic and abiotic conditions vary in space and time. As a result, different populations of a species are exposed to different selection pressures leading to adaptation to local conditions and intraspecific divergence (Kaltz & Shykoff, 1998; Manel *et al.*, 2003; Kawecki & Ebert, 2004; Kawecki, 2008). Locally adapted populations are, therefore, expected to outperform allochthonous populations (Kawecki & Ebert, 2004; Savolainen *et al.*, 2013). Local adaptation is particularly relevant in the face of the global changes our planet is exposed to, because locally adapted populations can become maladapted if environmental conditions change. Mathematical models indicate that climate change will increase the incidence of extreme temperatures, drought and flooding events (Zhao & Running, 2010; Sheffield *et al.*, 2012; Wheeler & von Braun, 2013). With global increase in mean temperatures, it is expected that species will move towards higher elevation and latitudes (Devictor *et al.*, 2012). This will result in decoupling of phenological relationships between species, and the intensity of selection pressure that biotic and abiotic conditions impose on organisms is also expected to change (Hughes, 2000). Thus, understanding how organisms adapt to environmental conditions and investigating how responses to environmental stresses co-vary with geographical gradients are central issues in exploring the ecological consequences of global change.

There is a wealth of experimental evidence for local adaptation in plants and animals (Bradshaw & Holzapfel, 2001; Savolainen *et al.*, 2007; Leimu & Fischer, 2008; Hereford, 2009; Leinonen *et al.*, 2009; Leinonen *et al.*, 2011). Local adaptation has been demonstrated along longitudinal, latitudinal, and elevational gradients in several plant species (Mikola, 1982; Olsson & Agren, 2002; Stinchcombe *et al.*, 2004; Montesinos-Navarro *et al.*, 2012; Alberto *et al.*, 2013; Debieu *et al.*, 2013). Important variables such as temperature and precipitation co-vary with these gradients, and the latter are, therefore, useful indicators of species adaptation to local environmental conditions through a continuous landscape of selection (Fukami & Wardle, 2005; Walker *et al.*, 2010; Halbritter *et al.*, 2013; Sundqvist *et al.*, 2013; Manzano-Piedras *et al.*, 2014). Traits involved in interactions between organisms and their environment have also been observed to display variation across gradients. For example, in several plant species, tolerance to abiotic stresses such as drought, salinity and low temperatures are known to vary with elevation and latitude (Baxter *et al.*, 2010; Hancock *et al.*, 2011; Alberto *et al.*, 2013; Paccard *et al.*, 2014). Resistance to biotic stresses such as insect herbivory is also known to vary along geographic gradients. Evidence suggests that herbivory is more intense at lower latitudes and elevations, due to increased herbivore diversity and, hence, plants will be under selection for increased defences (Sanders *et al.*, 2003; Andrew & Hughes, 2005; Moles *et al.*, 2011; Halbritter *et al.*,

2013; Sundqvist *et al.*, 2013; Rasmann *et al.*, 2014). A study on two piperaceous plant species, for example, reported higher incidence of herbivory towards the equator (Salazar & Marquis, 2012). Similarly, ecotypes of *Plantago lanceolata* and *Vicia sepium* from low elevations were more resistant to herbivory than plants from high-elevation ecotypes (Pellissier *et al.*, 2014). Although the hypothesis that "the lower the latitude and elevation, the higher the level of plant defences" (Andrew & Hughes, 2005; Moles *et al.*, 2011) has been supported in some studies, others failed to provide support and, therefore, further experiments are needed that address plant responses throughout large geographic areas. As firmly rooted organisms, plants are exposed to a wide diversity of biotic and abiotic stresses which commonly occur simultaneously (Atkinson & Urwin, 2012; Kissoudis *et al.*, 2014; Suzuki *et al.*, 2014). Much progress has been made in understanding how plants adapt to their environment and what the underlying genetic mechanisms are. Yet, we still know rather little about how plants integrate and respond to multiple simultaneous stresses, and how this is shaped by local adaptation.

Several methodologies have been developed to investigate local adaptation, including reciprocal transplants and common garden experiments (Kawecki & Ebert, 2004). When screening populations from a large geographical area, however, the former approach is often impractical for legal, logistic or ethical reasons, and hence growing different genotypes in a common environment is a good alternative. Under these circumstances, variation in phenotypic responses can be assessed. This approach is particularly useful because it allows to test the role of a particular agent as driver of population differentiation (Kawecki & Ebert, 2004; Alberto *et al.*, 2013; Manzano-Piedras *et al.*, 2014). Despite the limitations of inferring local adaptation from correlative approaches in a common environment (Savolainen *et al.*, 2013), this method represents a powerful tool for detecting selection along environmental gradients (Gomaa *et al.*, 2011; Montesinos-Navarro *et al.*, 2011; Montesinos-Navarro *et al.*, 2012; Pico, 2012; Debieu *et al.*, 2013; Manzano-Piedras *et al.*, 2014).

Model organisms like *A. thaliana* have become attractive for ecologists and evolutionary biologists because large collections of natural accessions with known genomic sequences are available (Shindo *et al.*, 2007; Fournier-Level *et al.*, 2011; Weigel, 2012). In *A. thaliana,* variation in several life-history traits along latitudinal and altitudinal gradients have been demonstrated, including (i) variation in flowering time, seed dormancy and growth rate (Stinchcombe *et al.*, 2004; Montesinos-Navarro *et al.*, 2011; Debieu *et al.*, 2013), (ii) variation in resistance against abiotic stresses such as salt and drought stress (McKay *et al.*, 2003; Baxter *et al.*, 2010; Juenger, 2013; Easlon *et al.*, 2014), and (iii) variation in defence-related traits. For example, *A. thaliana* displays a latitudinal and longitudinal gradient for diversity in glucosinolate profiles, a main class of defensive metabolites in the Brassicaceae (Brachi *et al.*,

2015) and through a longitudinal gradient, this correlates with the abundance of two aphid species, i.e. *Brevicoryne brassicae* and *Lipaphis erysimi* (Zust *et al.*, 2012).

An important trait in *A. thaliana* is that two different life-cycle strategies are found among accessions: summer and winter annuals (Pigliucci, 1998; Koornneef *et al.*, 2004; Shindo *et al.*, 2007). Winter annuals are accessions that germinate in late summer and autumn, overwinter as rosette and flower in spring. Winter annuals require a vernalisation period in order to flower. On the other hand, summer annuals or rapid cyclers germinate in spring and flower in summer and do not require vernalisation for flowering (Koornneef *et al.*, 2004; Stinchcombe *et al.*, 2004; Shindo *et al.*, 2007). Furthermore, the winter annual life-cycle is mostly expressed in temperate areas, whereas the summer annuals occur in warmer regions (Johanson *et al.*, 2000; Michaels *et al.*, 2003). This plasticity in life-cycle strategies has been hypothesized to be the basis for the highly adaptive capacity of *A. thaliana* (Shindo *et al.*, 2007). For instance, there is a strong positive genetic correlation between flowering time and water use efficiency such that, relative to early flowering accessions, late flowering ones display higher water use efficiency (McKay *et al.*, 2003; Juenger, 2013; Easlon *et al.*, 2014). Despite the different phenological, morphological and stress responses observed in *A. thaliana* accessions displaying different life-cycle strategies, these differences have rarely been considered when studying local adaptation and responses to abiotic and biotic stresses.

In this study, we used a collection of 308 *A. thaliana* accessions from European origin and exposed them in a controlled environment to diverse ranges of abiotic and biotic stresses. Abiotic stress consisted of drought, whereas biotic stresses were imposed by two specialist leaf-chewing insect herbivores (caterpillars of *Pieris rapae* and *Plutella xylostella*), two generalist piercing-sucking insect herbivores (the aphid *Myzus persicae* and the thrips *Frankliniella occidentalis*). Moreover, stress combinations were imposed by combining drought plus *P. rapae* and the necrotrophic fungus *Botrytis cinerea* plus *P. rapae*. The rationale for choosing these (combined) stresses is that *A. thaliana*'s response to *P. rapae*, *P. xylostella, F. occidentalis, B. cinerea* and drought as single stresses is highly diverse, yet at the same time regulated by the plant hormones JA and/or ABA (De Vos *et al.*, 2005; Broekgaarden *et al.*, 2007; Verhage *et al.*, 2011), while the response to the aphid *M. persicae* is regulated by the plant hormone SA (De Vos *et al.*, 2005).

We addressed the following questions: (1) Is there heritable genetic variation in *A. thaliana*'s responses to the different stresses studied? (2) Does the proportion of winter and summer annuals vary along latitude, longitude and elevation? (3) Do winter and summer annuals differ in their responses to these stresses? (4) Do *A. thaliana* plants from different latitudes, longitudes and elevations differ in their responses to these

stresses? Life-history theory predicts that for a specific genotype, the expression of a particular defensive or resistance trait will constrain the expression of others, so we also asked: (5) Are *A. thaliana* responses against specific stresses negatively correlated with others: i.e. are there trade-offs?

Materials and Methods

Arabidopsis thaliana populations and molecular markers

In this study we included 350 *Arabidopsis thaliana* (L.) Heynh. accessions from the Hapmap population (http://naturalvariation.org/hapmap) (Figure S1). In order to avoid geographical outliers, however, our analyses were limited to the European accessions found at a latitude ≥ 30, longitude between -50 and 50 and elevation ≤ 2000 m (Figure S2). This resulted in a subset of 308 accessions on which all further analyses were performed. The Hapmap population has been genotyped for 250K bi-allelic SNPs (Baxter *et al.*, 2010; Platt *et al.*, 2010; Chao *et al.*, 2012) and after quality control and imputation this SNP-set was reduced to a set of 214,051 SNPs. Genome-wide association analysis (GWA) for this data set are not presented because it was out of the scope of this paper. Results of a GWA analysis for a larger data set of accessions and stress conditions will be published elsewhere. We evaluated the effects of geographical gradients on plant responses because these gradients commonly correlate with environmental conditions. To get a deeper understanding of these correlates, we obtained a set of environmental variables from the locations where the *A. thaliana* accessions had been collected (Supplementary Data 1). Sources from where environmental data were obtained are summarized in Table S1. These variables were then correlated with latitude, longitude and elevation through Spearman correlation tests (Figure S3).

Genetic diversity in European accessions from the Hapmap population

Determination of geographically informative genetic groups was done as follows. Principal component analysis was performed on the scaled 0,1 molecular marker matrix of dimensions *n* x *m*, where *n* is the number of individuals and *m* the number of markers (Odong *et al.*, 2013; van Heerwaarden *et al.*, 2013). Spatial autocorrelation of individual PCs was determined by Moran's I statistic. Geographic coordinates were converted to spatial weight classes by the functions *graph2nb* and *graph2nb* in the R package *spdep*. The principal components with a P value above 0.001 were retained as spatially informative and used to calculate a Euclidean distance matrix. A dendrogram was produced by Ward clustering and genetic groups were assigned by splitting the dendrogram into groups using the R function *cutree*. Phenotypic differences between genetic groups were tested by ANOVA after correction for life-cycle strategy.

Classification of accessions as winter and summer annuals

Flowering time and flowering time after vernalisation under greenhouse conditions have been recorded for this plant population as reported by Bac-Molenaar *et al.* (2015b). Accessions were classified as winter annuals if vernalisation was required for flowering (flowering time ≥ 75 days) or as summer annuals otherwise (Supplementary Data 1).

Plants, insects and fungi

Arabidopsis thaliana plants were grown in a controlled environmental chamber at 24 ± 1 °C, 70 ± 10 % relative humidity, 200 µmol m^{-2} s^{-1} photosynthetically active radiation from fluorescent lights (TL-D 58W/840; www.philips.com) and a diurnal cycle of 8:16 L:D. In all experiments, seeds were vernalized at 4 °C for 5 d to induce even germination. Plants were individually grown in 0.08 liter pots (http://uk.poeppelmann.com/teku/home/). Plants were grown in a pasteurized (4 h at 80 °C) commercial *Arabidopsis* potting soil, which was mixed 1:1 (v/v) with autoclaved sand in experiment (1). In experiments (2), (3) and (4) (see later) plants were grown in pasteurized (4 h at 80 °C) commercial potting soil. Individual pots were accommodated in trays, randomly distributed within the growth chamber. Plants were watered three times per week by adding water to the tray. Once per week they received *Steinernema feltiae* entomopathogenic nematodes (Entonem; http://www.koppert.nl/) to prevent infestation by fungus gnats.

Pieris rapae L. (Small Cabbage White butterfly; Lepidoptera; Pieridae) were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) in a growth chamber at 21 ± 1 °C, 50 - 70 % relative humidity and a diurnal cycle of 16:8 L:D.

Plutella xylostella L. (Diamondback Moth; Lepidoptera; Yponomeutidae) were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) in a growth chamber at 22 ± 1 °C, 40-50% relative humidity and a diurnal cycle of 16:8 L:D.

The Western flower thrips (*Frankliniella occidentalis* (Pergande)) used in this study were originally collected from *Chrysanthemum* flowers and reared in glass bottles on green common bean pods (*Phaseolus vulgaris*) in climate cabinets at 25 ± 1 °C, 50-70% relative humidity and a diurnal cycle of 16:8 L:D.

Myzus persicae (Sulzer) (Green Peach Aphid; Hemiptera: Aphididae) were reared on radish plants, *Raphanus sativus* L., at 19 ±2 °C, 50-70% relative humidity and a diurnal cycle of 16:8 L:D.

The necrotrophic fungus *Botrytis cinerea,* strain B0510 (Van der Ent *et al.*, 2008) was grown on half-strength PDA plates containing penicillin (100 μg mL^{-1}) and streptomycin (200 μg mL^{-1}) for 2 weeks at room temperature. Spores were collected

and re-suspended in half-strength potato dextrose broth (Difco Laboratories) to a final density of 1.0 x 10^5 spores mL^{-1}. After a 3-h incubation period, the spores were used for inoculation (Thomma *et al*., 1998; Pre *et al*., 2008; Van der Ent *et al*., 2008).

Experimental design and treatments

We performed four different experiments in which plants were exposed to the following stressors (Figure S4): experiment (1): drought, damage by *P. rapae* caterpillars alone or preceded by drought or infestation by *B. cinerea;* experiment (2): damage by *P. xylostella* caterpillars; experiment (3): damage by the cell-content feeding thrips *F. occidentalis*, and experiment (4): infestation by the phloem-feeding aphid *M. persicae.*

For **experiment (1)**, bioassays were performed in 10 temporal blocks. Each block consisted of approximately 37 random accessions. One first layer of control to correct for variation within temporal blocks was the inclusion in all blocks of the same three accessions (CS28780;Tsu-0, CS76113;Col-0 and CS76129;Fei-0). The spatial location of each plant was recorded. Within temporal blocks, plants were allocated in trays and the position of the tray in the rearing chamber recorded as its position in either of the six racks, each with four shelves. The position of each plant in the trays was also recorded in terms of X and Y coordinates. In each temporal block, accessions were exposed to the following five treatments with a total of six replicates per accession and treatment: (a) no stress, (b) drought stress, (c) *P. rapae* herbivory, (d) drought plus *P. rapae* herbivory or (e) *B. cinerea* infection plus *P. rapae* herbivory (Figure S4A). Plants were grown under similar conditions during the first three weeks. Drought stress was imposed by withholding water for seven days during the third week while the rest of the plants was watered every two days with 1L of water per tray. *Botrytis cinerea* inoculation was carried out 24 h prior to *P. rapae* inoculation. Plants were inoculated with *B. cinerea* by pipetting 5 µL of spores suspended in half-strength PDB (Difco Laboratories) at a concentration of 1 × 10^5 spores mL^{-1} onto two of the leaves in the rosette. In order to ensure successful infection by *B. cinerea,* plants were kept at 100% RH for 24 h. Four-week-old plants were exposed to herbivory by *P. rapae* as single or combined stress. Infestation with this species was carried out by placing two newly hatched first instar (L1) caterpillars on one of the leaves; they were allowed to feed for 5 days. Subsequently, plant rosette fresh weight was quantified for all treatments.

Experiment (2) was performed in four temporal blocks. Within blocks, accessions were randomly distributed over 40 trays with nine accessions per tray. In this experiment, accession Col-0 was included to control for possible positional effects within the chamber. Each tray contained both control and treatment plants for Col-0 and nine other accessions. Plants were randomized within the trays. One replicate

per accession was screened at a time. Within blocks, accessions were exposed simultaneously to either (a) no stress or (b) herbivory by *P. xylostella* (Figure S4B). Plants were 4 weeks old when they were inoculated with two L2 larvae. Larvae were allowed to feed for 5 days after which plant rosette fresh weight was quantified for all plants.

Experiment (3) was performed in five temporal blocks. Each block was divided into three sub-blocks, representing three consecutive days. Within a block, accessions were randomly allocated to groups of 20 accessions (18 groups per block). Each sub-block consisted of 5 groups (100 accessions), with the exception of the last sub-block (8 groups, 150 accessions). Within blocks, one replicate per accession was screened at a time. Leaves from 5-week-old plants were cut and transferred to Petri dishes (diameter 5 cm; BD falcon, Product Number: 351006) containing a film of 1% agar. The petiole was inserted into the agar film (Figure S4C). Leaves were exposed to three juvenile (second larval instar, L2) *F. occidentalis* for 6 days (Figure S4C). Feeding damage was estimated in mm^2 after 6 days by counting the number of small 'silver damage' feeding spots, where one small feeding spot accounts for 3 mm^2 damage (bigger spots were counted as 2-5 small spots).

Experiment (4) was performed in three temporal blocks, each divided in four sub-blocks, representing four consecutive days. Two to three replicates were assayed per accession. Three-week-old plants were inoculated with one 0-24h-old *M. persicae* nymph per plant. Individual plants were placed on the inverted lid of a Petri dish in trays with a soap-diluted water barrier to prevent aphids from moving between plants. Each tray contained 16 plants. Fourteen days after infestation the number of aphids per plant were counted (Figure S4D).

Statistical analysis

Genotypic means

For each genotype we estimated a genotypic mean response to each stress (Jimenez-Gomez *et al.*, 2010; Filiault & Maloof, 2012; Riedelsheimer *et al.*, 2012). These estimated means (or predicted values) were extracted from linear mixed effect models that were fitted with the ASReml package in R (Butler, 2009).

Experiment 1: $Y = \mu + GEN + TRT + GEN \times TRT +$ random terms + e,
Experiment 2: $Y = \mu + GEN + TRT + GEN \times TRT +$ random terms + e,
Experiment 3: $Y = \mu + GEN +$ random terms + e,
Experiment 4: $Y = \mu + GEN +$ random terms + e.

In all these models Y represents the measured variable in each experiment (i.e. *A. thaliana* rosette fresh weight, thrips silver damage or aphid reproduction), and e is the

residual error. TRT is the treatment factor and *GEN* is genotype (accession) which were included as fixed factors. Random terms were included to correct for temporal and positional effects. Below, B refers to blocks, SB to sub-blocks, R to racks, S to shelves, T to trays, X to x-coordinate within tray, and Y y-coordinate within tray. The random part of experiment 1 consisted of random terms for B, R, S, B×R×S, B×R×S×T, B×R×S×T×X, and B×R×S×T×Y. For experiment 2 it consisted of random terms for B, B×T, B×T×X, and B×T×Y, and for experiments 3 and 4 random terms for B, and B/SB. In the first two experiments, predicted means from the fitted models were used to estimate variables that capture the response to each stress. The response to each stress consisted of *A. thaliana* fresh weight in the form of percentage reduction relative to control plants not exposed to stress. In the treatment where plants were exposed to both drought and herbivory by *P. rapae,* percentage weight reduction was relative to plants that experienced drought. From here onward, we will refer to the response to each stress as *P. rapae*, Drought, Drought&*Pieris*, *Botrytis*&*Pieris* and *P. xylostella* respectively. Feeding damage by thrips will be referred to as *F. occidentalis* and number of aphids as *M. persicae*. These variables are summarised in Supplementary Data1. Furthermore, these variables were used in downstream analyses as response variables in phylogenetic mixed models and to estimate heritability as explained in the section below.

Phylogenetic mixed models, heritability estimations

When comparing traits among species or among different populations of the same species, one needs to control for the non-independence of data points (correlated residuals) due to shared ancestry. This may be achieved by statistical methods such as phylogenetic mixed models or animal models (Hadfield, 2010; Hadfield & Nakagawa, 2010). A phylogenetic mixed model is a type of mixed effects model where, in addition to any other fixed or random effects, a pedigree representing the genealogy of the individuals is included in the model. This genealogy is then transformed into a variance-covariance matrix of relatedness between individuals and included in the model as a random additive genetic effect (Kruuk, 2004; Postma & Charmantier, 2007). In our study such genealogy was based on the kinship matrix obtained from the 214,051 *A. thaliana* SNPs (Atwell *et al.*, 2010) as is common in genetic association studies. By doing so, we accounted for the non-independence of plant phenotypic responses among genetically similar plant accessions. This is particularly relevant, for example when specific genotypes aggregate in a particular geographic location not because of the action of natural selection but because of genetic drift. To test hypotheses about the effect of plant life-cycle strategy and geographic location on plant responses to stress we built a different phylogenetic mixed model for each stress. Phenotypic responses of the plants were included as response variable with a Gaussian distribution. As predictors we included (i) plant life-

cycle strategy as a categorical variable (winter vs summer annual) and (ii) latitude, (iii) longitude and (iv) elevation as continuous variables (phylogenetic model type 1). The effect of environmental gradients (latitude, longitude and elevation) on *A. thaliana* plant life-cycle strategy were also modelled with similar phylogenetic mixed models but with plant life-cycle strategy included as a categorical response variable (phylogenetic model type 2). To explore potential trade-offs among plant responses to different stresses, a different phylogenetic mixed model was also built for each of the single stresses studied here. In each phylogenetic mixed model we included plant responses to a particular stress as a response variable with a Gaussian distribution, and as predictors we included responses to the other stresses and plant life-cycle strategy (phylogenetic model type 3). All phylogenetic mixed models were fitted with Bayesian Markov chain Monte Carlo (MCMC) techniques implemented in the MCMCglmm package in R (Hadfield, 2010; Hadfield & Nakagawa, 2010). In the phylogenetic mixed models types 1 and 3 the MCMC chain ran for 150,000 iterations. To prevent autocorrelation among subsequent iterations the chain was sampled every 50 iterations with the first 50,000 removed as burn-in. In the phylogenetic mixed model type 2, all MCMC parameters where ten times larger so that the chain ran for 1.5 million iterations. Autocorrelation between consecutive values was always lower than 0.1, and convergence of the chains was confirmed by visual inspection so that there were no trends in the chain and posterior distributions were not skewed. Fixed effects are presented as the posterior mean (PM) with the credible intervals (CI) of the estimate, with significance reported as the pMCMC statistic (Hadfield, 2010; Hadfield & Nakagawa, 2010). In a Bayesian model, probability distributions need to be specified via specific priors for the fixed effects and the covariance matrices. Because we did not have any *a priori* knowledge on the distribution of our data, we used flat priors. For phylogenetic mixed models type 1 and 3, we used priors with scale = 0.002 and degree of belief = 0.002 (i.e. V=1, nu=0.002). For the phylogenetic mixed models type 2 we used an inverse-Wishart prior with scale = **I***0.002 and degree of belief = 2.002 (i.e V=diag(1)*(0.002/2.002), nu=2.002). Narrow sense heritability for each response was also estimated with the heritability package in R (Kruijer *et al.*, 2015).

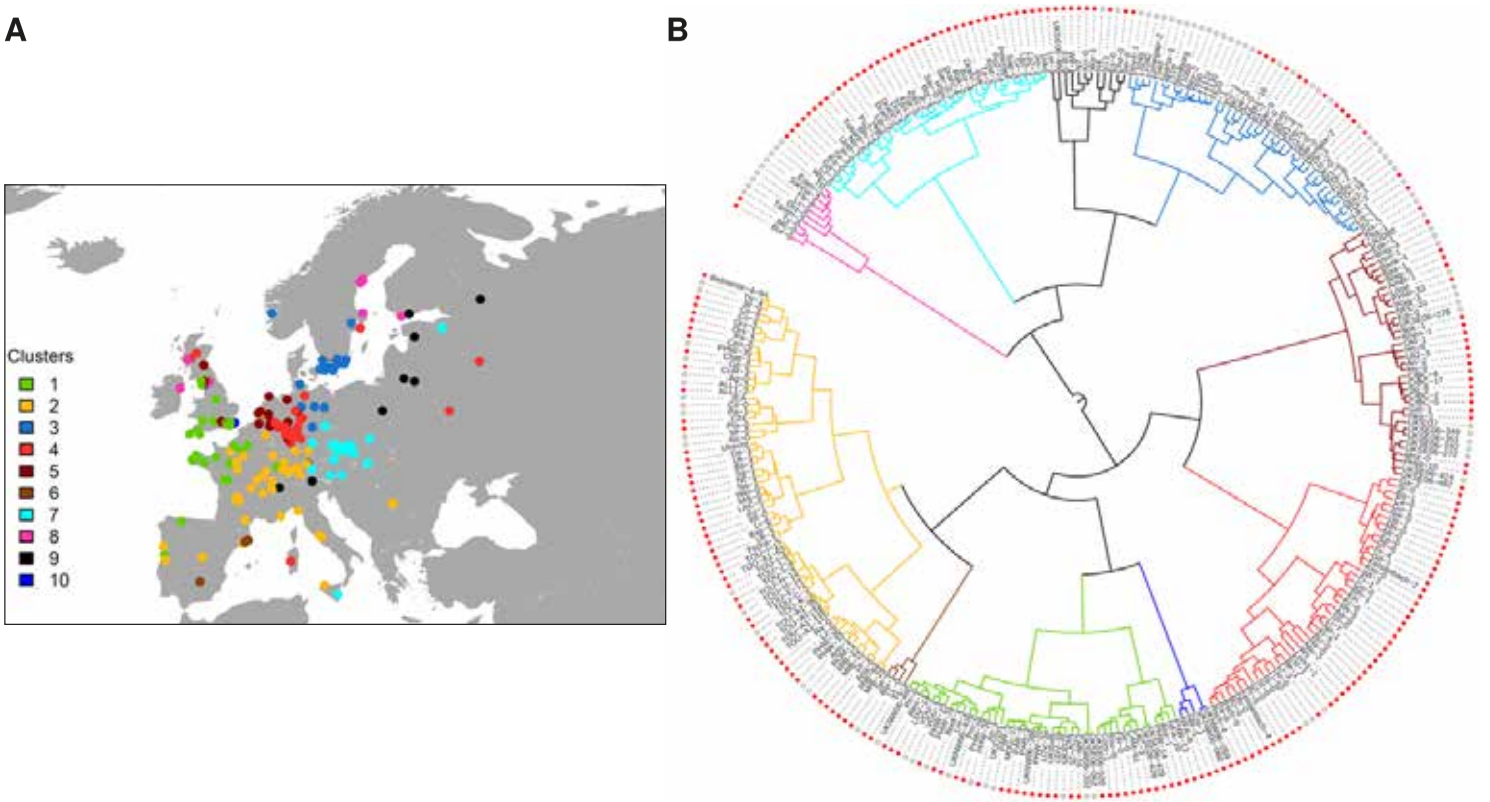

Figure 1. Population structure in the European accessions from the Hapmap population. Clusters of accessions were generated based on kinship matrix. A) Geographic distribution of genetic clusters is shown in colours. B) Dendrogram shows the relationship among accessions. Branch colours correspond to the colours in the map. Life-cycle strategy is indicated by red circles (summer annuals) and white circles (winter annuals). Dendrogram was generated with itol (http://itol.embl.de/index.shtml).

Results

Population structure of the European accessions of the Hapmap population

We first examined the patterns of population structure of the European accessions in the Hapmap population. The first nine genetic principal components showed strong geographic autocorrelation and were used to subdivide the European accessions into ten geographically distinct genetic groups (Figure 1).

Heritability of *A. thaliana* responses to abiotic and biotic stresses

An important condition for natural selection to act upon a trait (and therefore to allow local adaptation), is that this trait has phenotypic variation which is genetically determined. This condition was met for *A. thaliana* responses to different stresses as the different traits measured showed substantial heritable variation (Table 1). The largest trait variation was observed for the response to *P. xylostella* (CV = 138%) while the response to *M. persicae* displayed the lowest variation (CV= 20%). No relationship was observed between degree of trait variation and heritability. The largest heritability was observed for feeding damage by thrips (*F. occidentalis*) (h^2 = 0.90), while plant biomass reduction after *P. xylostella* feeding had the lowest heritability (h^2 = 0.25). The response to Drought&*Pieris* exhibited less variation and a lower heritability (CV = 33%, h^2 = 0.39) than the response to *P. rapae* alone (CV= 43%, h^2 = 0.60). The response to *Botrytis*&*Pieris* (CV= 117%, h^2 = 0.67) had more variation and a higher heritability than the response to *P. rapae* alone.

Table 1. Summary of trait values for *A. thaliana* resistance to abiotic and biotic stresses.

Response trait	Experiment	Min.	Mean	Max.	CV	N	h^2	va	ve
Drought	1	-25.94	19.22	50.07	75	308	0.41	83.32	119.96
P. rapae	1	-5.03	32.40	87.80	43	308	0.60	114.79	76.72
Drought&*Pieris*	1	-3.39	48.25	143.80	33	308	0.39	96.15	153.52
Botrytis*&*Pieris	1	-31.44	14.42	90.13	117	308	0.67	190.83	95.41
P. xylostella	2	-24.86	14.15	78.16	138	265	0.25	94.08	287.25
F. occidentalis	3	0.00	21.98	56.51	43	308	0.90	77.46	8.28
M. persicae	4	13.12	27.89	44.12	20	299	0.34	10.57	20.58

h^2 = Narrow sense heritability, va= Additive genetic variance, ve= residual variance, CV = Coefficient of variation (%). N = number of observations after subset for European accessions.

Effect of life history strategy, genetic structure and environmental gradients on stress responses in *A. thaliana*

We classified *A. thaliana* accessions as winter and summer annuals based on their flowering time. Of the 308 accessions analysed, 89 did not produce flowers after 75 days and were therefore classified as winter annuals, while 219 behaved as summer annuals (Supplementary Data 1). Within this collection of genotypes, winter annuals were more often found in lower elevations and higher latitudes, whereas longitude was not a significant explanatory variable (Figure S5, Table 2). Furthermore, the two life-history strategies were not equally distributed among genetic groups. For example, cluster 8 and cluster 3 had mostly winter annuals while cluster 10 comprised summer annuals exclusively (Figure 1B, Figure S5B). Phenotypic differences between genetic groups were tested by ANOVA after correction for life-cycle strategy. Overall, stress responses did not differ significantly between genetic clusters, with the exception of the response to *F. occidentalis* ($p < 10^{-6}$). For this stress, accessions belonging to clusters 1 and 3 suffered particularly low and high damage respectively (Figure S6).

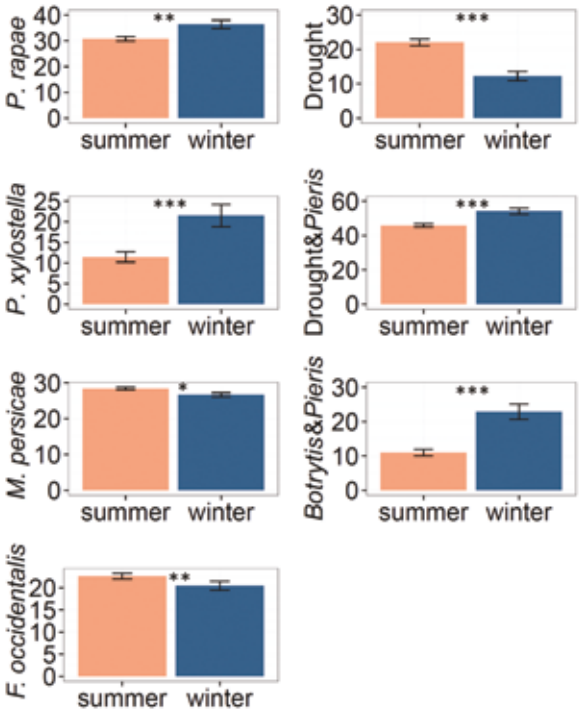

Figure 2. Stress responses of *A. thaliana* accessions belonging to either winter annual or summer annual life-cycle strategies. Accessions that required vernalisation for flowering were classified winter annuals (blue, n = 89), the rest were classified summer annuals (orange, n = 219). Bars show mean value ± SE. Stress responses to *P. rapae* caterpillars, *P. xylostella* caterpillars, Drought, Drought&*Pieris* and *Botrytis*&*Pieris* are represented by the percentage of biomass reduction in *A. thaliana* in response to each stress. Response to *M. persicae* is represented by the number of aphids produced. Response to *F. occidentalis* thrips is represented by the amount of feeding damage in mm^2. Bayesian p-values are indicated as * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P < 0.001$.

Table 2. Bayesian phylogenetic mixed model analysis to assess differences in life-cycle strategy and geographical gradients for each stress. For each variable the posterior mean and 95% credible intervals (in parentheses) are presented. For the fixed effects the Bayesian p-value is also presented, and significance indicated in bold text.

Response trait	Life-cycle strategy		Elevation		Latitude		Longitude		Plant genealogy
	PM	*P*	PM	*P*	PM	*P*	PM	*P*	PM
Drought	**-9.21 (-12.59 - -5.62)**	**< 0.001**	0.003 (-0.004 - 0.010)	0.389	-0.12 (-0.56 - 0.27)	0.566	**0.229 (-0.004 - 0.426)**	**0.043**	1.51 (<0.001 - 7.133)
P. rapae	**4.61 (1.38 - 8.18)**	**0.007**	-0.005 (-0.012 - 0.001)	0.167	0.12 (-0.29 - 0.53)	0.575	-0.03 (-0.24 - 0.19)	0.768	4.22 (<0.001 - 21.26)
Drought&*Pieris*	**7.37 (3.10 - 11.13)**	**< 0.001**	-0.002 (-0.011 - 0.005)	0.534	0.23 (-0.24 - 0.68)	0.352	-0.09 (-0.32 - 0.14)	0.432	2.34 (<0.001 - 11.81)
Botrytis&*Pieris*	**10.68 (6.76 - 14.81)**	**< 0.001**	-0.004 (-0.013 - 0.003)	0.329	0.25 (-0.23 - 0.73)	0.326	-0.11 (-0.38 - 0.12)	0.383	3.05 (<0.001 - 14.65)
P. xylostella	**10.40 (4.86 - 15.75)**	**< 0.001**	0.004 (-0.007 - 0.015)	0.444	-0.05 (-0.74 - 0.58)	0.867	-0.07 (-0.45 - 0.25)	0.683	5.49 (<0.001 - 28.58)
F. occidentalis	**-3.50 (-6.00 - -1.31)**	**0.004**	-0.003 (-0.008 - 0.001)	0.149	0.02 (-0.29 - 0.32)	0.893	0.16 (-0.03 - 0.35)	0.102	38.99 (0.029 - 99.53)
M. persicae	**-1.75 (-3.20 - -0.19)**	**0.018**	-0.001 (-0.003 - 0.002)	0.629	-0.08 (-0.27 - 0.09)	0.357	0.008 (-0.118 - 0.123)	0.851	3.78 (<0.001 - 17.17)
Life-cycle strategy	NA	NA	**-0.26 (-0.55 - -0.01)**	**0.031**	**15.58 (4.21 - 29.39)**	**0.003**	-0.971 (-6.61 - 4.31)	0.732	0.01 (<0.001 - 0.02)

PM = Posterior mean, *P* = Bayesian p-value. Plant genealogy was included as random effect in the models, thus p-values are not reported.

A different phylogenetic mixed model was built for each stress factor, to explore whether plant responses varied between winter and summer annuals and among accessions located along geographic gradients. We found that life-cycle strategy significantly affected all responses measured (Figure 2, Table 2). The percentage of *A. thaliana* biomass reduction as a result of feeding by *P. rapae* and *P. xylostella* caterpillars was 6% and 10% larger in winter than in summer annuals, respectively. The percentage of biomass reduction by the combined stress Drought&*Pieris*, and *Botrytis*&*Pieris* was 8% and 12% larger in winter than summer annuals, respectively. The percentage of biomass reduction in *A. thaliana* caused by drought was 10% lower in winter annuals than in summer annuals. Feeding damage by thrips (*F. occidentalis*) was 8% lower in winter annuals than in summer annuals, and the number of offspring produced by aphids (*M. persicae*) was 6% lower on winter annuals than on summer annuals.

We hypothesised that latitude, longitude and elevation of the locations from which the accessions had been collected would affect plant responses and hence these variables were also included in the phylogenetic mixed models as predictors. Although the three gradients were highly correlated with several environmental variables measured at each sampling location (Figure S3), we found only a single significant association with *A. thaliana* responses to stress (Table 2). In particular, we found a significant, positive association between longitude and percentage of biomass reduction caused by drought (Table 2, Figure S7). In all phylogenetic mixed models for plant responses to stress, and in accordance with our heritability estimates, the importance of *A. thaliana* genealogy was moderate, but relatively higher for the models on plant responses to thrips (*F. occidentalis*) (Table 2). Furthermore, a longitudinal gradient was observed for flowering time and a latitudinal gradient was observed for flowering time after vernalisation (Table S2, Figure S7).

Trade-offs in *A. thaliana* responses to stress

We built a separate phylogenetic mixed model for each of the single stresses and assessed whether they correlated with the other stresses. Because of its importance, plant life-cycle strategy was also included as a co-factor in these models. Among the different stresses, we found a clear negative relationship between response to drought and to *P. rapae* herbivory (Posterior Mean = -0.12, $P = 0.049$), which indicates that these two responses trade off (Figure 3 and Table 3). Responses to *P. rapae* herbivory were also negatively correlated (Posterior Mean =-0.42, $P = 0.005$) with reproduction by the aphid *M. persicae*.

Table 3. Bayesian phylogenetic mixed model analysis to assess correlations among single stresses. For each variable the posterior mean, 95% credible intervals and Bayesian P-values are presented. Significance is indicated in bold text.

Response trait	Life-cycle strategy		Drought		*P. rapae*		*P. xylostella*		*F. occidentalis*		*M. persicae*	
	PM	*P*	PM	*P*	PM	*P*	PM	*P*	PM	*P*	PM	*P*
Drought	**-6.70 (-10.77 - -2.86)**	**<0.001**			**-0.13 (-0.26 - -0.01)**	**0.032**	-0.08 (-0.16 - 0.01)	0.075	0.14 (-0.03 - 0.32)	0.133	0.09 (-0.21 - 0.38)	0.551
P. rapae	2.26 (-2.09 - 6.13)	0.276	**-0.12 (-0.24 - 0.01)**	**0.049**			0.05 (-0.03 - 0.14)	0.227	0.12 (-0.06 - 0.30)	0.187	**-0.42 (-0.70 - -0.13)**	**0.005**
P. xylostella	7.7 (2.42 - 13.35)	**0.003**	-0.16 (-0.32 - 0.04)	0.084	0.12 (-0.06 - 0.28)	0.196			-0.15 (-0.40 - 0.10)	0.262	<0.001 (-0.41 - 0.42)	0.988
F. occidentalis	**-3.72 (-6.39 - -1.11)**	**0.011**	0.05 (-0.03 - 0.12)	0.198	0.06 (-0.02 - 0.13)	0.153	-0.03 (-0.08 - 0.03)	0.314			-0.05 (-0.25 - 0.13)	0.649
M. persicae	-1.09 (-2.93 - 0.43)	0.191	0.01 (-0.03 - 0.07)	0.524	**-0.07 (-0.12 - -0.02)**	**0.002**	<0.001 (-0.04 - 0.04)	0.973	0.01 (-0.08 - 0.08)	0.809		

PM = Posterior mean, *P* = Bayesian P-value.

Discussion

Geographic patterns in genetic diversity of European accessions

We have studied plant responses in 308 European *A. thaliana* accessions. These accessions displayed a strong isolation by distance (IBD) which implies that, relative to distantly located accessions, accessions from the same location are genetically similar. Our findings are in line with several studies that have reported strong similar IBD patterns in *A. thaliana* accessions from Europe (Sharbel *et al.*, 2000; Koornneef *et al.*, 2004; Ostrowski *et al.*, 2006; Schmid *et al.*, 2006; Platt *et al.*, 2010), although such isolation is weaker among the accessions introduced in North America (Platt *et al.*, 2010). Many factors can contribute to isolation by distance patterns, but *A. thaliana* populations are suggested to become genetically isolated at a fast rate due to the self-pollinating nature of the species (Alonso-Blanco & Koornneef, 2000). This trait is thought to be also important in explaining why *A. thaliana* populations are highly inbred with large genetic variation within and among populations (Nordborg *et al.*, 2005; Horton *et al.*, 2012). This kind of population structure has implications for our study, especially when inferring local adaptation. If similar genotypes are clustered in space, correlations between genotype and environment may arise due to spatial clustering and lead to misleading interpretations (Platt *et al.*, 2010).

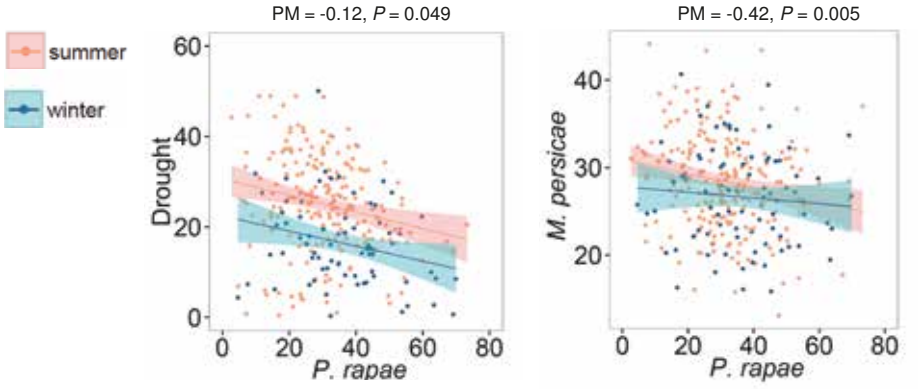

Figure 3. Trade-off between response to abiotic and biotic stresses among *A. thaliana* accessions. Accessions that required vernalisation for flowering were classified winter annuals (blue), the rest were classified summer annuals (orange). Stress responses to *P. rapae* and Drought, are represented by the percentage of biomass reduction in *A. thaliana* in response to each stress. Response to *M. persicae* is represented by the number of aphids produced. Lines represent a linear regression fit and shades the 95% confidence interval. These lines were just used for better appreciation of the data since correlations tests were carried out using a linear mixed model as described in Materials and Methods. Posterior means (PM) and (*P*) Bayesian P-values are indicated.

For example, Allard *et al.* (1972) provided a classical example of local adaptation in *Avena barbata* in California, but a deeper analysis revealed that these populations were not locally adapted but spatially structured (Latta & Gardner, 2009). In our study, we corrected for these potentially misleading associations by implementing a phylogenetic mixed model that accounted for the genetic resemblance among individuals and, therefore, their spatial co-location.

Arabidopsis responses to abiotic and biotic stresses differ between life-history strategies

Two life-history strategies have been described in *A. thaliana*: winter and summer annuals (Pigliucci, 1998; Koornneef *et al.*, 2004; Shindo *et al.*, 2007). The winter annual is considered the ancestral state because loss and reduced function alleles at two genes (*FRI* and *FLC*) confer the summer annual phenotype (Michaels *et al.*, 2003; Amasino, 2004). It is generally accepted that summer annuals occur closer to the equator while winter annuals occur in temperate regions (Johanson *et al.*, 2000; Michaels *et al.*, 2003). Despite this general assumption, no clear geographical patterns were observed for the occurrences of these two life strategies in an earlier study (Shindo *et al.*, 2007). Here, we observed a higher proportion of summer annuals at high elevations and southern latitudes. In line with this observation, an increase of summer annuals with increasing altitude in Spain (400 -1700 m) and the Swiss alps (600-2700 m) has been reported (Pico, 2012; Luo *et al.*, 2015). The predominance of summer annuals at high elevations has been attributed to the extended and cold winters that *A. thaliana* may be able to withstand as seed but not as rosette. On the other hand, several studies have reported a predominance of summer annuals at low altitudes along elevational gradients in the Iberian peninsula (100 – 1600 m) (Montesinos-Navarro *et al.*, 2011; Mendez-Vigo *et al.*, 2013) that was explained by the extreme drought conditions in summer that may select for early flowering (Luo *et al.*, 2015). Furthermore, we also observed genetic clusters consisting mostly of winter annuals at northern latitudes. This is also consistent with literature reports where non-random distribution of strong winter annuals has been observed in Scandinavia (Shindo *et al.*, 2005).

Independent of the biogeographic distribution of life-cycle strategies, our study revealed that life cycle strategy is the most important factor explaining plant responses to most of the stresses studied here, excepts for resistance to thrips. We found that the degree of damage to thrips (*F. occidentalis*) was strongly influenced by geographic-genetic structure. This was also reflected by the high heritability values estimated for thrips resistance. Interestingly, this geographic-genetic structure resembles the geographic-genetic distribution of glucosinolates, a well-established defence mechanisms against generalist herbivores, including thrips (Zust *et al.*, 2012; Brachi *et al.*, 2015).

Several authors have suggested that at higher latitude herbivore pressure is lower and hence plants are less defended (Pennings & Silliman, 2005; Salazar & Marquis, 2012; Halbritter *et al.*, 2013). Assuming that winter annuals are more common in these habitats, we expected that relative to summer annuals, winter annuals would be less defended against herbivory (Pennings & Silliman, 2005; Salazar & Marquis, 2012; Halbritter *et al.*, 2013). We found that winter annuals were more susceptible to damage by the two chewing specialist caterpillars *P. rapae* and *P. xylostella* than summer annuals. However, the opposite was observed for the two piercing-sucking generalist herbivores: the phloem-feeding aphid *M. persicae* and the cell-content feeding thrips *F. occidentalis*. Although we only tested four different insect herbivore species, these results add to the growing body of literature that suggests that the degree of specialisation (Mathur *et al.*, 2011; Ali & Agrawal, 2012; Barrett & Heil, 2012) and insect feeding guild (De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007; Bidart-Bouzat & Kliebenstein, 2011) may exert different selective pressures on plants.

Arabidopsis thaliana accessions displaying different life-cycle strategies cope with drought stress in a different manner. For instance, winter annuals employ drought avoidance (i.e. mechanisms that maintain the internal water status under limited water conditions such as stomatal closure and increased root growth) and summer annuals employ drought escape (i.e. shift in phenology that allows plants to grow and reproduce by avoiding activity during periods of water scarcity) (McKay *et al.*, 2003; Des Marais *et al.*, 2012; Juenger, 2013; Easlon *et al.*, 2014). Furthermore, a link has been observed between life-cycle strategies and heat tolerance, such that late-flowering plants are more sensitive to heat stress (Bac-Molenaar *et al.*, 2015a). We, therefore, predicted that under our controlled experimental conditions summer annuals would be less adapted to drought than winter annuals. Our hypothesis was confirmed. When exposed to drought stress, summer annuals gained less weight than winter annuals. A strong correlation between flowering life history and drought resistance has already been observed in the laboratory in *A. thaliana* (McKay *et al.*, 2003; Juenger, 2013; Easlon *et al.*, 2014). In fact, winter annuals have higher water use efficiency than summer annuals (Juenger, 2013; Lovell *et al.*, 2013). The association between these two traits has been suggested to be partially caused by alleles of the *FLC* and *FRI* genes (McKay *et al.*, 2003; Scarcelli *et al.*, 2007). A recent study that exposed a large collection of *A. thaliana* accessions to controlled moderate drought found the opposite, i.e. that summer annuals were more resistant than winter annuals (Bac-Molenaar *et al.*, 2015b). The discrepancy between these studies may arise from different levels or time patterns of drought stress applied. For instance, it has been determined that severe drought and moderate drought elicit different physiological and molecular responses in *A. thaliana* (Skirycz *et al.*, 2011). In addition, studies on other taxa also found evidence that flowering-time genes can

have pleiotropic effects on other traits such as water use efficiency in *Brassica rapa* (Franks, 2011), vegetative biomass in *A. barbata* (Latta & Gardner, 2009) and size at reproduction in *B. rapa* (Haselhorst *et al.*, 2011).

Here, we tested the hypothesis that *A. thaliana* responses to stress would vary with elevation, latitude and longitude. The only significant correlation found was between longitude and *A. thaliana* responses to drought: drought resistance (i.e. reduced weight loss when experiencing the stress) increased eastwards. Interestingly, flowering time in European *A. thaliana* accessions has also been found to correlate with longitude, where the proportion of early flowering accessions increases eastwards (Samis *et al.*, 2008). In this study we also observed a longitudinal gradient for flowering time decreasing eastwards. Interestingly, experimental evolution experiments in several plant species demonstrated that wet soil and late-season drought conditions selected for early flowering accessions which displayed low water use efficiency. On the other hand, early-season drought selected for higher water use efficiency (Heschel & Riginos, 2005; Sherrard & Maherali, 2006). Large physiological and transcriptional changes upon drought have been reported between winter and summer annuals in *A. thaliana* (Des Marais *et al.*, 2012), further underlining the importance of life history in responses to stress. Future efforts should be devoted to understanding at the mechanistic level how accessions displaying distinct life-cycle strategies cope with abiotic and biotic stresses.

Plant responses against different stresses are traded off

Upon stress, plants are able to elicit defence or resistance mechanisms that are specific for the attacker or adverse abiotic condition (Reymond *et al.*, 2000; De Vos *et al.*, 2005; Verhage *et al.*, 2011; Appel *et al.*, 2014; Stam *et al.*, 2014). However, plant responses to a specific stress are usually modulated at the individual or population level by co-occurrence with other stresses, because plants have limited resources and are locally exposed to different selection forces. In addition, life-history theory predicts that the defensive repertoire is also genetically constrained so that adaptation to different stresses can trade off. For example, abiotic stresses tend to have a negative impact on how plants deal with pathogens or herbivores (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). We hypothesised that the different *A. thaliana* accessions would be limited in their responses to stresses so that they would be negatively correlated. A negative correlation was observed between responses to *P. rapae* herbivory and drought stress or aphid reproduction on the plants. Trade-offs are expected when the defences are specifically tailored and are costly to the plant (Erb *et al.*, 2011). This suggests that defences elicited by drought and *P. rapae* are specific and costly. An example of specific defences that constrain each other is the interaction between jasmonic acid (JA) and salycilic acid (SA) signalling pathways

(Sendon *et al.*, 2011; Van der Does *et al.*, 2013; Caarls *et al.*, 2015). Caterpillar feeding and drought induce a common set of responses at least at the transcriptional level (Reymond *et al.*, 2000; Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Vos *et al.*, 2013a), but at the metabolic level clear differences can be recorded (Weldegergis *et al.*, 2015). Recently, it has been shown that JA signalling is required to increase ABA levels under water stress conditions (De Ollas *et al.*, 2015) and that ABA is involved in plant defence against caterpillar feeding (Vos *et al.*, 2013b). A plausible explanation for the observed trade-off between resistance to drought and feeding by the specialist herbivore *P. rapae* may be resource allocation. Furthermore, extensive down-regulation under drought stress has been observed for genes that are up-regulated by *P. rapae* herbivory, suggesting a mechanistic explanation for this trade-off (Coolen *et al.*, 2015).

Conclusion

We exposed a large collection of well genotyped European *A. thaliana* accessions to diverse biotic and abiotic and combined stresses. We have identified heritable genetic variation for responses to the different stresses, as estimated by marker-based heritability. Furthermore, we found that plant life-cycle strategy is a major determinant of responses to the different stresses we applied in this study. Moreover, an extensive trade-off between *A. thaliana*'s response to *P. rapae* herbivory and drought and between the responses to *P. rapae* and aphids was observed. Finally, we found an effect of drought on biomass reduction across a longitudinal gradient. Future experiments, should aim to understand the mechanisms of how accessions with different life strategies deal with different stresses.

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Supplementary Information (See Appendix)

Table S1. Summary of the climate variables mined for this study that vary along geographical gradients.

Variable	Resolution	URL	Reference
Annual aridity	1km	http://www.cgiar-csi.org	a
PET month 3-6	1km	http://www.cgiar-csi.org	a
Annual relative humidity	50km	http://www.sage.wisc.edu/atlas/maps.php	b
Elevation	10km	http://www.sage.wisc.edu/atlas/maps.php	c
Annual mean temperature	1km	http://www.worldclim.org	d
Max temperature of warmest month	1km	http://www.worldclim.org	d
Min temperature of coldest month	1km	http://www.worldclim.org	d
Annual precipitation	1km	http://www.worldclim.org	d

PET = Potential evapotranspiration, Min= Minimum, Max= Maximum.

a Trabucco, A., and Zomer, R.J. 2009. Global Aridity Index (Global-Aridity) and Global Potential Evapo-Transpiration (Global-PET) Geospatial Database. CGIAR Consortium for Spatial Information. Published online, available from the CGIAR-CSI GeoPortal at: http://www.csi.cgiar.org.

b New, M.G., M. Hulme and P.D. Jones, 1999: Representing 20th century space-time climate variability. I: Development of a 1961-1990 mean monthly terrestrial climatology. J. Climate. 12, 829-856.

c National Oceanic and Atmospheric Administration (NOAA) and U.S. National Geophysical Data Center, TerrainBase, release 1.0 (CD-ROM), Boulder, Colo.

d Hijmans, R.J., S.E. Cameron, J.L. Parra, P.G. Jones and A. Jarvis, 2005. Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology 25: 1965-1978.

Table S2. Bayesian phylogenetic mixed model analysis to assess differences in flowering time without and after vernalization and geographical gradients. For each variable the posterior mean and 95% credible intervals (in parentheses) are presented. For the fixed effects the Bayesian P-value is also presented, and significance indicated in bold text. Because flowering type was estimated based on flowering time, this variable was excluded from the models of flowering time.

	Elevation		**Latitude**		**Longitude**		**Plant genealogy**
	PM	*P*	PM	*P*	PM	*P*	PM
Flowering time	-0.004 (-0.014 - 0.006)	0.444	0.28 (-0.37 - 1.03)	0.477	**0.47 (0.01 - 0.84)**	**0.019**	8020 (5879 - 10084)
Flowering time vern.	-0.001 (-0.002 - 0.002)	0.888	**0.20 (0.01 - 0.37)**	**0.024**	0.02 (-0.10 - 0.13)	0.777	143.82 (41.29 - 268.11)

Flowering time vern. = Flowering time after vernalization, PM = Posterior mean, *P* = Bayesian P-value. Plant genealogy was included as random effect in the models, thus P-values are not reported.

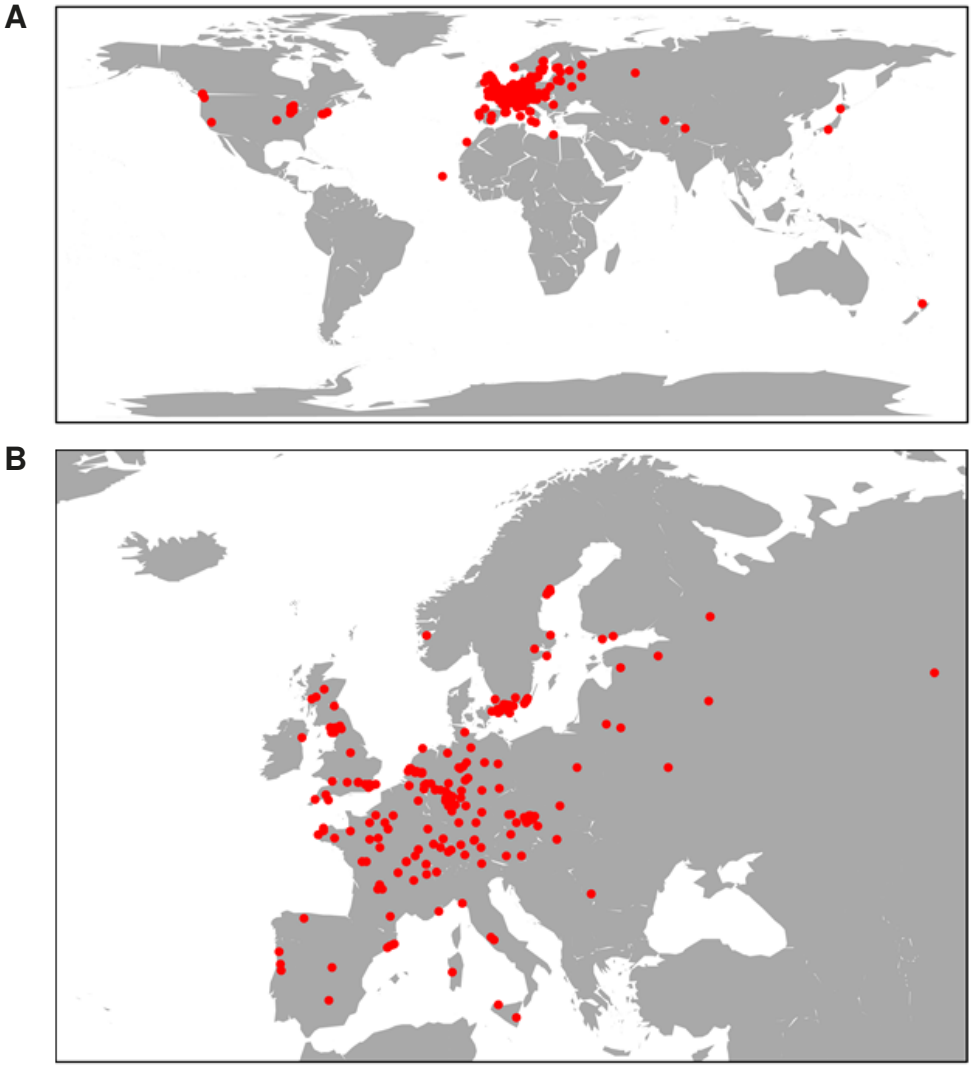

Figure S1. Geographic distribution of *Arabidopsis thaliana* accessions from the Hapmap population. (A) Worldwide distribution; (B) European distribution. Locations are shown from which the accessions of the Hapmap population have been collected. The Hapmap population was chosen to represent the geographic range of *A. thaliana*. The plant species is native to Europe and Asia. This native range is represented in the Hapmap population by 320 accessions. Furthermore, *A. thaliana* has been naturalized at many places around the world and this is represented in the Hapmap population by 33 accessions from North America, 2 accessions from Japan, 1 from Libya, 1 from India, 1 from New Zealand, 1 from Cape Verde Island and 1 from the Canary Islands.

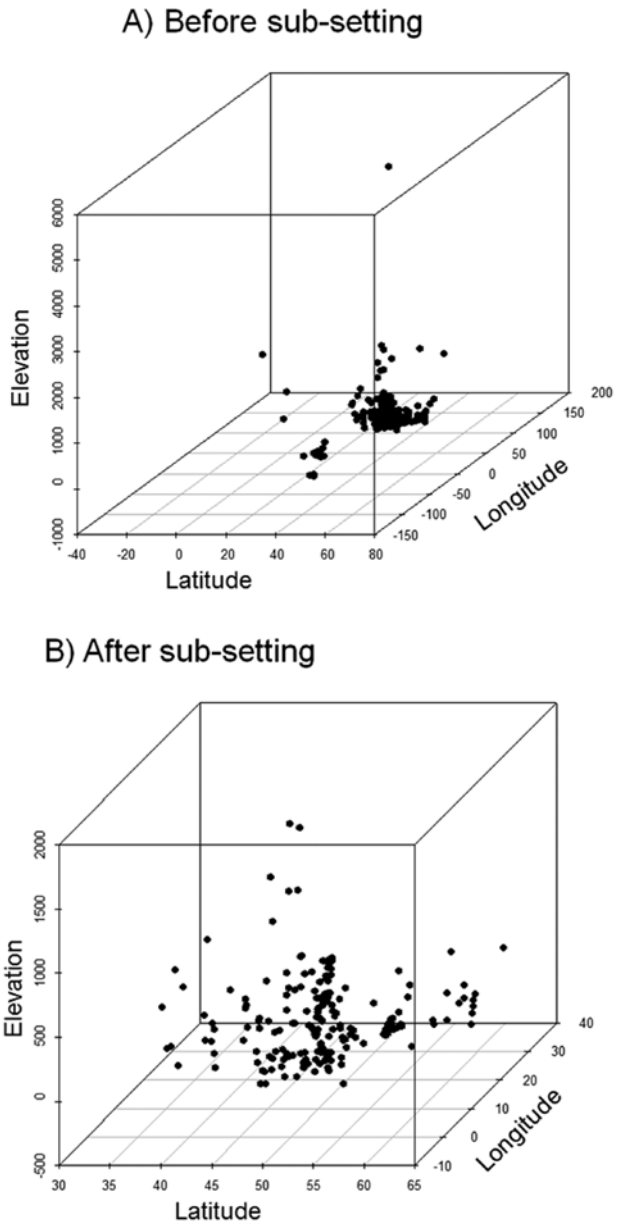

Figure S2. Subset of accessions for Europe. We limited the analysis to accessions from Europe (1) To avoid associations to be affected by geographical outliers and (2) Europe represents the centre of genetic diversity in *A. thaliana*, while North American accessions most likely were introduced by humans and are not genetically diverse from Euro-Asia. A) All accessions. B) Accessions limited to a Latitude ≥ 30, Longitude between -50 and 50 and elevation ≤ 2000 m.

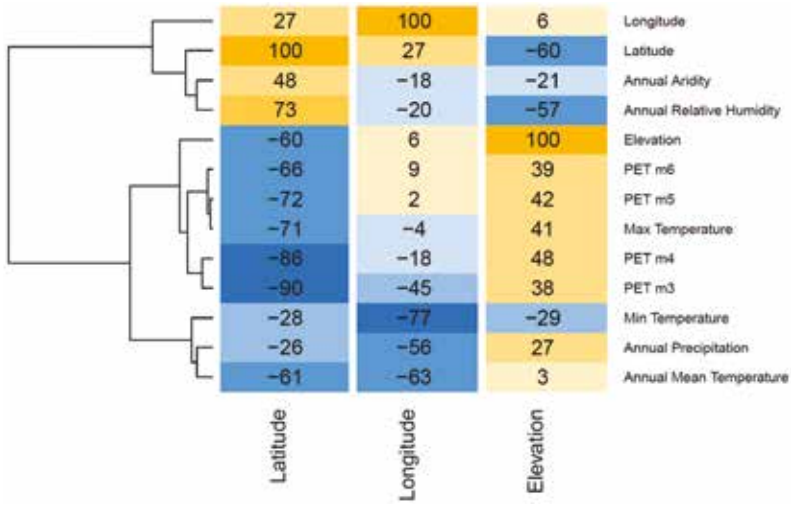

Figure S3. Correlation matrix between geography and climate variables for the 308 *A. thaliana* accessions investigated. Heatmap displays Spearman correlation coefficients multiplied by 100. Negative (blue) and positive (gold) correlations are indicated. Climate variables are clustered according to Ward's minimum variance method. Max temperature = maximum temperature during the warmest month. Min temperature = minimum temperature during the coldest month, PET = Potential evapotranspiration; m 3-6 correspond to the month of the year.

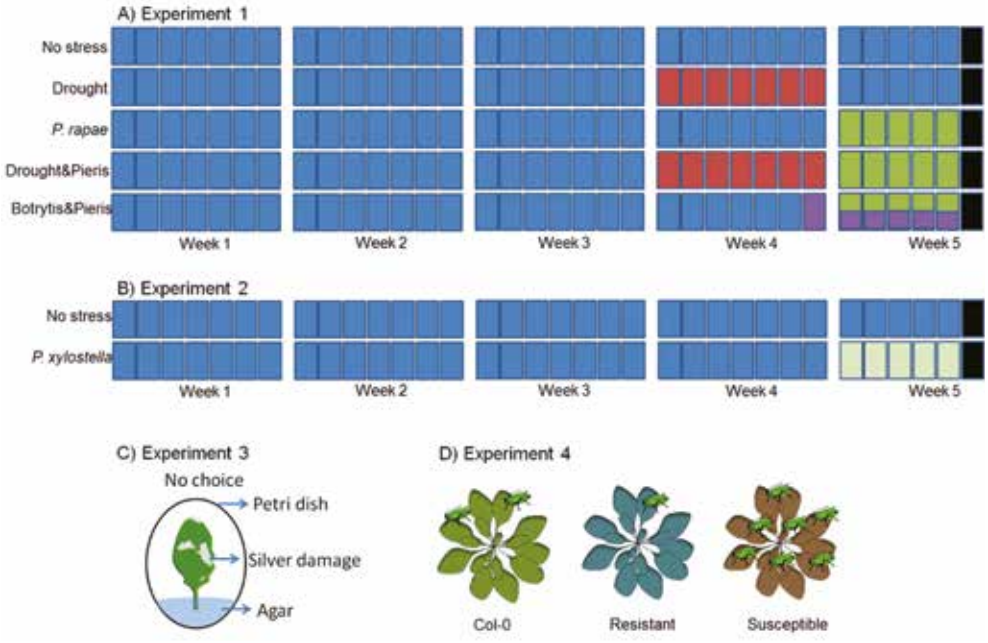

Figure S4. Experimental Design and Treatments Scheme. A) In experiment (1) the effects of drought and herbivory by *P. rapae* caterpillars either as single treatment or preceded by drought stress or pathogen infestation were evaluated. Blue indicates that the plants were growing in no-stress conditions. Drought stress period is indicated in red. *Botrytis cinerea* time of inoculation is indicated in purple. *Pieris rapae*

inoculation is indicated in dark green. The moment of plant response assessment is indicated in black. B) In experiment (2) the effects of herbivory by *P. xylostella* caterpillars was evaluated. Blue indicates that the plants were growing under no-stress conditions. *Plutella xylostella* time of inoculation is indicated in light green. The moment of plant response assessment is indicated in black. C) In experiment (3) the effects of thrips infestation were evaluated by measuring the amount of feeding damage. In experiment (4) aphid reproduction on the accessions was evaluated.

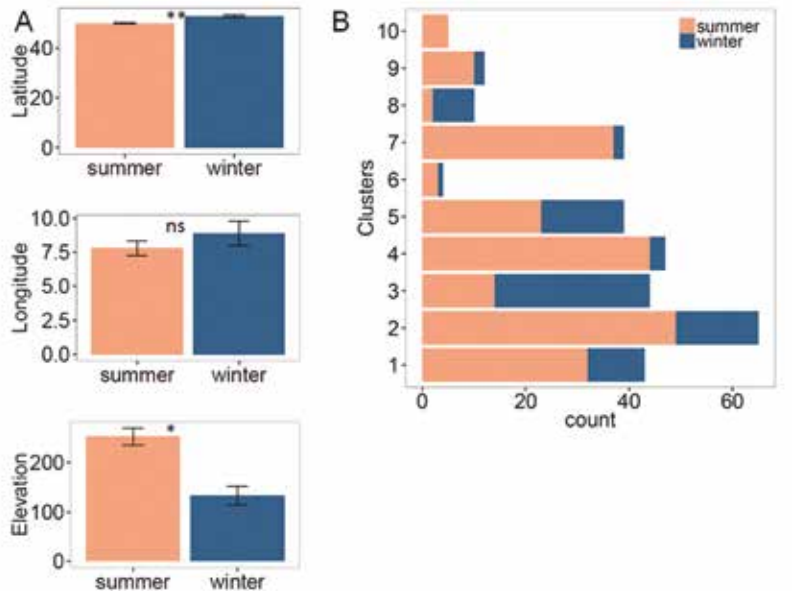

Figure S5. Spatial and genetic distribution of summer annual (219 accessions) and winter annual (89 accessions) life histories in *A. thaliana*. A) Geographical distribution. B) Proportion of summer and winter annuals per genetic group. Bayesian p-values are indicated as ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P < 0.01$.

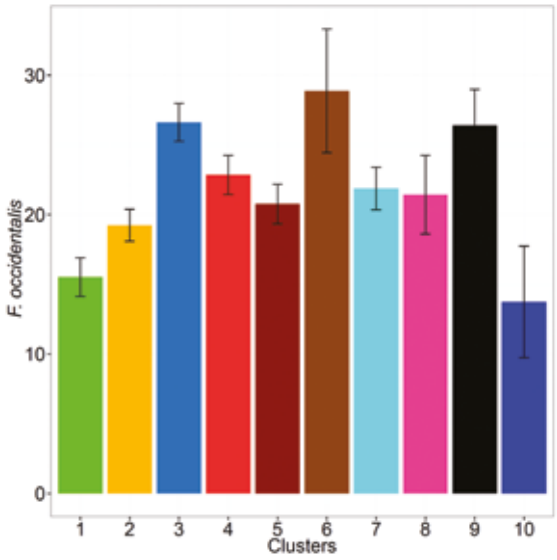

Figure S6. Feeding damage (mm²) by thrips per genetic clusters. Colours in clusters represent the same colours as in Figure 1 in the main text. Bars show mean value ± SE.

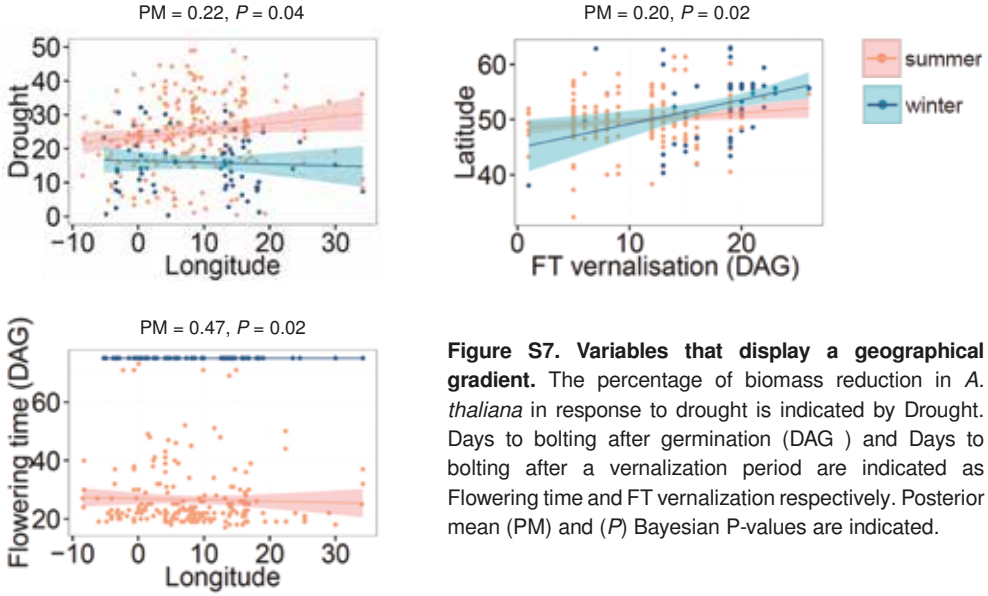

Figure S7. Variables that display a geographical gradient. The percentage of biomass reduction in *A. thaliana* in response to drought is indicated by Drought. Days to bolting after germination (DAG) and Days to bolting after a vernalization period are indicated as Flowering time and FT vernalization respectively. Posterior mean (PM) and (*P*) Bayesian P-values are indicated.

Chapter 3

Transcriptome dynamics of *Arabidopsis* during sequential biotic and abiotic stresses

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Abstract

In nature, plants have to cope with a wide range of biotic and abiotic stress conditions that often occur simultaneously or in sequence. Previously, adaptive plant stress responses and their corresponding signaling pathways have been mainly investigated at the single stress and single time point level. In this study, we aimed to gain a deeper understanding of the interaction between different biotic and abiotic stress response pathways when activated in sequence. Using RNA-seq, we analyzed the dynamics of whole-transcriptome profiles of *Arabidopsis thaliana* plants exposed to (1) infection by the necrotrophic fungus *Botrytis cinerea,* (2) herbivory by *Pieris rapae*, or (3) drought stress by water withhold. In addition, changes in the dynamics of the transcriptome profiles as induced by these three stresses were analyzed when the plants had been exposed before to either one of the other two stresses. Each of the single stresses induced specific expression profiles that showed dynamic changes over time. Interestingly, when two stresses were applied in sequence, the plants swiftly adapted their transcriptome to the second stress applied, irrespective of the nature of the first stress. Despite the great overlap in transcriptome profiles of plants that received a certain stress with or without pre-exposure to another stress, significant first-stress-signatures could be identified in the sequential stress profiles. Using a bioinformatics approach for the analysis of the behaviour of co-expressed gene clusters, genes and biological processes specifically affected by single and sequential stresses were highlighted. These analyses showed that first-stress-signatures in second stress transcriptional profiles were remarkably often related to responses to phytohormones, strengthening the notion that hormones are global regulators of positive and negative interactions between different types of stress. Because prior stresses can affect the level of tolerance against a subsequent stress, the first-stress-signatures in the transcriptomes of plants exposed to sequential stresses provide important leads for the identification of molecular players that are decisive in the interactions between stress response pathways.

Keywords: RNA-seq, combined stresses, multiple stresses, gene clusters, co-regulated genes, *Botrytis cinerea*, *Pieris rapae*, drought.

Introduction

Plants are continuously threatened by a wide range of harmful microbial pathogens and insect herbivores. Besides these biotic stresses, plants are also exposed to extreme abiotic environmental conditions such as drought, heat, cold, water logging, high salinity or toxicity. Adaptive plant responses to single biotic and abiotic stresses have been extensively studied. Both biotic and abiotic stress responses are associated with the action of the phytohormones jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA), and to a lesser extent with cytokinin, brassinosteroids and auxin (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Giron *et al.*, 2013; O'Brien & Benková, 2013; Kazan & Lyons, 2014; Broekgaarden *et al.*, 2015). JA and ET are generally involved in defense against pathogens with a necrotrophic lifestyle, whereas defenses against biotrophs are commonly controlled by SA (Glazebrook, 2005). ABA is associated with plant development and abiotic stresses (Yamaguchi-Shinozaki & Shinozaki, 2006), such as drought, but its role in modulating JA-dependent defenses against insect herbivores and SA-dependent defenses against pathogens is becoming increasingly evident (Yasuda *et al.*, 2008; Verhage *et al.*, 2011; Vos *et al.*, 2013b). Antagonistic and synergistic interactions between hormonal signal-transduction pathways is thought to provide the plant with a regulatory potential to adapt to its complex biotic and abiotic environment while utilizing its resources in a cost-efficient manner (Pieterse *et al.*, 2012; Vos *et al.*, 2013a; Vos *et al.*, 2015).

In natural and agricultural settings, plants often have to cope with multiple stress conditions at the same time. In the context of climate change, it is highly likely that the frequency and complexity of these multi-stress conditions will increase and further threaten crop yield. Abiotic stresses can significantly affect plant responses to biotic stresses and vice versa, depending on the timing, nature, and severity of the stresses (Atkinson & Urwin, 2012; Appel *et al.*, 2014). How plants regulate and prioritize their adaptive response when exposed to multiple stresses is largely unknown. Several studies have investigated plant responses to different stress factors occurring simultaneously or sequentially (Mohr & Cahill, 2003; De Vos *et al.*, 2006; Van Oosten *et al.*, 2008; Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Santino *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015). From these studies, the picture emerged that different stress signaling pathways are interconnected in a network that is under control of key regulators of adaptive responses such as MAP kinases, transcription factors and the above-mentioned stress-related hormones (Pieterse *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011; Caarls *et al.*, 2015). In order to gain insight in the complexity of the plant response to

combinatorial stresses, several recent studies investigated changes in the *Arabidopsis* transcriptome in response to simultaneous exposure to abiotic and biotic stresses (Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015). Generally, the responses to the single stresses were different from those to the double stresses. However, these studies often focused on a single time point, representing only a snapshot of the transcriptional changes that are induced by a single or combinatorial stress. The influence of one stress over the other may simply have an effect on the timing of the response to the second stress. Due to the shift in timing, one may detect large transcriptional differences in combinatorial stress situations in comparison to the respective single stresses, while over time these differences may be much smaller or vice versa.

In order to gain detailed insight into how plants cope with multiple stresses simultaneously, we here investigated in detail how a first stress influences the nature and dynamics of the transcriptional response that is induced by a second stress. We chose to study the response of the model plant species *Arabidopsis thaliana* (*Arabidopsis*) to two biotic stresses (infection by the necrotrophic fungus *Botrytis cinerea* and herbivory by larvae of *Pieris rapae*) and to one abiotic stress (drought stress by water withhold). These stresses were chosen because in previous studies it was demonstrated that the plant hormones JA, ABA, and/or ET were involved in adaptive plant responses to these respective stresses. We hypothesized that combining these stresses may lead to hormonal signal interactions that potentially affect the outcome of the response to the second stress. Several previous studies have identified thousands of *Arabidopsis* genes that change in expression in response to the selected single stresses (Reymond *et al.*, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2005; Ferrari *et al.*, 2007; Huang *et al.*, 2008; Rowe *et al.*, 2010; Birkenbihl *et al.*, 2012; Windram *et al.*, 2012; Rehrig *et al.*, 2014; Clauw *et al.*, 2015), but their dynamic behavior during multi-stress conditions is virtually unknown.

B. cinerea is considered the second most important plant pathogen (Dean *et al.*, 2012), infecting over 200 cultivated plant species and causing significant economic damage to crops worldwide. Moreover, *B. cinerea* has become an important model for studying interactions between plants and necrotrophic pathogens (van Kan, 2006; Laluk & Mengiste, 2010). As a necrotroph, *B. cinerea* kills plant tissue prior to feeding by using different mechanisms that cause plant decay, e.g. enzymatic degradation of the cell walls, generation of toxic reactive oxygen compounds, or secretion of host non-selective toxins. JA and ET participate in the defense response of *Arabidopsis* against *B. cinerea* (Thomma *et al.*, 1998; Thomma *et al.*, 1999; Diaz *et al.*, 2002; Geraats *et al.*, 2002; Rowe *et al.*, 2010; El Oirdi *et al.*, 2011), while ABA and SA have a negative effect on *B. cinerea* resistance (El Oirdi *et al.*, 2011; Liu *et al.*, 2015).

Insect herbivores consume over 15% of the plant biomass produced annually in temperate and tropical ecosystems making insect herbivory a major conduit by which energy flows through food webs (Cyr & Pace, 1993; Agrawal, 2011; Johnson, 2011). The Small Cabbage White butterfly *P. rapae* is one of the most destructive pests of cruciferous plants because it has adapted to the glycoside toxins known as glucosinolates that are produced by the crucifers as chemical defenses (Hopkins *et al.*, 2009). *Arabidopsis* and other plants activate additional defense responses that reduce the performance of the leaf-chewing *P. rapae* caterpillars on pre-infested plants (De Vos *et al.*, 2006). It has been shown that this herbivore- or wound-induced resistance also extends systemically to undamaged plant parts (Howe & Jander, 2008). JA is an important primary signal in herbivore-induced local and systemic defenses in various plant–herbivore interactions, while ABA has a modulating role in the JA-responsiveness (Bodenhausen & Reymond, 2007; Howe & Jander, 2008; Soler *et al.*, 2013; Vos *et al.*, 2013b).

Drought is one of the most frequently experienced abiotic environmental stresses in plants. Low water availability in the rhizosphere leads to a reduction in leaf stomatal conductance and growth (Schachtman & Goodger, 2008). Adaptive responses to drought also involve metabolic, osmotic, and structural adjustment, as well as the production of proteins with DNA damage control and repair functions (Ingram & Bartels, 1996). ABA accumulation is essential for the adaptation to drought, but also ABA-independent regulatory systems are involved in drought stress-responsive gene expression. In the latter, JA and ET have been implicated as important regulators (Bray, 1997; Shinozaki *et al.*, 2003; Riera *et al.*, 2005; Huang *et al.*, 2008).

In this study, we used RNA-seq to analyze the dynamics of the transcriptome changes that occurred in *Arabidopsis* over four time points in response to *B. cinerea* infection, *P. rapae* feeding, drought stress, and all six combinations of sequential double stresses. Our results show that irrespective of the first stress, *Arabidopsis* is capable of swiftly shifting its transcriptome when it encounters a second stress. Over time, this second stress transcriptome is highly similar to that of plants that did not receive a first stress, but contains clear first-stress-signatures, which may play a role in the phenotypic interaction between consecutive stresses.

Materials and Methods

Plant material and growing conditions

Seeds of *Arabidopsis thaliana* accession Col-0 were sown in cultivation containers containing autoclaved river sand. Sand was supplied with half-strength Hoagland solution with sequestreen. In order to keep a high humidity for germination, cultivation containers were enclosed in a tray with transparent lid, supplied with water. Seeds were vernalized for two days at 4°C in the dark to overcome remaining dormancy and induce a homogeneous germination. After two days, the tray was moved to a growth chamber with an 8-h day/16-h night rhythm under 21°C, 70% relative humidity (RH), and a light intensity of 100 µmol/m^2/s. After approximately ten days, the lid from the tray was opened and gradually removed for transplanting the seedlings to soil. Seedlings were transplanted to individual pots containing a mixture 1:1 (v/v) of river sand and sowing soil. Plants pots were watered at the bottom three times per week. At an age of 3 weeks the plants were supplied once with Hoagland solution.

Rearing conditions *P. rapae*

Pieris rapae (Lepidoptera, Pieridae) caterpillars were reared on cabbage plants (*Brassica oleracea* convar. capitata var. alba) under greenhouse conditions (24°C, with natural daylight). Butterflies were supplied with flowering plants such as *Lantana camara* for their food and nutrient requirements. When flowers were scarce an additional feeding solution (20% honey and 10% sucrose) was offered to the butterflies. Inbreeding of the population was minimalized by adding wild butterflies and caterpillars from the Dutch Flevopolder, to the existing population.

Cultivation of *B. cinerea*

Botrytis cinerea strain B05.10 (Staats & Van Kan, 2012) was grown on half-strength Potato Dextrose Agar (PDA; Difco Laboratories) plates containing penicillin (100 µg/ml) and streptomycin (200 µg/ml) for 2 weeks at room temperature. Spores were collected and re-suspended in half-strength PDA to a final density of 1 x 10^5 spores/ml. After a 3-h incubation period, the spores were used for inoculation.

Single and sequential double stress treatments

Single and sequential double stress treatments were applied according to the schedule shown in Fig. 1. For single and sequential double stress treatments with *B. cinerea* as the second stress, developmental leaf number 8 of 5-week-old plants were inoculated with *B. cinerea* by pipetting four 5-µl droplets of spore suspension (1×10^5 spores/ml). Plants were kept at 100% relative humidity for the whole time period of *B.*

cinerea infection. Pre-treatment with drought was achieved by withholding water for 7 d after which plants were re-watered and allowed to recover for 24 h before plants were inoculated with *B. cinerea*. *P. rapae* pre-treatment was performed 1 d prior to *B. cinerea* inoculation by allowing a single *P. rapae* caterpillar to feed on the plant for 24 h. Only plants with undamaged leaves number 8 were used for inoculation with *B. cinerea* as second stress. Leaf number 8 was harvested at 6, 12, 18, and 24 h after inoculation with *B. cinerea.*

For single and sequential double stress treatments with *P. rapae* herbivory as second stress, *P. rapae* first-instar (L1) larvae were starved for 1 h after which they were transferred directly to developmental leaf number 8 of 5-week-old plants (two caterpillars per plant). Pre-treatment with drought was achieved by withholding water for 7 d after which plants were re-watered and allowed to recover for 24 h before *P. rapae* larvae were transferred to the plants. *B. cinerea* pre-treatment was performed 1 d prior to transfer of *P. rapae* to the plants by inoculating leaves 6 and 7 with a 5-µl droplet of *B. cinerea* spore suspension containing 1×10^5 spores/ml and placing the plants at 100% RH for 24 h. Leaf number 8 was harvested at 3, 6, 12 and 24 h after the start of *P. rapae* feeding. When leaf number 8 was not damaged by *P. rapae* (because it had moved to another leaf)*,* the next-closest *P. rapae*-damaged leaf was harvested.

For single and sequential double stress treatments with drought as the second stress, 4-week-old plants were refrained from watering for 7 d. After 7 d of water withhold, plants were re-watered and allowed to recover for 24 h. *B. cinerea* pre-treatment was performed at day 0 of the drought period by inoculating leaves 6 and 7 with a 5-µl droplet of *B. cinerea* spore suspension containing 1×10^5 spores/ml and placing the plants at 100% RH for 24 h. *P. rapae* pre-treatment was performed at the same time as the *B. cinerea* pre-treatment by allowing a single *P. rapae* caterpillar to feed on the plant for 24 h. Only plants with undamaged leaves number 8 were used for harvest. Leaf number 8 was harvested at 5, 6, 7, and 7+1 d after the onset of water withhold (with 7+1 representing the time point of 24 h after re-watering).

For each treatment and time point, 3 biological replicates were used for RNA-seq analysis. Each of the three biological replicates consisted of four pooled “number 8” leaves harvested from four similarly-treated plants. For all treatments in which *B. cinerea* inoculation was used as first or second stress, a mock-treatment was performed in which plants were inoculated with droplets of half-strength PDA and placed at 100% RH for 24 h. For all treatments without *B. cinerea,* controls consisted of untreated plants. For all timepoints and treatments, 3 biological replicas consisting of pools of four “number 8” leaves that were harvested at the same time were used in the RNA-seq analysis. After harvest, leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C.

Experimental design

The experiment was carried out in a fully randomized factorial design with two factors; time and treatment. The climate chamber space was divided in three blocks, in which time was randomized. Within every time point, treatments were assigned randomly to the plants. Leaf samples were randomly collected by a random persons assigned to a certain treatment within a time point. RNA extraction was carried out in batches of approximately 20 randomly chosen samples.

RNA extraction, library preparation, and RNA-seq alignment

RNA was extracted using Plant RNeasy Plant Mini Kit (Qiagen), according to the manufacturers instructions. All samples were treated with DNAase I on column using the Qiagen RNase-Free DNase Set. Quality and quantity of total RNA were initially measured with a NanoDrop ND-1000 (Nanodrop, Delaware, USA). Quality of RNA was also checked using the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabChip. For the library preparation we used only samples with RIN values ≥6. The sample preparation was performed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina. This protocol allows to identify strand-specific transcripts. First, poly-A RNA was isolated from the total RNA using a Poly-T oligo-attached magnetic beads. Subsequently, Poly-A RNA was fragmented using divalent cations under elevated temperature. First strand cDNA was synthesized using random primers. Strand specificity was achieved by replacing dTTP with dUTP in the second Strand Marking Mix (SMM), followed by second strand cDNA synthesis using DNA polymerase I and RNase H. Samples were sequenced with an Illumina Hi-seq 2000 sequencer, using three sequencing runs. Samples were randomly assigned to 7 lanes of the Illumina flow cells within each run. Alignment of the RNA-seq data and downstream processing was performed as described by van Verk *et al.* (2013). Differential gene expression was calculated for each pair of relevant treatments and mock/control using DESeq2 (Love *et al.*, 2014).

GO-term analysis

GO-Term enrichment analysis was performed using GO term finder (Boyle *et al.*, 2004) using an *A. thaliana* gene association file downloaded from ftp.geneontology.org on May 2nd 2013. Over-represented GO categories are determined as categories having a P-value ≤ 0.01 using the hypergeometric distribution with Bonferroni correction for multiple testing. Heatmaps were generated using the bioinformatics toolbox in Matlab R2014a.

Clustering

Hierarchic clustering of the core set of single stress DEGs was performed on log2 fold-change expression values using the R function hclust with a cosine similarity metric and average linkage. The cutree function was used with a visually determined cut height to partition the resulting dendrogram into clusters. Clustering of the core set of single stress DEGs and shared main treatment datasets was performed using model-based clustering package mclust version 4 in R (Fraley *et al.*, 2012) with the number of clusters determined by the Bayesian information criterion. To identify groups of genes that may be co-regulated across the three main treatments, Wigwams (Polanski *et al.*, 2014) was used to identify co-expressed genes spanning subsets of the drought, *P. rapae* and *B. cinerea* time series datasets. Wigwams clustering was performed using log2 expression values for the union of genes DE in all three main single treatments. Wigwams was provided with a list of genes that are DE in each condition so that Wigwams will then only place genes in a module if it is DE in all the conditions under consideration at a given time. All other arguments were kept as default.

Results

Experimental approach for RNA-seq analysis of *single* and *sequential stress* time series

In order to capture a maximal dynamic range of the stress responses, the response to each of the three main stresses was monitored in a different time frame of four time points, depending on how quickly the stress response developed (Fig. 1). The transcriptional response to each stress and at each time point was compared to a non-treated control (for treatments not involving *B. cinerea*) or a mock-treated control (for all treatments involving *B. cinerea*) that was harvested at the same time as the treatment. For the induction of drought stress, 4-week-old *Arabidopsis* plants that had previously been watered with equal amounts of water were subsequently withheld from water for 7 days. At day 5 of water withhold, drought-stressed plants were clearly smaller and darker green than the watered control plants, a phenotype that progressed further on day 6 and 7, when they were at the point that they started wilting (data not shown). The transcriptome time series were chosen at 5, 6 and 7 days after water withhold, and at day 8 (7+1d), which was one day after re-watering. The recovery response at day 8 was chosen as the fourth time point of the drought time series because (1) this recovery response after drought stress is interesting by itself, and (2) at this time point the sequential treatment with *P. rapae* and *B. cinerea* was executed (for both of which a re-watering recovery period of one day was required). For the induction of *P. rapae* stress, we chose a time span between 3 and 24 h after infestation because previous studies demonstrated that this would yield a maximal dynamic range of transcriptional responses (Reymond *et al.*, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2005; Verhage *et al.*, 2011). For the induction of *B. cinerea* stress, we chose a time span between 6 and 24 h after inoculation, because previous studies showed that the earliest transcriptional changes can be observed around 6 h after application of the inoculum, while at 24 h after inoculation massive changes in gene expression can be detected (Windram *et al.*, 2012; Vos *et al.*, 2015). Prior to applying the second stress, further development of the first stress was stopped by re-watering the plants (first stress drought), changing the 100% relative humidity condition to 70% (first stress *B. cinerea*), or removing the caterpillar (first stress *P. rapae*). Developmental leaf number 8 was used for applying *P. rapae* or *B. cinerea* as second stress. For all treatments, leaf number 8 was harvested for RNA-seq analysis. When leaf number 8 was not damaged by *P. rapae,* the next closest *P. rapae*-damaged leaf was harvested. Three biological replicates per treatment/time point were subjected to RNA-seq. Each of the three biological replicates consisted of four “number 8” leaves that were pooled to form one sample. After harvest, leaves were processed and subjected to RNA-Illumina sequencing. On average, 14.6 million reads (range 8.5 – 29.8 million) were generated per sample with >90% of sequences aligning to the *Arabidopsis* genome after quality filtering (Van Verk *et al.*, 2013).

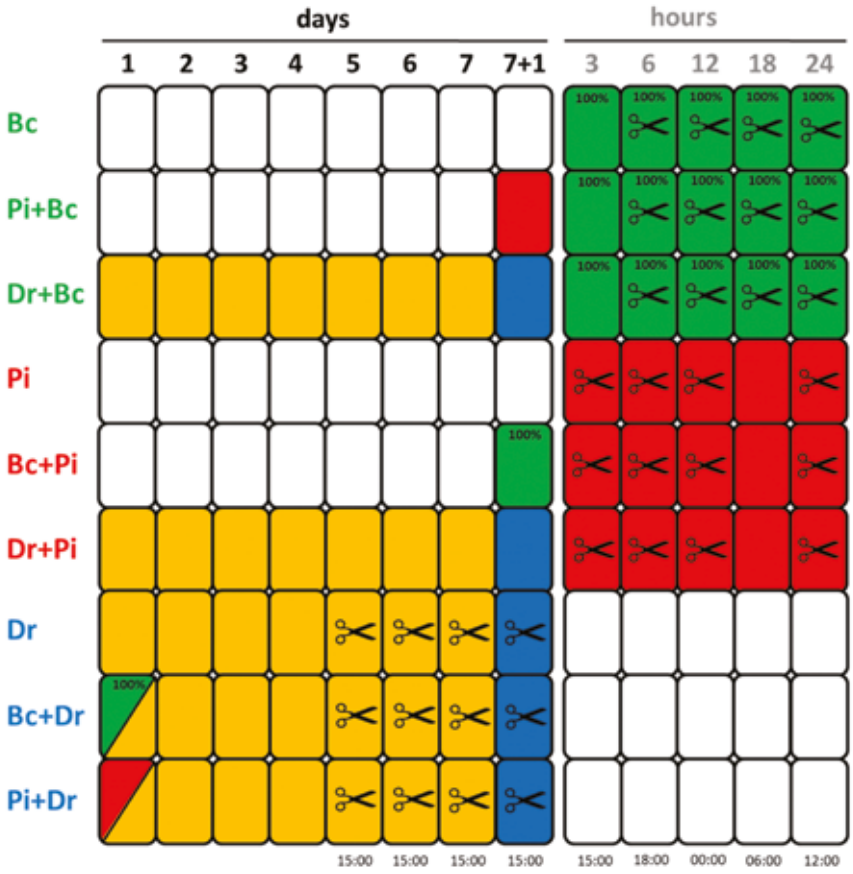


Figure 1. Experimental schedule of treatments and harvests for transcriptome time series of single and sequential double stresses. The schedule shows the timing of treatments and time points of harvest for the three main treatments, *B. cinerea* (Bc, green), *P. rapae* (Pi, red) and Drought (Dr, orange), and the respective pre-treatments. Each single and sequential double stress had its own mock/control at each time point. Stresses were stopped by either lowering relative humidity to 70% (after 24 h in case of Bc pre-treatment), removing caterpillars from plants (after 24 h), or re-watering after a 7-d period of drought (7+1; blue). In case the second stress was drought, the pretreatments with *B. cinerea* and *P. rapae* were performed at day 0, right after the last moment of watering. 100%; period of 100% RH instead of standard 70% RH; time indications at the bottom indicate time of the day at which leaves were harvested. Scissors indicate time point of harvest.

Time series transcriptome profiling following *single* and *sequential stresses*

In this study, our aim was to analyze the dynamic transcriptome changes that are triggered by the single stresses and investigate how the nature and dynamics of the transcriptome profiles were affected by pre-exposure to each of the other two stresses. First, a set of differentially expressed genes (DEGs) derived from each single stress time series was selected according to their significance in fold-change

expression (false discovery rate (FDR) <0.05) and a threshold level of at least 2-fold change in comparison to the respective control (Supplemental Table S1). For drought stress, the set of DEGs was determined for the first three time points (without the 24 h after re-watering time point). The first observation that can be made from the RNA-seq results is that over time there are clear differences in the number of genes that are significantly up- or down-regulated during the different single stress conditions (Fig. 2). For *B. cinerea* (total 2128 unique DEGs) and *P. rapae* (total 4026 unique DEGs), a strong increase in the number of up-regulated genes is observed over time, while relatively few genes are down-regulated. Upon exposure to drought stress (total 4136 unique DEGs), relatively more genes become down-regulated than up-regulated. A prior stress did not dramatically change the number of up- or down-regulated genes relative to the single stresses (Fig. 2). Clustering the union of DEGs of the single stress sets (total 7393 unique DEGs), and subsequent Gene Ontology (GO) analysis using the AmiGO software package (Carbon *et al.*, 2009) of overrepresented biological processes in each cluster highlights the differentially regulated biological processes during the plant response to the single stresses (Fig. 3).

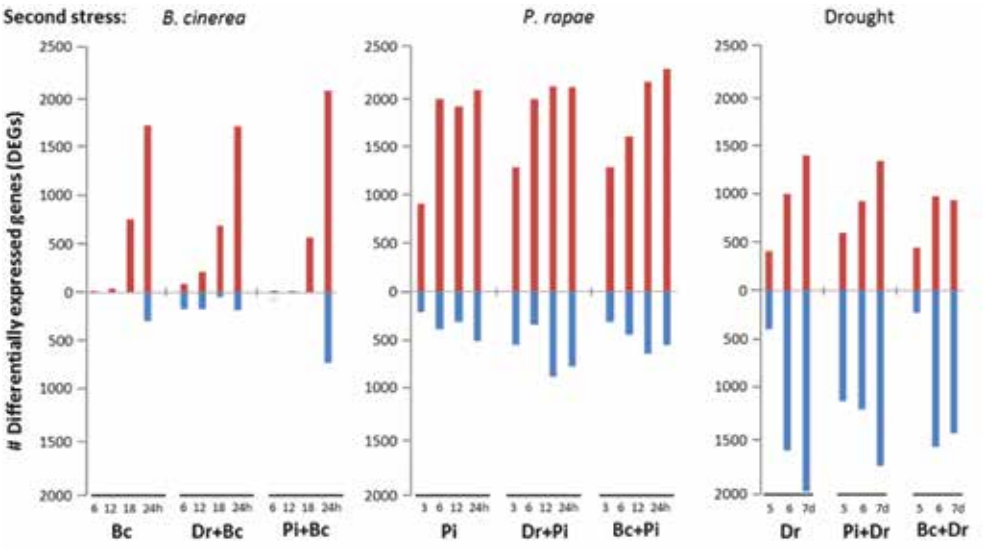


Figure 2. Numbers of DEGs at different time points in single and sequential stress responses. Graphs show the number of up-regulated (red bars) and down-regulated genes (blue) for all single stresses and their corresponding sequential double stresses at different time points after harvest (FDR <0.05; >2-fold). Bc, *B. cinerea*; Pi, *P. rapae*; Dr, drought; Dr+Bc, Pi+Bc, Dr+Pi, Bc+Pi, Pi+Dr, and Bc+Dr, respective sequential double stresses.

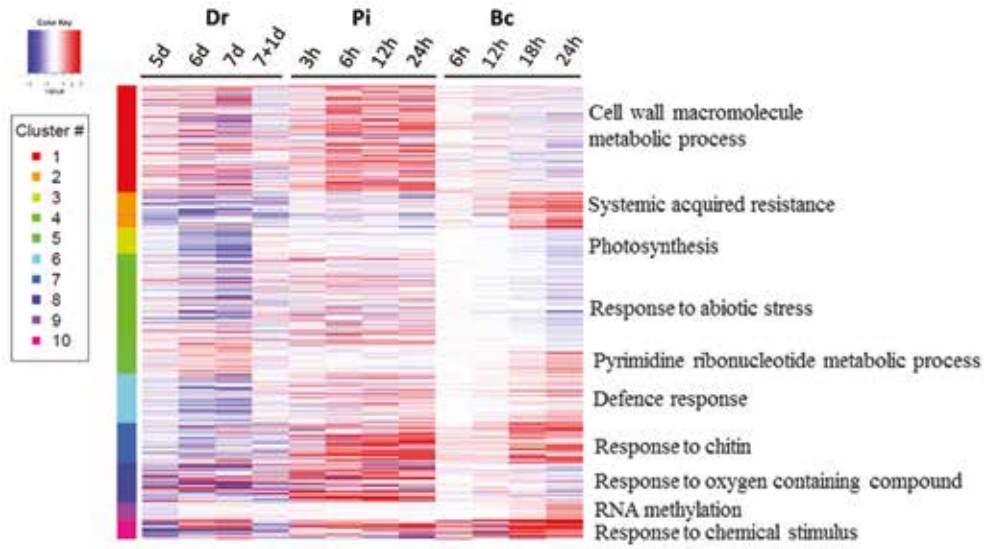


Figure 3. Clustering of the union of DEGs of the single stress responses. Heatmap showing the expression patterns of the union of differentially expressed genes (DEGs) in the three single stresses (FDR <0.05; >2-fold; total 7393 unique genes). In blue the down-regulated genes and in red the up-regulated genes. DEGs of all the single stresses combined were clustered using Mclust yielding 10 gene clusters (colored bars on the left). On the right side, the most significant GO terms based on AmiGO term analysis are shown for the clusters.

Similarities between core DEGs of *single stress* responses

To gain insight in the uniqueness of the transcriptional response to the three single stresses, we compared their DEGs. Figure 4A shows that there is a large overlap between the DEGs of the single stress responses, ranging from 1714 genes shared between the drought and *P. rapae* sets, to 794 genes between the drought and *B. cinerea* sets, and 781 genes between the *P. rapae* and *B. cinerea* sets. A core set of 392 genes was differentially expressed in response to all three single stresses. These 392 shared core DEGs could be clustered into 9 gene clusters (Fig. 4B). Analysis of the expression profiles of these 9 clusters showed that they are clearly different in response to the different single stresses and are often regulated in opposite directions. Only the cluster 6 genes (enriched for GO term “response to oxygen compound”) are clearly regulated in the same direction during all three stress conditions (Fig. 4B). GO term analysis of overrepresented biological processes in each cluster highlights the differentially regulated biological processes in the clusters (Fig. 4B). The fact that the same genes are differentially regulated during the three single stresses, albeit in different directions, suggests that they may act as a point of convergence under conditions when these stresses are applied in sequence.

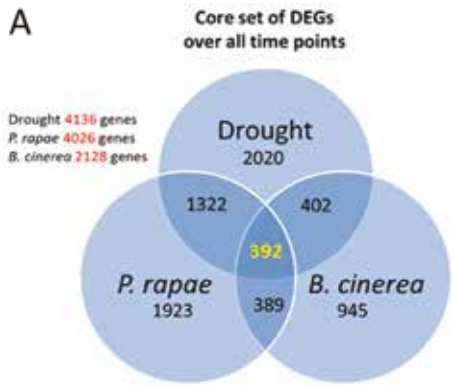
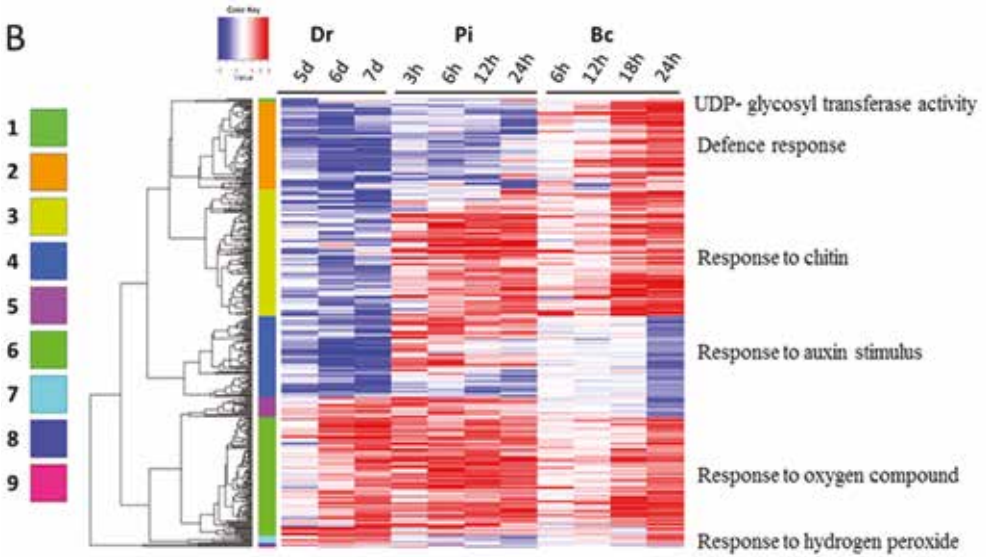


Figure 4. Overlap between the DEGs of the single stress responses. (A) Venn diagram showing the overlap between the DEGs (FDR <0.05; >2-fold) of each of the single stress responses. The total number of DEGs per single stress over all time points is shown in red. (B) Hierarchical clustering of the 392 core DEGs that are shared between the three single stresses (Cosine similarity metric; clusters are color coded in the square boxes on the left). In blue the down-regulated genes and in red the up-regulated genes. The most significant overrepresented GO terms based on AmiGO term analysis are shown on the right.



B. cinerea data set: effect of herbivory and drought stress on dynamics of *B. cinerea*-induced gene expression

To investigate the effect of *P. rapae* feeding and drought stress on the dynamics of the transcriptome changes that are induced by *B. cinerea* infection, we analyzed the dynamics of the global expression patterns of all DEGS from the *B. cinerea* single stress and the sequential stresses with *B. cinerea* as the second stress (Fig. 5; Supplemental Table S1). Clustering of the *B. cinerea* DEGs yielded 6 clusters of co-expressed genes. GO term analysis of overrepresented biological processes in each cluster highlights the main differentially regulated biological processes. *B. cinerea* infection induced a relatively large number of genes related to GO term "response to chitin" (Fig. 5, cluster 3), reflecting recognition of fungal chitin by the plant immune system, and "response to ethylene" (Fig. 5, cluster 2), reflecting the high level of ET emission that is related to plant responses to *B. cinerea* infection. In addition, *B. cinerea* repressed genes associated

with the GO term “multidimensional cell growth” (Fig. 5, cluster 6), highlighting the antagonistic relationship between plant growth and defense. Interestingly, global expression patterns over time in the sequential double stress treatments appear very similar to the ones of the *B. cinerea* treatment alone. This suggests that *Arabidopsis* swiftly reprogrammes its transcriptome to the response to *B. cinerea* infection, thereby largely overruling the effect of the prior stresses herbivory and drought.

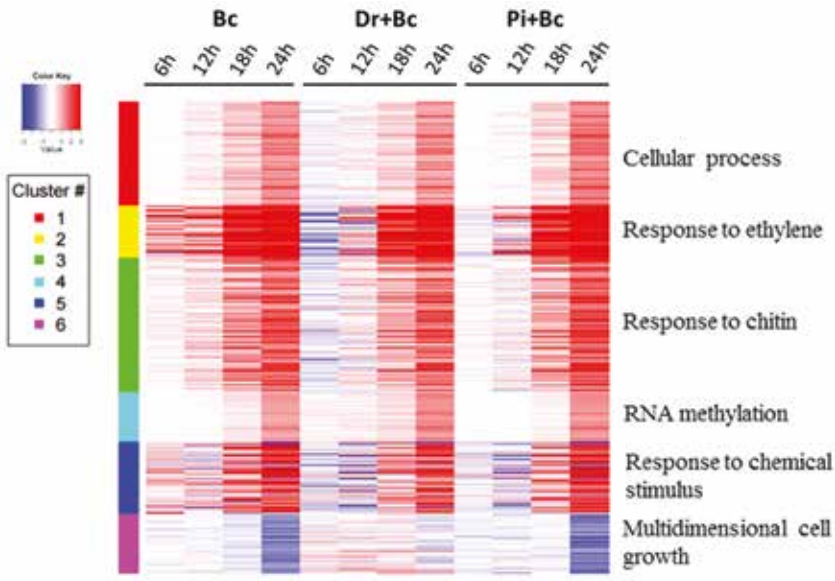


Figure 5. Dynamics of the expression of *B. cinerea* DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the *B. cinerea*-induced DEGs during infection of *Arabidopsis* by *B. cinerea* on mock pre-treated (Bc), drought pre-treated (Dr+Bc), or *P. rapae* pre-infested (Pi+Bc) *Arabidopsis* plants (FDR <0.05; >2-fold; total 4381 unique genes). In blue the down-regulated genes and in red the up-regulated genes. The union of DEGs of all treatments combined were clustered using Mclust yielding 6 clusters (colored bars on the left). On the right side, the most significant GO terms based on AmiGO term analysis are shown for the clusters.

In order to identify co-regulated genes of which the expression pattern in response to *B. cinerea* infection were affected in plants that prior to pathogen inoculation experienced either herbivory or drought stress, we used the bioinformatics tool Wigwams (Polanski *et al.*, 2014). The Wigwams algorithm identifies gene modules showing evidence for co-regulation in multiple time series gene expression data sets and identifies signatures of condition-dependent regulatory mechanisms in co-regulated gene sets. Wigwams identified 32 modules of co-regulated genes in the *B. cinerea* data sets. Analysis of these clusters for co-expression under the single and sequential double stress conditions (Bc, Dr+Bc, and Pi+Bc) revealed gene modules of which the expression patters were clearly affected in one or both of the double treatments in comparison to the *B. cinerea*

treatment alone (Examples shown in Fig. 6; Full set in Supplemental Fig. S1). The identities of the genes in these Wigwams gene modules are given in Supplemental Table S2. Among the *B. cinerea*-responsive genes of which the expression pattern is clearly different when plants were exposed to one of the other stresses, are the chitin-responsive transcription factor gene *WRKY53* (At4g23810) (Zhang *et al*., 2015) and the hypersensitive response-related gene *HYPERSENSITIVE INDUCED REACTION2* (*HIR2*; At3g01290) (Qi *et al*., 2011). They are part of Bc Wigwams module 4, which is enriched for genes related to the GO term "regulation of plant-type hypersensitive response'' (data not shown). Genes in Bc module 4 are suppressed at 12 h after *B. cinerea* inoculation in *P. rapae* pre-treated plants. As a nectrotoph, *B. cinerea* uses the hypersensitive response as a strategy to spread the infection (Govrin & Levine, 2000). Hence, suppression of this module by *P. rapae,* may reduce development of the disease in the sequential double stress.

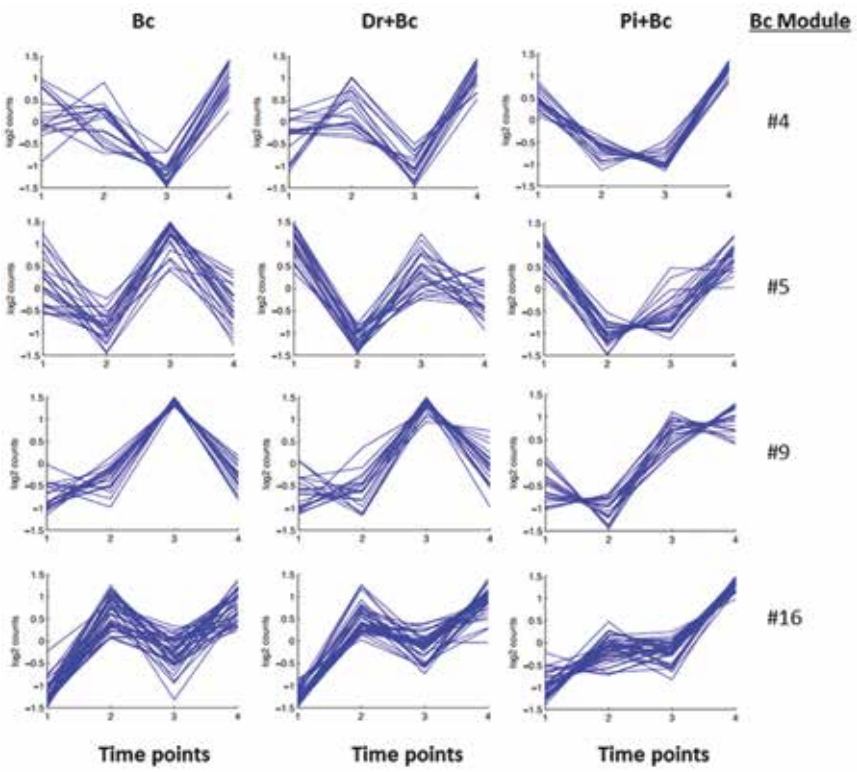


Figure 6. Expression patterns of selected Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules is depicted that contain significantly co-expressed gene clusters for the single stress *B. cinerea* (Bc), and of which the expression pattern changed in one or both of the sequential stresses drought-*B. cinerea* (Dr+Bc) and *P. rapae-B. cinerea* (Pi+Bc). The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. Time points 1, 2, 3 and 4 represent 6, 12, 18 and 24 h after *B. cinerea* infection.

In order to gain insight into the biological processes that are affected when *B. cinerea* infection is preceded by either drought stress or herbivory, we determined the level of significance of overrepresentation of all the GO terms for all the *B. cinerea* DEGs at the time point that they became differentially expressed using AmiGO. For this, we first identified the time point of first differential expression of all *B. cinerea* DEGs, divided them over up- and down-regulated genes, and performed GO term analysis on them. Figure 7 shows the timing and strength of the onset of significant GO term enrichment in the single and sequential double stress conditions. In the *B. cinerea* single stress data set, GO terms "response to ethylene", "ethylene biosynthesis process", "response to wounding", and "respiratory burst" are clearly enriched in the up-regulated gene clusters, reflecting the importance of these processes in defense against this necrotrophic pathogen. In the down-regulated gene sets, GO terms "growth" and "cell wall organization and biogenesis" are overrepresented, highlighting the negative effect of pathogen infection on plant growth. Several GO terms become more strongly enriched in the sequential double stresses. For instance, GO term "response to ethylene" (bottom Fig. 7) is much stronger enriched in the up-regulated gene sets of the sequential double treatments than in that of the *B. cinerea* single treatment. The same holds true for the GO terms "response to fungus", "response to jasmonic acid", "response to salicylic acid", "regulation of hydrogen peroxide regulated metabolic process", "camalexin metabolic process", "response to water deprivation", "response to abscisic acid", and "defense response by callose deposition". Notably, biological processes related to hormone action prevail in the *B. cinerea*-responsive processes that are sensitive to modulation by prior exposure to one of the other stresses.

Effect of drought stress or herbivory on *B. cinerea* resistance

Both drought stress and herbivory imposed a first-stress-signature in the dynamics of the *B. cinerea*-induced transcriptome profiles. Wigwams analysis gained insight into the identity of the genes related to these first-stress-signatures (Figs. 6), whereas analysis of GO term enrichment provided global insight into the biological processes that were affected by the prior stress treatment (Fig. 7). To investigate the effect of the two first stresses on the outcome of the response to *B. cinerea* infection, we assessed the level of disease resistance in single and sequential double stress treatments. Inoculation of 5-week-old *Arabidopsis* Col-0 plants with *B. cinerea* resulted in the development of spreading lesions in about 60% of the inoculated leaves (Fig. 8A). Plants that were exposed to drought stress prior to *B. cinerea* inoculation showed a mild, but not significant increase in the percentage of leaves with spreading lesions (~70%), although the lesions were clearly larger than in the *B. cinerea* single-stress treatment (Fig. 8B). Interestingly, plants that were exposed to herbivory prior to *B. cinerea* inoculation showed a high level of resistance against *B. cinerea* infection (average ~35% spreading lesions). Together

these results indicate that a first stress can have strong effects on the outcome of the adaptive stress response to a second stress, depending on the nature of the first stress.

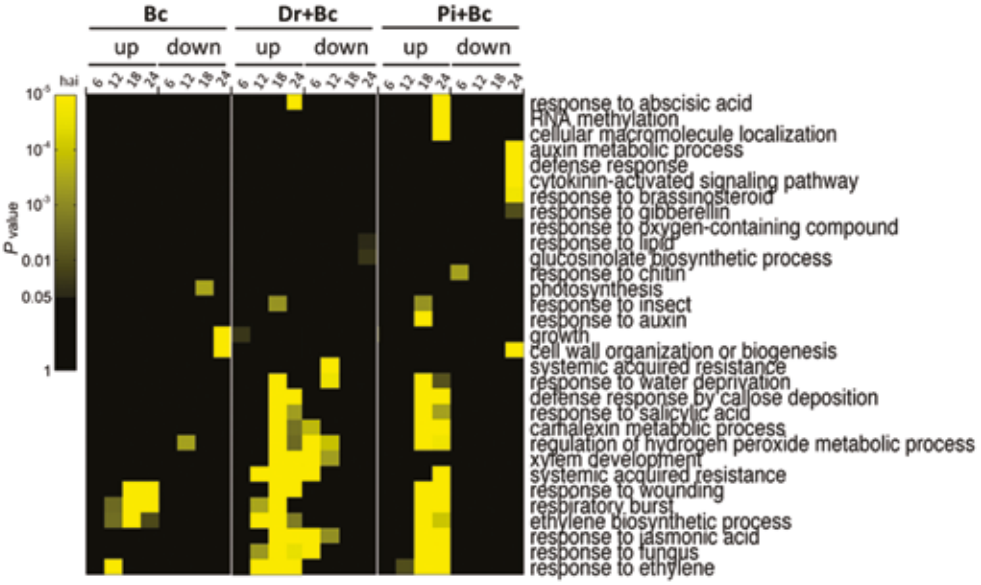


Figure 7. Timing of GO term overrepresentation patterns in *B. cinerea* single and sequential stress data sets. Heatmap represents the P-values of GO term overrepresentation in up- or down-regulated gene clusters in *Arabidopsis* at the given stress conditions and time points. Color index of P-values represents level of significance. On the right, overrepresented GO terms based on AmiGO term analysis. hai, h after *B. cinerea* infection.

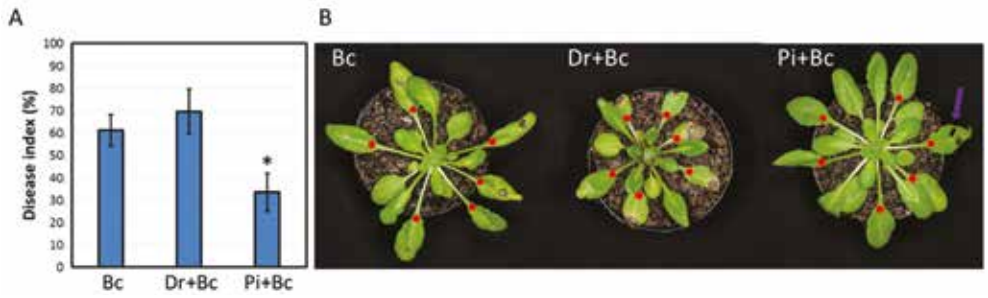


Figure 8. Effect of drought stress and herbivory on *B. cinerea* resistance in *Arabidopsis*. (A) Disease index (% of spreading lesions) of *B. cinerea* disease symptoms on *Arabidopsis* accession Col-0 plants. On each plant, six leaves were inoculated with *B. cinerea* spores. Three d later, the average number of leaves with spreading lesions was determined per plant. Asterisk indicates statistically significant difference from single stress (Bc) treatment (n=6 plants; Student's t-test; P<0.05). (B) Photographs of *B. cinerea* disease symptoms, 3 d after inoculation. Bc, *B. cinerea* inoculated plants; Dr+Bc, *B. cinerea*-inoculated plants that prior to inoculation received a drought treatment for 7 days, followed by a re-watering phase of one day; Pi+Bc, *B. cinerea*-inoculated plants that prior to inoculation were exposed to herbivory by *P. rapae* larvae for 24 h. Red dots, *B. cinerea*-inoculated leaves; purple arrow, damage caused by *P. rapae* feeding.

P. rapae data set: effect of drought stress and *B. cinerea* infection on dynamics of *P. rapae*-induced gene expression

In order to investigate the effect of drought stress and *B. cinerea* infection on the dynamics of the transcriptome changes that are induced by *P. rapae* feeding, we analyzed the dynamics of the global expression patterns of all DEGs from the *P. rapae* single stress and the sequential stresses with *P. rapae* as the second stress (Fig. 9; Supplemental Table S1). Clustering of the *P. rapae* DEGs using Mclust yielded 7 clusters of co-expressed genes. GO term analysis of overrepresented biological processes in each cluster highlights the main differentially regulated biological processes in the clusters. As expected, *P. rapae* feeding induced many genes related to GO term "response to wounding" (Fig. 9, cluster 6), reflecting induced defenses that are triggered by herbivory.

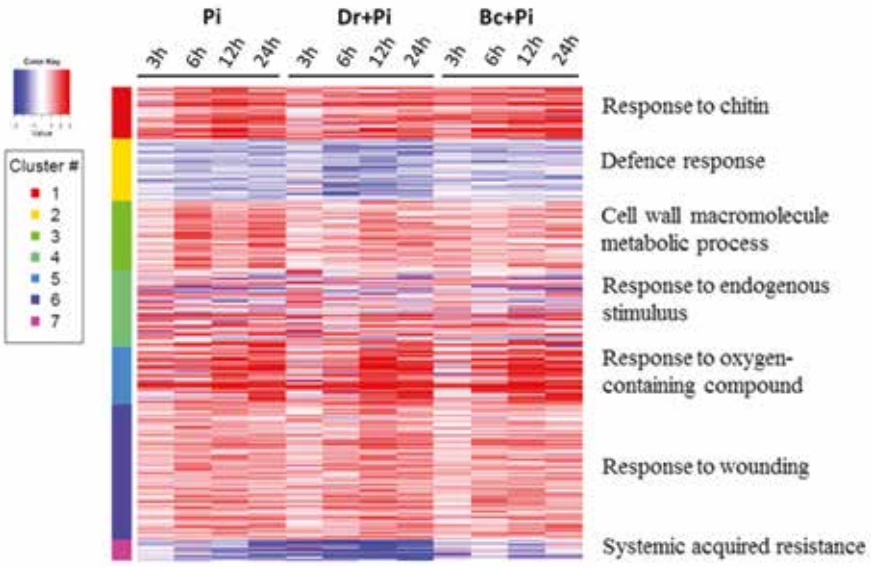


Figure 9. Dynamics of the expression of *P. rapae* DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the *P. rapae* DEGs during feeding of *P. rapae* on control (Pi), drought pre-treated (Dr+Pi) or *B. cinerea* pre-infected (Bc+Pi) *Arabidopsis* plants (FDR <0.05; >2-fold; total 8847 unique genes). In blue the down-regulated genes and in red the up-regulated genes. The union of DEGs of all treatments combined were clustered using Mclust yielding 7 clusters (colored bars on the left). On the right side, the most significant GO terms based on AmiGO term analysis are shown for the clusters.

In addition, *P. rapae* feeding repressed SA-related genes associated with GO term "Systemic acquired resistance" (Fig. 9, cluster 7), reflecting the antagonistic relationship between JA- and SA-dependent defenses. In line with this, also genes related to "Defense response" (Fig. 9, cluster 2), amongst which many pathogen defense-related genes, are strongly down-regulated by *P. rapae* feeding. In line with

what we observed in the *B. cinerea* single and sequential double stress data sets, global gene expression patterns over time in the sequential double stress treatments was very similar to those inflicted by the *P. rapae* treatment alone, again suggesting that *Arabidopsis* is capable of rapidly reprogramming its transcriptome to the last stress encountered, thereby largely overruling the effects of the prior stresses.

To identify co-regulated genes whose expression pattern in response to herbivory is affected by *B. cinerea* infection or drought stress, the set of *P. rapae* DEGs was analyzed with the Wigwams algorithm. Wigwams identified 85 modules of co-regulated genes in the *P. rapae* set of DEGs. Analysis of these clusters for co-expression under the single and sequential double stress conditions (Pi, Bc+Pi, and Dr+Pi) revealed gene modules of which the expression patterns were clearly affected in one or both of the sequential double stress treatments in comparison to the *P. rapae* treatment alone (Examples shown in Fig. 10; Full set in Supplemental Fig. S2). These gene modules represent signatures of a previous stress in the *P. rapae*-global gene expression profile, and may thus be functionally related to the effect of the first stress on the outcome of the plant response to *P. rapae* feeding. The identities of the genes in the *P. rapae*-related Wigwams gene modules are given in Supplemental Table S2. It is beyond the scope of this paper to discuss the identity of the genes in detail, but examples of *P. rapae*-induced genes of which the expression pattern is strongly affected by prior drought stress or *B. cinerea* infection are the transcription factor gene *MYB12* (At2g47460) and *FLAVONOL SYNTHASE1* (*FLS1*; At5g08640) in Pi Wigwams module 25 (Fig. 10; Supplemental Table S2), which is enriched for genes related to the GO terms “flavonoid biosynthetic process” and “flavonoid metabolic process” (data not shown). Both *MYB12* and *FLS1* are involved in the biosynthesis of flavonoids, and have an important role in defense against herbivores (Mehrtens *et al.*, 2005; Li *et al.*, 2013; Ali & McNear, 2014). The genes in Pi module 25 are suppressed early in the *P. rapae* response when plants were pre-treated with *B. cinerea* or drought, which may influence the level of resistance against this herbivore in the sequential double stress.

To obtain a global view on the herbivory-associated biological processes that are affected by drought stress or *B. cinerea* infection, the level of significance of overrepresentation of all the GO terms for the *P. rapae* set of DEGs was assessed. Figure 11 shows the timing and strength of the onset of significant GO term enrichment in the single and sequential double stress conditions. Figure 11 shows that the majority of the *P. rapae*-affected biological processes are only marginally affected by a prior stress. For instance GO term “response to wounding” (bottom Fig. 11) shows a similar enrichment pattern in the upregulated genes of the single and sequential double stress time series. However, several GO terms show clearly different enrichment patterns in the double stress conditions. Examples are the GO terms “response to jasmonic acid” and “jasmonic acid biosynthetic process” which are significantly stronger enriched in the Bc+Pi and Dr+Pi

data set in comparison the *P. rapae* single stress data set. The same holds true for GO terms “response to ethylene”, “ethylene biosynthesis process”, “response to abscisic acid”, and “response to auxin”, which are significantly more overrepresented in the up-regulated gene sets of the sequential double stresses. Also GO terms “response to salicylic acid” and “systemic acquired resistance” stand out, with overrepresentation in the up-regulated genes of the Bc+Pi sequential double stress and overrepresentation in the down-regulated genes of the Dr+Pi combination. Overall, these data indicate that *B. cinerea* infection or drought treatment prior to *P. rapae* infestation, predominantly affects timing of the biological processes that are related to the response of the plant to JA, ABA, ET and SA, corroborating the notion that different stresses interact via the hormone-regulated signaling network.

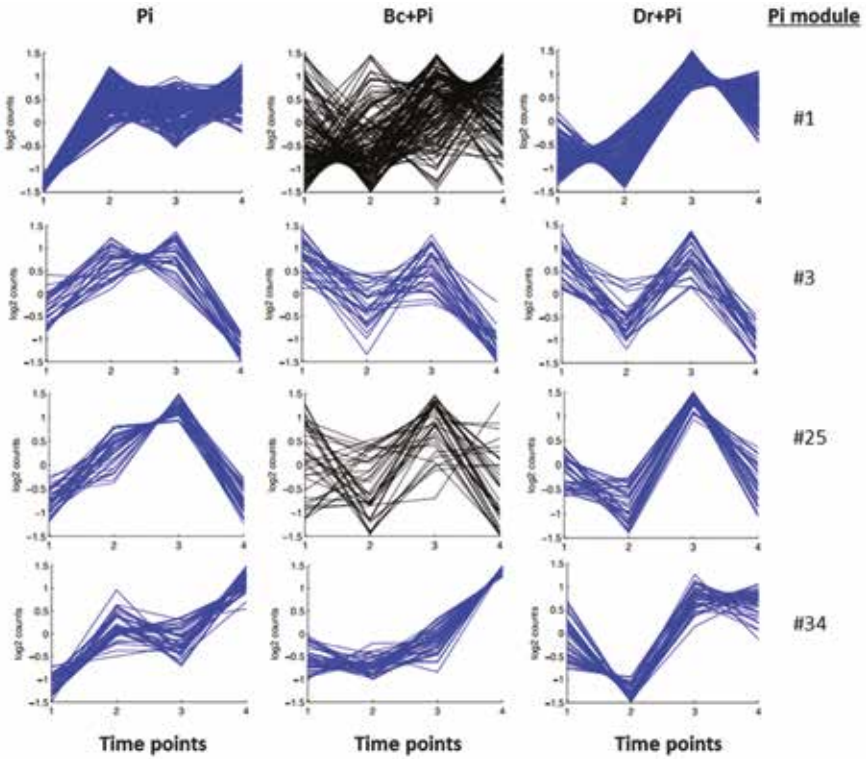


Figure 10. Expression patterns of selected Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules is depicted that contain significantly co-expressed gene clusters for the single stress *P. rapae* (Pi), and of which the expression pattern changed in one or both of the sequential stresses *B. cinerea*-*P. rapae* (Bc+Pi) and drought-*P. rapae* (Dr+Pi). The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 3, 6, 12 and 24 h after *P. rapae* infestation.

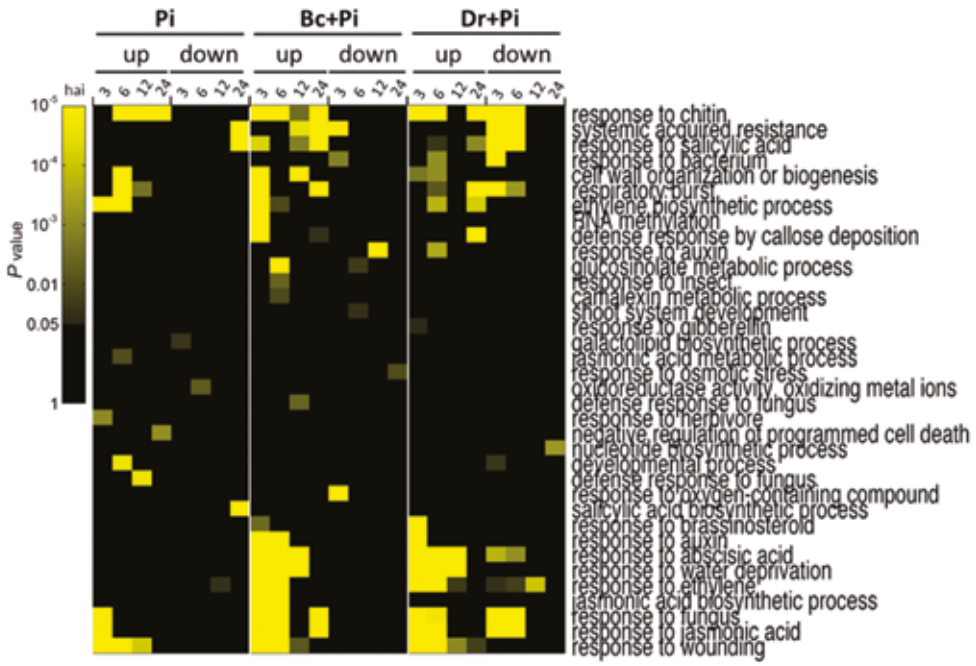


Figure 11. Timing of GO term overrepresentation patterns in *P. rapae* single and sequential double stress data sets. Heatmap represents the P-values of GO term overrepresentation in up- or down-regulated gene clusters in *Arabidopsis* at the given stress conditions and time points. Color index of P-values represents level of significance. On the right, overrepresented GO terms based on AmiGO term analysis. hai, h after *P. rapae* infestation.

Drought data set: effect of herbivory and *B. cinerea* infection on dynamics of drought stress-induced gene expression

Also for drought stress we investigated the effect of the other two stresses on the dynamics of the transcriptome changes that are induced by this abiotic stress. We analyzed the dynamics of the global expression patterns of all DEGs from the drought single stress and the sequential stresses with drought as the second stress (Fig. 12; Supplemental Table S1). Clustering of the drought DEGs yielded 8 clusters of co-expressed genes. GO term analysis of overrepresented biological processes in each cluster highlights the main differentially regulated biological processes. As expected, drought stress induced a relatively large number of genes related to GO term “response to water deprivation” and “response to abiotic stimulus” (Fig. 12, clusters 7 and 8). Another feature that stands out is the fact that drought stress is associated with the down-regulation of a large number of genes, many of which are associated with biological processes such as “photosynthesis”, “defence response”, “DNA replication” and “immune system process” (Fig. 12, clusters 1, 2, 3, and 6), reflecting the fact that drought-stressed plants shift their strategy from energy-demanding processes related to growth and biotic stress defense to adaptation

to the abiotic stress condition. Interestingly, after 1 day of re-watering (7+1 d columns in Fig. 12), the drought-induced transcriptional changes that intensified over the 7-d period of water withhold, were to a large extend reset towards basal levels within 24 h. Like what we observed in the *P. rapae* and *B. cinerea* single and sequential double stress data sets, global gene expression patterns over time in the sequential double stress treatments was to a large extent similar to those inflicted by the drought treatment alone. Only cluster 6 genes, which are enriched for GO term "immune system process" seemed to be more down-regulated after 1 d of re-watering when drought-treated plants had been pre-treated with *B. cinerea* infection. Overall, we conclude that irrespective of the nature of the first stress, *Arabidopsis* is capable of rapidly reprogramming its transcriptome to the last stress encountered.

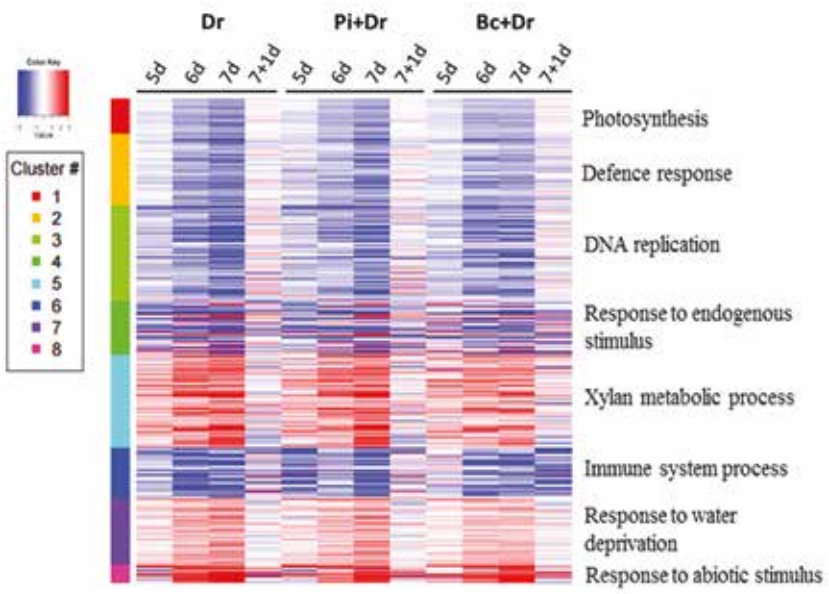


Figure 12. Dynamics of the expression of drought DEGs during single and sequential double stresses. Heatmap showing the expression pattners over time of the drought DEGs during a 7-d period of water withhold on non pre-treated control (Dr), *P. rapae* pre-infested (Pi+Dr) or *B. cinerea* pre-infected (Bc+Dr) *Arabidopsis* plants (FDR <0.05; >2-fold; total 6736 genes). In blue the down-regulated genes and in red the up-regulated genes. The union of DEGs of all treatments combined were clustered using Mclust yielding 8 clusters (colored bars on the left). On the right side, the most significant GO terms based on AmiGO term analysis are shown for the clusters.

Wigwams analysis of co-regulated gene clusters in the drought data sets identified 41 co-expressed gene modules. Analysis of these clusters for co-expression under the single and sequential double stress conditions (Dr, Bc+Dr, and Pi+Dr) revealed gene modules of which the expression patterns were clearly affected in one or both of the double-stress treatments in comparison to the drought treatment alone (examples in Fig. 13; full set in Supplemental Fig. S3). The identities of the genes in these Wigwams gene modules

are given in Supplemental Table S2. Again, a detailed description of the identified genes is not within the scope of this paper, but among the drought-responsive genes of which the expression pattern is clearly different when plants were exposed to *B. cinerea* infection or herbivory prior to the start of the drought period is *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*; At3g48090) (Falk *et al.*, 1999) in Dr module 21 (Fig. 13; Supplemental Table S2), in which genes related to GO term "innate immune response" are overrepresented (data not shown). Prior herbivory by *P. rapae* suppressed the expression level of this co-regulated gene module up to day 5 of the drought treatment. Previously, mutant *eds1* plants were shown to display reduced drought tolerance (Chini *et al.*, 2004)2004. Hence, suppression of *EDS1* in herbivore pre-treated plants may affect the level of drought tolerance in the sequential double stress.

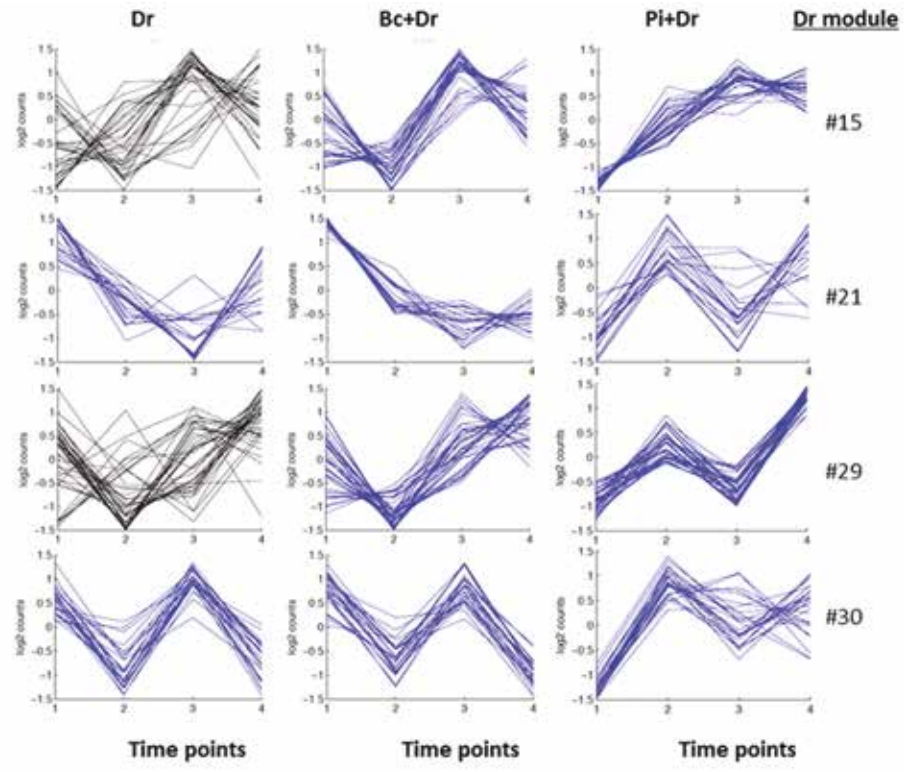


Figure 13. Expression patterns of selected Wigwams modules from the drought set of DEGs during single and sequential stress conditions. A selection of Wigwams modules is depicted that contain significantly co-expressed gene clusters for the single stress drought (Dr), and whose expression pattern changed in one or both of the sequential stresses *B. cinerea*-drought (Bc+Dr) and *P. rapae*-drought (Pi+Dr). The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 5, 6, 7 and 7+1 d after onset of water withhold, in which the 7+1 d time point represents plants that were re-watered for 1 d after the 7-d drought period.

Also for the drought DEGs, we analyzed the timing and level of significance of overrepresentation of all the GO terms in the single and sequential double stress time series (Fig. 14). In the drought single stress data set, GO terms “response to water deprivation”, “response to wounding”, “jasmonic acid metabolic process”, and “cell wall organization or biogenesis” are clearly enriched in the upregulated genes, while in the down-regulated gene set, GO terms “photosynthesis”, “shoot system development” and “nitrogen compound metabolic process” prevail, highlighting the biological processes that are engaged or affected during drought stress. GO terms “phenylpropanoid biosynthetic process”, “response to ethylene”, “response to abscisic acid”, “response to osmotic stress”, and “response to jasmonic acid”, become significantly more overrepresented in the sequential double stress, especially when the drought stress treatment was preceded by herbivory. In the down-regulated gene sets, GO terms “response to auxin”, “response to fungus”, “response to chitin”, “systemic acquired resistance” and “response to salicylic acid” become more significantly enriched in the double stress combinations. Like in the *P. rapae* and *B. cinerea* data sets, biological processes related to hormone action become relatively often differentially enriched in the sequential double treatments in comparison to the single stress treatment.

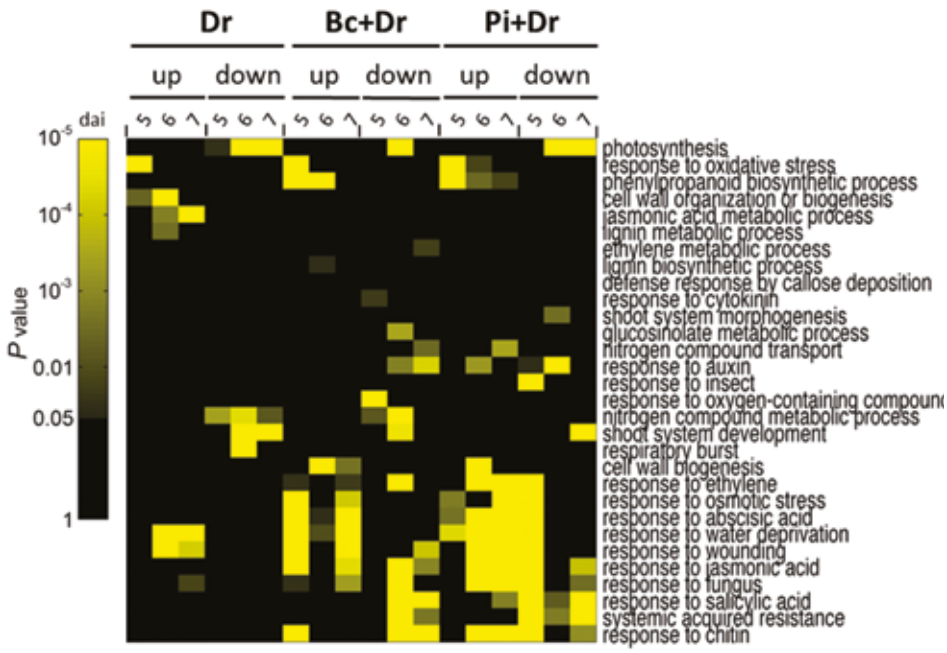


Figure 14. Timing of GO term overrepresentation patterns in drought single and sequential stress data sets. Heatmap represents the P-values of GO term overrepresentation in up- or down-regulated gene clusters in *Arabidopsis* at the given stress conditions and time points. Color index of P-values represents level of significance. On the right, overrepresented GO terms based on AmiGO term analysis. dai, d after water withhold.

Effect of stress interactions on plant resistance

For all three main stresses tested, prior treatment with one of the other stresses imposed a first-stress-signature in the dynamics of their transcriptome profiles. Wigwams analysis provided insight into the identity of the genes related to these first-stress-signatures (Figs. 6, 10 and 13), whereas analysis of GO term enrichment provided global insight into the biological processes that were affected by the prior stress treatment (Figs. 7, 11 and 14). As an example we showed the effect of prior drought stress and herbivory on the level of resistance against *B. cinerea* infection (shown in Fig. 8). The plant resistance assays with the reciprocal sequential stress treatments will be reported elsewhere.

Discussion

Plants are often exposed to different abiotic and biotic stresses, which can occur simultaneously or sequentially. How plants selectively adapt their response to this complexity of stresses is largely unknown. In this study, we aimed to gain insight into how plants respond do different biotic and abiotic stresses when previously exposed to another stress, using a necrotrophic pathogen, an insect herbivore, and drought as main stress factors. By analyzing the dynamics of the *Arabidopsis* transcriptome over four consecutive time points in response to the single stresses and the six possible sequential double stresses we were able to show that 1) up to 40% of the DEGs in a given single stress are also differentially regulated in one or both of the other two stresses, albeit often in different directions; 2) irrespective of the nature of the first and second stress applied, the *Arabidopsis* transcriptome is rapidly rewired to follow the pattern induced by the second stress; 3) the Wigwams algorithm identified first-stress-signatures of co-expressed genes that behave differently in the sequential double stress profile in comparison the single stress treatment; 4) plant hormone-related biological processes play a dominant role in the interaction between different stresses; 5) a prior stress can have positive or negative effects on the outcome of a sequential second stress (e.g. opposite effects of drought and herbivory on *B. cinerea* resistance).

Transcriptome profiles of different single stresses show significant overlap

The expression profiles of the single stresses caused by *B. cinerea* infection, *P. rapae* infestation, and drought are clearly different in timing and numbers of up- and down-regulated genes (Fig. 2). Nevertheless, up to 40% of the DEGs of any of the single stress profiles are also differentially expressed in one of the other single stress profiles (Fig. 4A). When clustering the union of all DEGs from the three single stress profiles, it becomes clear that genes related to the usual suspects in stress-related processes are overrepresented in the predominantly upregulated gene sets (e.g. GO terms related to abiotic stress and defense), while genes related to GO term "photosynthesis" become overall down-regulated (Fig. 3), confirming the notion that plants under stress prioritize adaptive responses over growth (Vos *et al*., 2013a; Vos *et al*., 2015). Clustering of the 392 DEGs that are differentially expressed in all three single stress conditions clearly shows that the expression profiles of the 9 clusters are often in different directions (up or down), depending of the nature of the stress (Fig. 4B). Only cluster 6, containing an overrepresentation of genes related to the GO term "response to oxygen compound" shows a general upregulation of genes in all three single stress conditions, highlighting that this biological process is central to stress responses in general. Overall, these results indicate that a significant proportion of the stress-related transcriptome is engaged by all three stresses tested. The observation

that the specific segments of the core stress-related transcriptome are differentially up or down regulated, depending on the type of stress, suggests that these segments may function in the antagonistic or synergistic effects that combinations of stresses can have on the outcome of the stress response.

Arabidopsis rapidly rewires transcriptome to latest stress encountered

Analysis of the dynamics of the single and sequential double stress transcriptome profiles showed in all possible double stress combinations that the transcriptome profiles in the sequential double stresses were remarkably similar to those of the respective single stresses (Fig. 5, 9, and 12). Also drought-stressed plants largely reset the drought-induced changes within 24 h after re-watering (Fig. 12). Apparently, plants are highly plastic in their capacity to adapt to changes in their biotic and abiotic environment, and swiftly rewire their transcriptome to the latest stress encountered. Nevertheless, it has been demonstrated that prior exposure to biotic or abiotic stress conditions can have dramatic effects on the outcome of a second stress. Classic examples of this are the different forms of induced resistance that are triggered by pathogens, insect herbivores and beneficial microbes, that change the outcome of the defense response against a subsequent invasion by another pathogen or insect in a positive or negative manner (De Vos *et al.*, 2004; Howe & Jander, 2008; Van Oosten *et al.*, 2008; Pieterse *et al.*, 2014). The same holds true for the effects of abiotic stresses, which have also been shown to alter the level of resistance against biotic stresses (Fujita *et al.*, 2006). Indeed drought and herbivory changed the level of resistance against *B. cinerea* (Fig. 8), even though the global transcriptional profiles of the *B. cinerea* single and sequential double stresses did not differ dramatically (Fig. 5). It must thus be concluded that subtle first-stress-signatures in the double stress transcriptional profile can have significant effects on the outcome of the adaptive response to the second stress.

Previously, it was shown that herbivory on *Arabidopsis* by *P. rapae* results in a systemic increase in the levels of JA, and that this can prime systemic tissues for enhanced JA-dependent defenses (Vos *et al.*, 2013b). Since the JA/ET-regulated defense pathway plays a major role in resistance against *B. cinerea,* herbivory-induced priming may play a role in the enhanced resistance against *B. cinerea* (Fig. 8A). Drought typically increases the level of ABA in the plant. It has been shown that ABA antagonizes JA/ET-regulated defenses (Vos *et al.*, 2015), which may explain our observation that drought pre-treated plants develop larger lesions after inoculation with *B. cinerea* (Fig. 8B).

Wigwams analysis of co-expressed gene clusters identifies first-stress-signatures in the sequential double stress transcriptional profiles

When globally inspecting the transcriptional profiles of the single and sequential double stress profiles, it is difficult to pinpoint obvious effects of a first stress on the dynamics of the transcriptional response to a second stress. For the *B. cinerea*-related profiles (Fig. 5) one could identify Cluster 2 ("response to ethylene") and cluster 6 ("multidimensional cell growth") as being affected by herbivory and drought (Cluster 2) or drought (Cluster 6). Also in the *P. rapae*-related profiles (Fig. 9) and the drought-related profiles (Fig. 12) first-stress-signatures can be identified (e.g. Cluster 7 "systemic acquired resistance" in Fig. 9 and Cluster 6 "immune system process" in Fig. 12). Using the bioinformatics tool Wigwams (Polanski *et al.*, 2014), we were able to dissect the three stress-related profiles into gene modules that are co-regulated in time under different conditions. This approach highlighted gene modules whose expression patterns differed from the single stress profile in one or both of the sequential double stress profiles. A number of exemplary genes from these differential modules were highlighted, including *WRKY53* and *HIR2* in the *B. cinerea*-related profiles, *MYB12* and *FLS1* in the *P. rapae*-related profiles, and *EDS1* in the drought-related profiles. Future studies should reveal their role, and that of the many other genes identified in the first-stress-signatures, in shaping the outcome of the adaptive stress responses in the sequential dual stress conditions.

Hormone-related responses prevail in biological processes that are differentially enriched in the double stress transcriptional profiles

Zooming in on the biological processes that are differentially enriched among the transcriptional profiles of the sequential double stresses in comparison to their respective single stress profiles, we monitored GO term enrichment at the time points of first differential expression of all genes. Plots of all biological GO terms that become significantly represented in the set of DEGs at a certain time point, provide a landscape of the timing of when these biological processes significantly change (Figs. 7, 11 and 14). Interestingly, among all biological processes that become clearly more enriched in the sequential double stresses over their respective single stresses are GO terms related to the response to the stress-related hormones JA, ABA, SA, and ET and occasionally to auxin. This observation suggests that responses to these hormones are likely to play a central role in the interaction between the signaling pathways that regulate the adaptive responses to the individual stresses. In the past, JA, ABA, ET, and SA have been demonstrated to be crucial positive or negative regulators of plant resistance against *B. cinerea* (JA, ET, and SA; Thomma *et al.*, 1998; Thomma *et al.*, 1999; El Oirdi *et al.*, 2011), *P. rapae* (JA and ABA; De Vos *et al.*, 2006; Bodenhausen & Reymond, 2007; Vos *et al.*, 2013b), and drought stress

(ABA; Yamaguchi-Shinozaki & Shinozaki, 2006). Hence, crosstalk between them may be decisive in the outcome of the adaptive response when two stresses are encountered sequentially. Future research, will be focused on biological validation of candidate genes in the Wigwams modules with putative major roles in shaping the outcome of sequential double stresses. Knowledge on how plants cope with different stresses simultaneously or in sequence will aid in breeding for multi-stress tolerant crops.

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Supplementary Information (See Appendix)

Supplemental Table S1: Differentially expressed *Arabidopsis* genes (AGI numbers of DEGs; FDR <0.05; >2-fold) in response to *P. rapae* infestation, *B. cinerea* infection, drought stress, and their six sequential combinations at four consecutive time points.

Supplemental Table S2: List of *Arabidopsis* genes (AGI numbers of DEGs; FDR <0.05; >2-fold) in Wigwams modules of co-expressed genes in the *B. cinerea, P. rapae,* and drought single and sequential double stress data sets.

Supplemental Figure S1. Expression patterns of all 32 Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions. Wigwams modules contain significantly co-expressed gene clusters in the *B. cinerea* set of DEGs from the single and sequential stresses. The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 6, 12, 18 and 24 h after *B. cinerea* inoculation.

Supplemental Figure S2. Expression patterns of all 85 Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions. Wigwams modules contain significantly co-expressed gene clusters in the *P. rapae* set of DEGs from the single and sequential stresses. The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 3, 6, 12 and 24 h after *P. rapae* infestation.

Supplemental Figure S3. Expression patterns of all 41 Wigwams modules from the drought set of DEGs during single and sequential stress conditions. Wigwams modules contain significantly co-expressed gene clusters in the drought set of DEGs from the single and sequential stresses. The modules represent standardized patterns of differential gene expression over time (log2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 5, 6, 7 and 7+1 d after water withhold, in which 7+1 d is one day after rewatering.

Chapter 4

Effect of prior drought and pathogen stress on *Arabidopsis* transcriptome changes to caterpillar herbivory

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Abstract

In nature, plants are exposed to biotic and abiotic stresses that often occur simultaneously. Therefore, plant responses to combinations of stresses are most representative of how plants respond to stresses in nature. We used RNA-seq to assess temporal changes in the transcriptome of *Arabidopsis thaliana* to herbivory by *Pieris rapae* caterpillars, either alone or in combination with prior exposure to drought or infection with the necrotrophic fungus *Botrytis cinerea*. Pre-exposure to drought stress or *Botrytis* infection resulted in a significantly different timing of the caterpillar-induced transcriptional changes. Additionally, the combination of drought and *P. rapae* induced an extensive downregulation of *A. thaliana* genes involved in defence against pathogens. Despite, the larger growth reduction observed for plants exposed to drought plus *P. rapae* feeding compared to *P. rapae* feeding alone, this did not affect weight gain of this specialist caterpillar. Plants respond to combined stresses with phenotypic and transcriptional changes that differ from the single stress situation. The effect of previous exposure to drought or *B. cinerea* on transcriptional changes to caterpillars is largely overridden by the stress imposed by caterpillars, indicating that plants prioritize insect defence over responses to drought or *B. cinerea* when applied in sequence.

Keywords: RNA-seq, transcriptome, combined stresses, multiple stresses, herbivory, abiotic stress.

Introduction

During their life cycle, plants suffer from a broad range of stresses (Buchanan *et al.*, 2000). These include abiotic stresses (e.g. drought, flooding, heat, cold, or nutrient deficiency) (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Roy *et al.*, 2011; Fahad *et al.*, 2015; Mickelbart *et al.*, 2015) and biotic stresses, imposed by other living organisms (e.g. bacteria, fungi, viruses, insects, or other plants) (Jones & Dangl, 2006; Howe & Jander, 2008; Dicke & Baldwin, 2010; Mithofer & Boland, 2012; Pieterse *et al.*, 2012; Dangl *et al.*, 2013; Pierik *et al.*, 2013; Stam *et al.*, 2014). Under natural conditions, these stresses do not occur in isolation but are commonly present simultaneously (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014). Due to the sessile nature of plants, they have evolved sophisticated mechanisms for tolerating or combatting stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Mickelbart *et al.*, 2015).

Plants have evolved mechanisms for perceiving microbial pathogens, insect herbivores and abiotic stresses (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Howe & Jander, 2008; Dangl *et al.*, 2013). Upon stress perception, plants can elicit defensive mechanisms in a stressor-specific manner (De Vos *et al.*, 2005; Kilian *et al.*, 2007; Bidart-Bouzat & Kliebenstein, 2011). Plant hormones have emerged as important players underlying specificity in plant stress responses (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Erb *et al.*, 2012; Pieterse *et al.*, 2012). For instance, salicylic acid (SA) especially mediates responses to phloem-feeding insects and biotrophic pathogens (De Vos *et al.*, 2005; Glazebrook, 2005), jasmonic acid (JA) mediates especially responses to chewing insects and necrotrophic pathogens (Reymond *et al.*, 2004; De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007) and abscisic acid (ABA) mediates responses to abiotic stresses such as drought, cold and heat stress (Yamaguchi-Shinozaki & Shinozaki, 2006; Kilian *et al.*, 2007; Huang *et al.*, 2008).

Phytohormonal signalling pathways are known to interact with each other in a phenomenon coined “crosstalk”. Crosstalk has been hypothesized to allow plants to respond in a fast, specific and cost-effective manner to stresses (Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Vos *et al.*, 2013a). Interactions between phytohormonal signalling pathways can be antagonistic and synergistic. For example, SA- and JA-mediated defences are known to exert negative effects on each other (Verhage *et al.*, 2010; Sendon *et al.*, 2011; Van der Does *et al.*, 2013; Caarls *et al.*, 2015), whereas ethylene (ET) and ABA have emerged as positive modulators of JA-mediated responses (Van der Ent *et al.*, 2008; Verhage *et al.*, 2010; Verhage *et al.*, 2011;

Vos *et al.*, 2013b). Responses to necrotrophic pathogens and herbivorous caterpillars are mediated by different branches of the JA signalling cascade, the ERF and MYC branches respectively (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). The ERF branch is activated by JA/ET through the transcription factors ERF1 and ORA59 and results in the expression of genes such as *PDF1.2* (Lorenzo *et al.*, 2003; Pre *et al.*, 2008; Verhage *et al.*, 2010). The MYC branch is activated by JA/ABA through the transcription factor MYC2*,* resulting in the expression of genes such as *VSP2* (Verhage *et al.*, 2011; Vos *et al.*, 2013b). The ERF and MYC2 transcription factors antagonistically regulate the two branches of the JA signalling pathway (Verhage *et al.*, 2011). Other hormones that mediate the responses of plants to environmental stresses include auxin (IAA), cytokinins, brassinosteroids, strigolactones and gibberellins (Erb *et al.*, 2012; Pieterse *et al.*, 2012; Giron *et al.*, 2013; Song *et al.*, 2014).

The simultaneous occurrence of stresses may modify the overall level of stress imposed on a plant. For instance, abiotic stresses tend to have a negative impact on plant responses to pathogens (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). Tomato plants exposed to simultaneous stress imposed by salinity and a pathogen were more susceptible to the pathogen than when exposed only to the pathogen (Kissoudis *et al.*, 2015) and drought promoted population growth of generalist aphids in *Arabidopsis thaliana* (Mewis *et al.*, 2012). Therefore, the question emerges how plants elicit an effective defence response when exposed to multiple stresses. To address this question, several recent studies have conducted whole transcriptome profiling using microarrays, of plants exposed to multiple abiotic and biotic stresses (Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Sewelam *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015). These studies have shed light onto plant responses to multiple stresses at the molecular level. One clear pattern that emerged is that responses to combined stresses cannot be predicted from the responses to individual stresses (Atkinson & Urwin, 2012; Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015).

Quantifying the complete set of transcripts under specific ecophysiological conditions is essential to understand the regulatory mechanisms involved in acclimation to those conditions. With the reduction in costs of NGS (Next Generation Sequencing), RNA-seq analysis is becoming feasible for large-scale transcriptomic analyses (Wang *et al.*, 2009; Van Verk *et al.*, 2013). RNA-seq analysis offers several advantages over microarrays: (1) there is no restriction to known genes, (2) unlimited dynamic range in quantitation, (3) more accurate expression level assessment, (4) less sophisticated normalization procedures needed, (5) no problems with cross-hybridization of similar transcripts (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). In

addition, RNA-seq analysis can extend the studies of transcriptomes to the analysis of splice variants.

Here, we used RNA-seq to assess temporal changes in the transcriptomic response of *A. thaliana* to herbivory by *Pieris rapae* caterpillars alone or combined with exposure to drought or the necrotrophic pathogenic fungus *Botrytis cinerea*. These stresses were chosen because the response of *A. thaliana* to these three stresses is highly diverse, while at the same time regulated by the plant hormones JA/ET (response to *P. rapae* and *B. cinerea*) and/or JA/ABA (response to drought). The main goals of this study were: (1) To characterize transcriptome changes of *A. thaliana* in response to herbivory by *P. rapae* caterpillars, (2) to investigate to what extent transcriptome signatures elicited by caterpillar herbivory change when plants had been previously exposed to drought or *B. cinerea* infection, (3) to identify genes specifically differentially expressed upon exposure to a combination of stresses, and (4) to identify putative splice variants differentially expressed in response to herbivory by *P. rapae* caterpillars alone or combined with previous exposure to drought or *B. cinerea*. To our knowledge, this is the first study using a platform independent from microarrays to address transcriptional responses of *A. thaliana* to herbivores. Thus, this study presents a valuable dataset for comparison with results obtained through microarray analysis and deriving general conclusions of how plants respond to herbivory by caterpillars.

Materials and Methods

Plants, insects and pathogens

Plant growth conditions. Arabidopsis thaliana (L.) Heynh. Col-0 seeds were sown in containers (10 cm length, 5 cm width, 5 cm height) containing pasteurized (80 °C for 4 h) sand that was humidified by adding 50 ml Hoagland solution. Seeds were sown at a density of approximately 100 seeds per container. In order to keep 100% RH during germination, the containers were enclosed in a tray with a transparent lid. Seeds were vernalized for two days at 4 °C in a dark room to overcome remaining dormancy and to induce even germination. Hereafter, the tray was moved to a controlled-environment chamber at 23 ± 1 °C, 70 ± 10 % relative humidity, 100 µmol m^{-2} s^{-1} photosynthetically active radiation and a diurnal cycle of 8:16 L:D. After one week, the lid was removed from the tray. Two-week-old seedlings were individually transplanted to 0.08 L pots (5 cm height × 5 cm diameter) containing a 1:1 mixture (v/v) of commercial potting soil and sand. Pots were watered at the bottom three times per week. Plants were subsequently grown under similar conditions until they were exposed to the treatments.

Herbivore rearing. Pieris rapae L. (Lepidoptera: Pieridae; Small Cabbage White butterfly) were routinely reared on cabbage plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in a greenhouse as described previously (Van Poecke *et al.*, 2001; De Vos *et al.*, 2005).

Pathogen cultivation. Botrytis cinerea strain B0510 was grown on one-half-strength PDA plates, containing penicillin (100 μg mL^{-1}) and streptomycin (200 μg mL^{-1}), for two weeks at room temperature. Spores were collected and suspended in half-strength potato dextrose broth (Difco Laboratories) to a final density of 1.0 x 10^5 spores mL^{-1}. After a 3 h-incubation period, the spores were used for inoculation (see treatment section) (Thomma *et al.*, 1998; Pre *et al.*, 2008; Van der Ent *et al.*, 2008).

RNA-seq experiment

Treatments

Plants were exposed to five treatments: a) control (C), b) mock (M), c) *P. rapae* herbivory (P), d) drought plus *P. rapae* (DP) and e) *B. cinerea* plus *P. rapae* (BP). Plants were grown under similar conditions for 32 days after germination (DAG) (see Figure 1). Plants were exposed to drought by withholding water for seven days: from 33-39 DAG. The drought treatment was followed by a 24-h recovery period (40 DAG). *B. cinerea* inoculation was also carried out at 40 DAG. Plants were inoculated with *B. cinerea* by pipetting 5 µl of spores suspended in half-strength PDB at a concentration

of 1 × 10^5 spores mL^{-1} on two leaves of the rosette. Plants were kept at 100% RH in order to ensure successful infection by *B. cinerea*. Plants exposed to *P. rapae* as single or combined stress were inoculated with 2 first instar (L1) *P. rapae* caterpillars on leaf number 8 (41 DAG). Caterpillars were allowed to move freely on the plant. Two controls were included in this experiment, a group of plants that did not experience any stress (Control) and a group of plants that were inoculated with 5 µl of mock solution on two leaves of the rosette (Mock). Mock solution consisted of the same medium that *B. cinerea* spores were suspended in. Plants treated with mock solution were also kept at 100% RH.

Experimental design

The experiment was carried in a full factorial design with two factors: time and treatment. Time had four levels (3, 6, 12, and 24 h) and treatment had five levels (control, mock, *P. rapae* herbivory, drought plus *P. rapae,* and *B. cinerea* plus *P. rapae*). The experiment was carried out in a growth chamber, following a flat table design with three blocks (Figure S1). Time was randomized within the three blocks. For every time point, treatments were assigned randomly. Each time and treatment combination consisted of 3 biological replicates. Each biological replicate consisted of pools of 4 plants. In total, we collected 60 samples (3 replicates × 5 treatments × 4 time points). RNA extraction was carried out in batches of 20 randomly chosen samples. Samples were sequenced single end (SE) 93 bp. on an Illumina Hi-Seq 2000 sequencer. Samples were sequenced in three runs. Within each run, samples were randomly assigned to 7 lanes of the Illumina flow cells.

Sampling

Leaf samples were collected from plants exposed to *P. rapae* herbivory as single and combined stress and its respective controls; this was done at 3, 6, 12 and 24 h after inoculation (HAI) with *P. rapae* (Figure 1). For plants under control and mock conditions, leaf number 8 was collected. For plants exposed to *P. rapae* as single or combined stress, leaf number 8 was collected when it showed caterpillar feeding damage. Otherwise, the leaf closest to leaf number 8 that displayed feeding damage was collected. Upon collection, leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C.

RNA extraction and library preparation

RNA was extracted using Qiagen Plant RNeasy Plant Mini Kit (cat. no. 74903). All samples were treated with DNAase I on column using the Qiagen RNase-Free DNase Set (cat. no. 79254). Quality and quantity of total RNA were initially measured with a NanoDrop ND-1000 (Nanodrop, Delaware, USA). RNA quality was also checked using the RNA Integrity Number (RIN) with The Agilent 2100 bioanalyzer using RNA Nano chips. For library preparation we used only samples with RIN values ≥ 6. The

sample preparation was performed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina. This protocol allows the identification of strand-specific transcripts. First, Poly-A RNA was isolated from the total RNA using Poly-T oligo attached magnetic beads. Subsequently, RNA was fragmented using divalent cations under elevated temperature. First-strand cDNA was synthesized using random primers. Strand specificity is achieved by replacing dTTP with dUTP in the second Strand Marking Mix (SMM), followed by second strand cDNA synthesis using DNA Polymerase I and RNase H.

Preparation of cDNA and validation of RNA-seq by quantitative RT-PCR

A total of 24 out of the 60 samples that were used for the RNA-seq analysis were selected to be validated by quantitative RT-PCR. These samples corresponded to 12 samples from control plants and 12 samples from plants exposed to *P. rapae* as single stress. RNA extracted from control plants and plants exposed to *P. rapae* as single stress was prepared as follows. A total of 100 ng of RNA for all samples was used as starting material. First-strand cDNA synthesis was carried out with iScript cDNA Synthesis Kit (Bio-Rad) with an oligo (dT) primer according to the manufacturer's protocol. PCR was carried out in a total volume of 25 µL, containing 5 µL of a 10x diluted cDNA, 12.5 µL of iQ SYBR Green Supermix (Bio-Rad) and 5 pmol of each primer. RT-PCR was carried out for *VSP2* (AT5G24770) (Pineda *et al.*, 2012) and *MYC2* (AT1G32640) (Anderson *et al.*, 2004). These genes are induced by *P. rapae* herbivory (Reymond *et al.*, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007; Broekgaarden *et al.*, 2007). As reference gene, we used *PP2A* (AT1G13320) (Czechowski *et al.*, 2005; Verhage *et al.*, 2011). The following PCR conditions were used: 3 min at 95.0 °C, followed by 40 cycles of 15 s at 95.0 °C and 45 s at 62.0 °C. Melting curves were recorded by heating the samples from 60 to 95˚C with a ramp speed of 1.9˚C min^{-1}. Reactions were performed in two technical replicates per sample. For *VSP2* and *MYC2,* ΔCt values were calculated using *PP2A* as reference gene. Primers used for RT-PCR are summarized in Table S1. The RPKM values were obtained by converting the raw counts from HTSeq-count (see below) using a custom R script in reads per kilobase per million reads (RPKM). ΔCt values were compared to $\log_2$ (RPKM_gene of interest)/RPKM_house keeping gene) using Pearson correlation (Figure S2). Both ΔCt and $\log_2$ (RPKM_gene of interest)/RPKM_house keeping gene) are equivalent to the $\log_2$-fold changes in expression for each gene relative to the same reference gene (Loraine *et al.*, 2013).

RNA-seq analysis

Gene expression quantification

RNA-seq reads were mapped to the *A. thaliana* genome version TAIR 10 using TopHat (Trapnell *et al.*, 2009) with standard parameters and the following adjustments: -N 3 --min-intron-length 50 --max-intron-length 5000 -g 1 -M -p 4 -G --read-edit-dist 3 --library-type. The number of reads mapped to each annotated *Arabidopsis* gene (TAIR10) were determined using HTSeq count (Anders *et al.*, 2014)2014. Finally, raw read counts were subject to a regularized log transformation, implemented in the DESeq2 package (Love, 2014), which normalizes counts for differences in sequencing depth across samples and produces expression values that are more suitable for clustering and visualization.

Differential gene expression

Differential gene expression analysis was carried out with the DESeq2 Bioconductor package in R (Anders & Huber, 2010; Love *et al.*, 2014). Raw counts, which are the number of read pairs aligned to each TAIR 10 gene model with a maximum of three mismatches were used for this analysis. Differentially expressed genes (DEGs) were computed per time point. For each pair of treatments, we compared the resulting read counts from three biological replicates. Per individual time point, we performed the following comparisons among treatments: (1) control vs *P. rapae*, (2) *P. rapae* vs drought plus *P. rapae* and (3) *P. rapae* vs *B. cinerea* plus *P. rapae.* DESeq2 fits a Generalized Linear Model (GLM) to the data, where counts per gene and sample are modelled using a negative binomial distribution. P-values were computed using a Wald test (Love *et al.*, 2014). Genes were considered to be differentially regulated in a given pair of treatments if they had a P-value ≤ 0.01 after Bonferroni correction and a $\log_2$-fold change ≤ -1 or ≥1.

Gene ontology analysis

We used Bingo, implemented into Cytoscape 3.1.1 platform (Maere *et al.*, 2005), to identify enriched gene ontology (GO) terms in our gene lists. In all cases we used the following parameter settings: hypergeometric test, with Benjamini-Hochberg FDR adjustment, $\alpha = 0.05$. As background, we used only genes that were expressed in at least one sample out of the 60 samples analyzed. GO term categories were reduced by semantic similarity using REVIGO (Supek *et al.*, 2011). The following settings were used: allowed similarity (small) and semantic similarity measure (Simrel).

Statistical analysis

Principal Component Analysis (PCA)

We used Principal Component Analysis *(*PCA) in the DESeq2 package in R (Love *et al.*, 2014). PCA was performed on the regularized $\log_2$-transformed data.

Orthogonal Projection to Latent Structures – Discriminant Analysis (OPLS-DA)

OPLS-DA was carried out on the regularized $\log_2$-transformed data with the software SIMCA P+ version 12 (Umetrics, Umeå, Sweden). The analysis shows the variable importance in the projection (VIP) of each variable (in this case, transcriptional data for the different genes), with variables having VIP values greater than 2 being most influential in the model (Eriksson *et al.*, 2006). One of the drawbacks of fitting a model with all variables (genes) is over-parametrization and poor predictability (Perez-Enciso & Tenenhaus, 2003). Thus, we also fitted a reduced model using only variables with VIP ≥ 2 as described in Perez-Enciso & Tenenhaus (2003) and Burguillo *et al.* (2014). Alternatively, one could use a t-test for each variable sequentially (Nguyen & Rocke, 2002). However, VIP is more appropriate since a t-test cannot be applied for more than two classes and VIP takes into consideration the effect of a variable (gene) on all categories and OPLS components (Perez-Enciso & Tenenhaus, 2003).

Phenotype expression experiments

Plant bioassay

An independent experiment was carried out to quantify the effect of herbivory by *P. rapae* alone or preceded by drought or *B. cinerea* on plant biomass. *A. thaliana* (Col-0) plants were exposed to five treatments: drought (D), herbivory by *P. rapae* (P), combinations of drought and *P. rapae* (DP) and *B. cinerea* and *P. rapae* (BP) and control (C). Pre-treatments before herbivory were applied in the same way as described for sample collection for RNA-seq analysis. For treatments involving herbivory, four-week-old plants were infested with two L1 *P. rapae* caterpillars. Caterpillars were allowed to feed on the plants for five days. At the end of the experiment, shoot fresh weight for the five treatments was quantified. A total of 65 biological replicates were included per treatment. Data were square-root transformed to satisfy the ANOVA assumptions of normality and homogeneity of variances. One-way ANOVA followed by Tukey Honestly Significant Difference (Tukey HSD) post-hoc tests for pairwise comparison were executed in R version 3.0.

Insect bioassay

An independent experiment was carried out to quantify the effect of drought or *B. cinerea* pre-treatment on insect performance. *A. thaliana* (Col-0) plants were pre-treated with drought, *B. cinerea* or no stress. Pre-treatments before herbivory were

applied in the same way as described for sample collection for RNA-seq analysis. Subsequently, plants were infested with one L1 *P. rapae* caterpillar. Caterpillars were allowed to feed on the plants for five days. At the end of the experiment, caterpillar weight was quantified. A total of 20 biological replicates were included per treatment. Data were square-root transformed to satisfy the ANOVA assumptions of normality and homogeneity of variances. One-way ANOVA was executed in R version 3.0.

Results

Arabidopsis transcriptional changes in response to herbivory by *P. rapae* alone or in combination with previous exposure to drought or *B. cinerea*

Gene expression levels were quantified by RNA-seq analysis for non-infested *A. thaliana* plants and for plants infested with *P. rapae* alone or preceded by drought or *B. cinerea* at four time points (3, 6, 12, and 24 h after insect infestation (HAI)). The dataset was analyzed by PCA (Figure 2). The first two principal components (PCs) explained 42% and 19% of the variation, respectively. The first PC separated non-infested from infested plants and the second component separated the time point 24 HAI from the remaining, earlier, time points (Figure 2A).

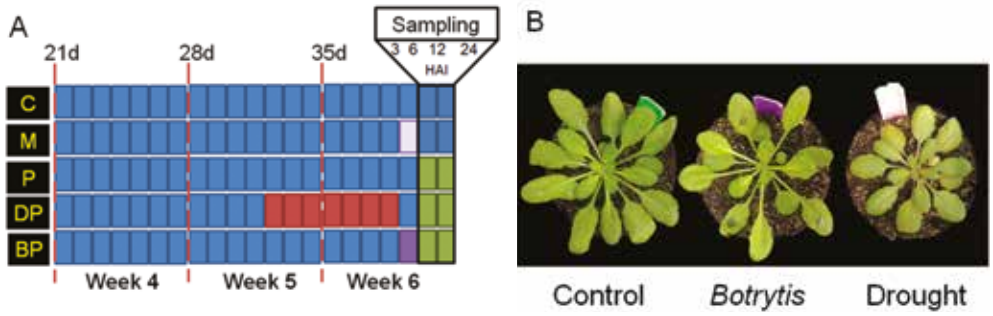

Figure 1. Experimental Design. (A) Treatment and sampling schedule. Plants were exposed to one of five treatments: Single stress imposed by *P. rapae* herbivory = P, combined stresses drought plus *P. rapae* = DP and *B. cinerea* plus *P. rapae* = BP, Control = C and Mock = M. Days after germination (DAG) are indicated in red dashed lines. Drought was imposed by not watering the plants for 7d followed by 24 h recovery after re-watering (red boxes). *B. cinerea* inoculation (dark purple box) was made 24 h prior to caterpillar inoculation (green boxes). Two types of controls were included: (1) Control, i.e. plants that were not exposed to stress (blue boxes) and (2) Mock, i.e. plants that were inoculated with the same medium used to inoculate the plants with *B. cinerea* spores (white box). Following the first stress, plants were inoculated with two neonate *P. rapae* caterpillars (green boxes) and samples for RNA-seq analysis were taken for all five treatments at 3, 6, 12 and 24 hours after inoculation (HAI). Three biological replicates were included per treatment and time point. Each biological replicate consisted of a pool of four plants. (B) Representative pictures of *A. thaliana* plants that had not been exposed to stress, exposed to *B. cinerea* infection or drought stress.

When comparing only samples from infested plants either with *P. rapae* infestation alone or *P. rapae* infestation preceded by exposure to drought or *B. cinerea* at the four different time points (Figure 2B), the first two PCs explained 43% and 24% of the variation, respectively. Both PCs contributed to the separation of samples by time points. Separation was also observed for plants infested with *P. rapae* alone or in combination either with drought or *B. cinerea* in a time-dependent manner. For instance, the largest separation was at 3 HAI, while at 24 HAI the samples were more similar to each other. Thus, the timing of the transcriptional response to *P. rapae*

caterpillars is significantly affected by prior drought or pathogen stress and that the differences diminished when the response developed with time.

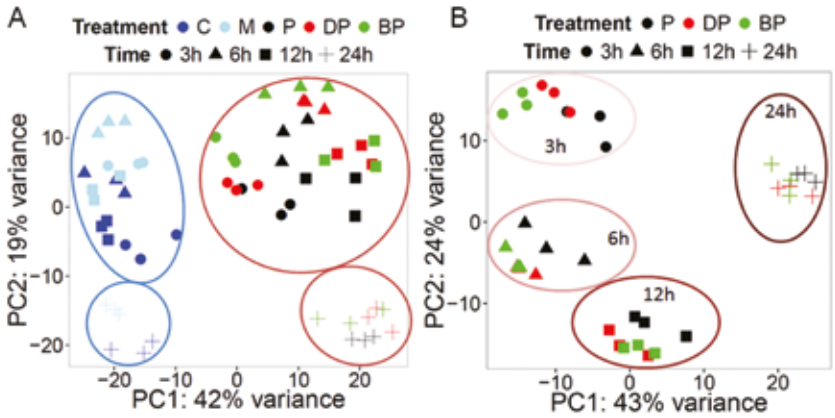

Figure 2. Principal component analysis of gene expression levels in *A. thaliana* plants that had been non-infested, infested with *P. rapae*, or infested by *P. rapae* after previous exposure to either drought or *B. cinerea* infection; samples had been taken at four different time points. Principal component analysis was executed with DESeq2 software on the regularized $\log_2$-transformed data. The first two principal components are plotted. (A) PCA on all treatments, and (B) PCA on the three treatments that included caterpillar feeding, while excluding the control and mock treatments. Colours indicate different treatments. Shapes indicate different time points. Percentages of variation explained by each PC are indicated along the axes. Single stress imposed by *P. rapae* herbivory = P; combined stresses drought plus *P. rapae* = DP, *B. cinerea* plus *P. rapae* = BP, Control = C and Mock =M.

Differential expression of *A. thaliana* genes in response to herbivory by *P. rapae* alone

Over the four time points of sample collection, a total of 3548 (2755 up- and 793 down-regulated) genes were differentially expressed upon herbivory by *P. rapae* at 1% FDR (P3, P6, P12 and P24 in Figure 3, Supplementary Data 1).The number of up- and down-regulated genes increased with time. Furthermore, the number of up-regulated genes was higher than the number of down-regulated genes at all time points (Figure 3B, Figure S3). A total of 59% and 31% of the differentially expressed genes (DEGs) were up- and down-regulated respectively at more than one time point (Table 1, Figure S3). Samples collected at 24 HAI had the highest proportion of genes that were differentially expressed at only one time point (hereafter called time-point specific expression): 16% for up- and 31% for down-regulated genes respectively. On the other hand, samples collected at 3 HAI and 12 HAI displayed the lowest proportion of time-point specific up- (4%) and down-regulated (10%) genes, respectively (Table 1). GO-term analysis revealed that up- and down-regulated genes were associated with 58 and 16 processes respectively (Supplementary Data 2). Up-regulated genes were associated with processes involved in secondary metabolism

(e.g. flavonoids, phenylpropanoids, phytoalexins, and glucosinolates), phytohormone signalling pathways (e.g. JA, ET, ABA, SA and IAA), cell-wall modification and abiotic stresses (e.g. drought and cold responses). Down-regulated genes were associated with processes related to plant defences, circadian rhythm and nitrate assimilation (Supplementary Data 2).

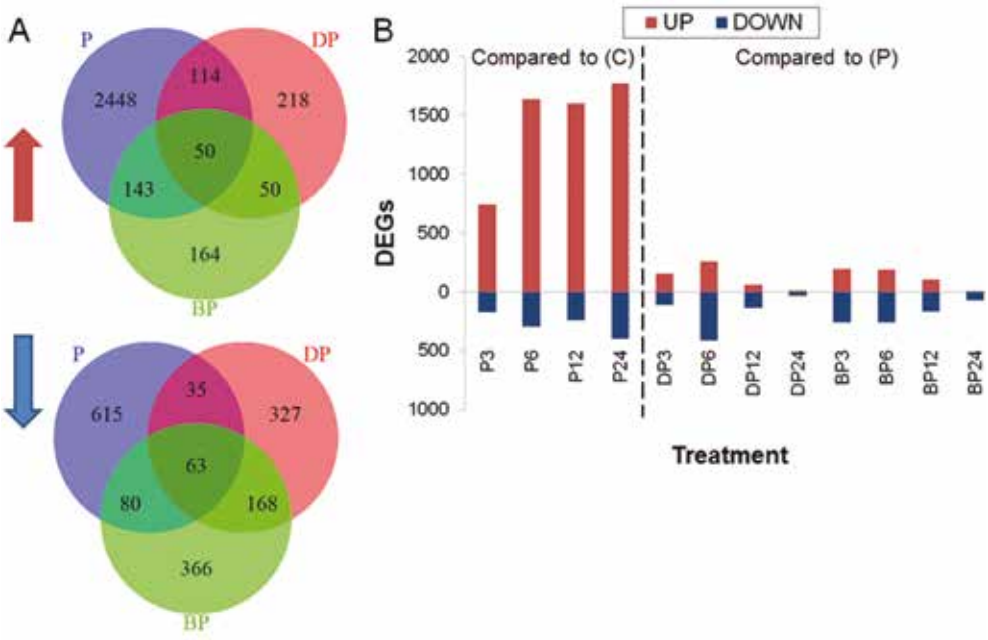

Figure 3. Differentially expressed genes (DEGs) in response to *P. rapae* herbivory alone or preceded by drought or *B. cinerea* over time. (A) Venn diagrams showing DEGs per treatment, P = Single stress *P. rapae*, DP = Combined stresses drought plus *P. rapae*. BP= Combined stresses *B. cinerea* plus *P. rapae*. (B) DEGs per time point and treatment combination. The time indications 3, 6 ,12 and 24 h refer to the numbers of hours after insect inoculation (HAI; see Figure 1) at which the samples were collected. Genes were considered to be differentially regulated if they displayed a $\log_2$-fold change ≥1 for up-regulated or ≤ -1 for down-regulated genes with respect to the reference condition and a P-value ≤ 0.01 after Benjamini-Hochberg correction for false discovery rate.Gene expression levels for the single stress *P. rapae* were compared with the untreated control (C). Gene expression levels for the double stresses drought plus *P. rapae,* or *B. cinerea* plus *P. rapae* were compared with the single stress *P. rapae* (P).

Differential gene expression of *A. thaliana* in response to herbivory by *P. rapae* preceded by exposure to drought

Over the four time points, a total of 1025 (432 up- and 593 down-regulated) genes were differentially expressed under combined drought and *P. rapae* stress compared to *P. rapae* alone at 1% FDR (Figure 3, Supplementary Data 3). A total of 26% of DEGs were shared with DEGs in plants exposed to *P. rapae* alone and 32% with DEGs in plants exposed to *B. cinerea* plus *P. rapae* (Figure 3A). The number of

up- and down-regulated genes increased from 3 HAI to 6 HAI, followed by a lower number at the subsequent time points (Figure 3B, Figures S3 and S4). A total of 12% and 15% of all DEGs were up- and down-regulated respectively at more than one time point (Table 1, Figure S3). Samples collected at 6 HAI displayed the highest proportion of time-point specific DEGs (51% up- and 56% for down-regulated genes respectively). On the other hand, samples collected at 24 HAI displayed the lowest proportion of time point-specific DEGs: 1% up- and 3% down-regulated genes (Table 1). GO-term analysis revealed that up- and down-regulated genes were associated with 22 and 38 processes respectively (Supplementary Data 4). Up-regulated genes were associated with processes involved in cytokinin metabolism and signalling, flavonoid, phenylpropanoid, and pigment biosynthesis. Down-regulated genes were associated with processes related to immune responses, response to salicylic acid, photosynthesis and protein phosphorylation (Supplementary Data 4).

Table 1. Proportion of genes up- and down-regulated upon *P. rapae* herbivory alone or when preceded by drought or *B. cinerea,* per time point. Number of genes differentially expressed genes specific for a specific time point are indicated. 'Common' reflects the number of genes differentially expressed at more than one time point.

		Pieris rapae		
Time	**Up**	**% Up**	**Down**	**% Down**
3h	114	4.1%	91	11.5%
6h	324	11.8%	129	16.3%
12h	255	9.3%	77	9.7%
24h	434	15.8%	248	31.3%
Common	1628	59.1%	248	31.3%
Total	2755	100.0%	793	100.0%
		Drought and *P. rapae*		
Time	**Up**	**% Up**	**Down**	**% Down**
3h	132	30.6%	81	13.7%
6h	222	51.4%	334	56.3%
12h	21	4.9%	69	11.6%
24h	5	1.2%	19	3.2%
Common	52	12.0%	90	15.2%
Total	432	100.0%	593	100.0%
		B. cinerea and *P. rapae*		
Time	**Up**	**% Up**	**Down**	**% Down**
3h	145	35.6%	294	43.4%
6h	121	29.7%	121	17.9%
12h	60	14.7%	49	7.2%
24h	4	1.0%	28	4.1%
Common	77	18.9%	185	27.3%
Total	407	100.0%	677	100.0%

Differential gene expression by *A. thaliana* in response to herbivory by *P. rapae* preceded by *B. cinerea* infection

Over the four time points, a total of 1084 (407 up- and 677 down-regulated) genes were differentially expressed after treatment with *B. cinerea* infection followed by *P. rapae* infestation, compared to plants exposed only to *P. rapae,* at 1% FDR (Figure 3, Supplementary Data 3). A total of 31% of these 1084 genes were shared with DEGs in response to *P. rapae* alone and 31% with DEGs found in the combined exposure to drought followed by *P. rapae* caterpillars (Figure 3A). The number of up-regulated and down-regulated genes decreased steadily over the time points (Figure 3B). Furthermore, the number of down-regulated genes was higher than the number of up-regulated genes at all time points (Figure 3B, Figures S3 and S4). A total of 19% and 27% were up- and down-regulated respectively at more than one time point (Table1, Figure S3). Samples collected at 3 HAI displayed the highest proportion of time-point specific DEGs (36% up- and 43% down-regulated genes respectively). On the other hand, samples collected at 24 HAI displayed the lowest proportion of specific up- (1%) and down-regulated (4%) genes respectively (Table 1). Up- and down-regulated genes were associated with 24 and 51 processes respectively (GO-term analysis, Supplementary Data 4). Up-regulated genes were associated with processes involved in lipid metabolism, response to temperature stimulus, wax metabolism, response to insects and regulation of anthocyanin metabolism. Down-regulated genes were associated with processes related to immune responses, flavonoid metabolism, protein phosphorylation, defence response by callose deposition in cell wall and indole glucosinolate metabolism (Supplementary Data 4).

Genes whose expression levels explain most of the differences between plants exposed to herbivory by *P. rapae* alone or preceded by exposure to drought or *B. cinerea*

We executed a discriminant analysis using OPLS-DA on the gene expression levels of *A. thaliana* plants that were non-infested, infested with *P. rapae* alone, or with *P. rapae* after exposure to drought or *B. cinerea*, at different time points (Figure 4). A full model identified two significant components explaining 6% and 5% of the variation, respectively. The first PC separates plants infested with *P. rapae* alone or exposed to *B. cinerea* plus *P. rapae* from plants exposed to drought plus *P. rapae* (Figure 4A). The second PC separates plants infested with *P. rapae* alone from those pre-infested with *B. cinerea* followed by infestation by *P. rapae* (Figure 4A). Subsequently, we fitted a reduced model including only genes having VIP values ≥ 2 (See Materials and Methods). A total of 420 genes were identified as having VIP values ≥ 2 in the full model (Supplementary Data 5). The reduced model identified two significant PCs, explaining 33% and 21% of the variance, respectively. The first PC separates plants exposed to drought plus *P. rapae* from plants exposed to *B. cinerea* plus *P. rapae* (Figure 4B).

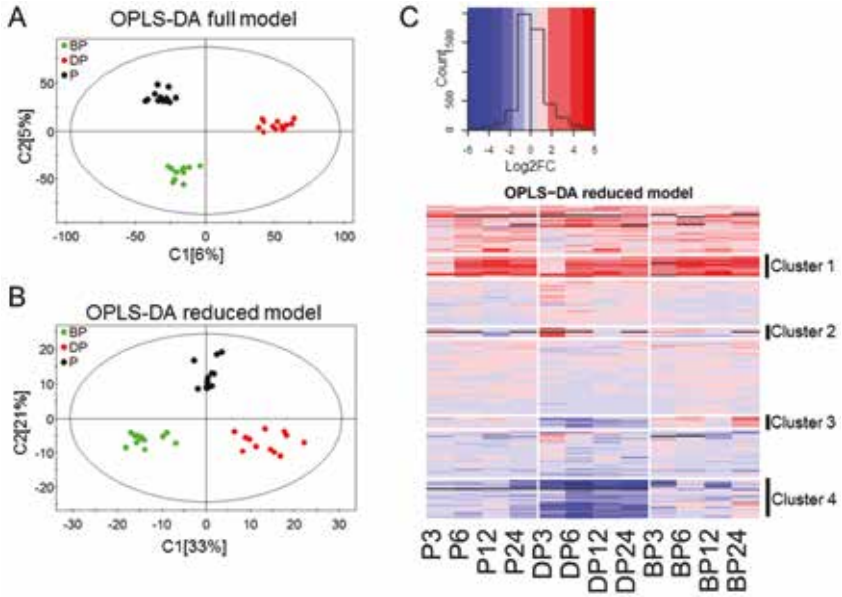

Figure 4. Discriminant analysis of gene expression levels for *A. thaliana* uninfested control plants, plants infested with *P. rapae* alone or with *P. rapae* infestation preceded by drought or *B. cinerea* infection at different time points. (A) OPLS-DA full model. Treatments are indicated in colours. (B) OPLS-DA reduced model. Treatments are indicated in colours. The reduced model was limited to genes with a VIP value ≥ 2 in the full model. (C) Heatmap showing the $\log_2$-fold changes of genes with VIP values in the reduced model being ≥ 0.8. Blue indicates down-regulated genes. Red indicates up-regulated genes. Black indicates missing values. P = P. *rapae* as single stress, DP = Combination of drought and *P. rapae*, BP = Combination of *B. cinerea* and *P. rapae*. The time indications 3, 6 ,12 and 24 h refer to the numbers of hours after insect inoculation (HAI) at which the samples were collected.

The second PC separates plants infested with *P. rapae* alone from plants exposed the two double stress treatments (Figure 4B). The expression patterns of the 420 genes with a VIP $\geq$ 2 are displayed in Figure 4C. Four clusters of genes were clearly different across treatments. Cluster 1 consists of 25 genes that were more up-regulated at 3 HAI in the *B. cinerea* plus *P. rapae* treatment compared to the other two treatments, i.e. drought plus *P. rapae* infestation or *P. rapae* infestation alone. Several genes involved in plant immunity belong to this cluster such as *BAP1* and *ERF104*. Cluster 2 consists of 13 genes that were up-regulated at 3 HAI in the drought plus *P. rapae* treatment compared to *B. cinerea* plus *P. rapae* or *P. rapae* alone. Among the genes in cluster 2 are two ABA receptors (*PYL4* and *PYL5*). Cluster 3 consists of 17 genes that were down-regulated at all time points in the drought plus *P. rapae* treatment, whereas it was induced by *B. cinerea* plus *P. rapae* or *P. rapae* alone. Several receptors were in this group of genes such as two Toll-Interleukin-Resistance (TIR) proteins (*AT1G57630, AT2G20142*). Cluster 4 consists of 52 genes that were down-regulated at all time points in the drought plus *P. rapae* treatment compared to *B. cinerea* plus *P. rapae* or *P. rapae* alone. Several genes involved in plant defences

against pathogens were present in this cluster, such as receptor-like proteins (*RLP39*, *RLP41*), receptor-like kinases (*CRK1*, *CRK37*,*CRK4*, *CRK6*, *CRK7*, *CRK8*, *WAK3*) and PR proteins (*PR-2*, *PR-5*).

Effect of herbivory by *P. rapae* alone or preceded by drought or *B. cinerea* infection on *A. thaliana* biomass

Because we observed that pre-treatment with drought or *B. cinerea* changed the timing of *A. thaliana* responses to *P. rapae*, we investigated if this shift in responses compromised plant immunity against *P. rapae*. We observed that *A. thaliana* plants exposed to stress imposed by drought, *P. rapae* alone or *P. rapae* herbivory preceded by drought or *B. cinerea* had a lower shoot fresh weight than control plants not exposed to stress ($P \leq 0.05$) (Figure 5A). Plants exposed to the combination of drought plus *P. rapae* feeding had a lower shoot fresh weight than plants exposed to *P. rapae* alone or plants exposed to *B. cinerea* plus *P. rapae* ($P < 0.05$). No difference in shoot fresh weight was observed between plants exposed to *P. rapae* alone or *B. cinerea* plus *P. rapae.* Interestingly, pre-treatment with drought or *B. cinerea* did not have an effect on caterpillar weight compared to caterpillars fed on plants not previously exposed to stress (Figure 5B). In conclusion, we observed that pre-treatment with drought or *B. cinerea.* followed by *P. rapae* elicited transcriptome changes that were different from those elicited by *P. rapae* alone and that these changes converged over time. Despite, these transcriptome differences, pre-treatment with drought, or *B. cinerea* did not compromise plant immunity against the most damaging stress imposed by the specialist caterpillar *P. rapae*.

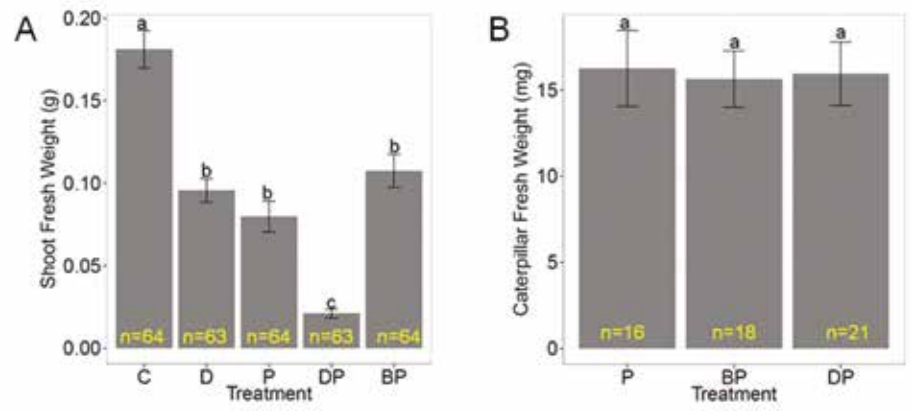

Figure 5. Phenotypic characterization of *A. thaliana* and *P. rapae* caterpillars when plants are exposed to *P. rapae* feeding alone or to *P. rapae* herbivory preceded by drought or *B. cinerea* infection. (A) *A. thaliana* rosette fresh weight after exposure to different stress treatments. C = Control, D = Drought, P = *P. rapae,* DP = drought plus *P. rapae* and BP = *B. cinerea* plus *P. rapae*. (B) *P. rapae* caterpillar weight after feeding for 5 days on plants that had not been exposed to stress (P), to drought (DP) or to *B. cinerea* infection (BP) prior to exposure to the caterpillars. Bars show mean values ± SE. Different letters above bars indicate significant differences between treatments (Tukey's HSD test, $P < 0.05$).

Discussion

Transcriptomic responses to herbivory by *P. rapae* caterpillars

Plants have evolved sophisticated mechanisms for detecting and responding to feeding by insect herbivores (Howe & Jander, 2008; Hogenhout & Bos, 2011; Mithofer & Boland, 2012). Herbivore-induced plant defences can be divided in three phases: perception, signal-transduction and response (Heidel-Fischer *et al*., 2014). JA-mediated responses have emerged as important components of plant defences against chewing herbivores (Reymond *et al*., 2000; Reymond *et al*., 2004; De Vos *et al*., 2005). For instance, Reymond *et al.* (2004) estimated that for 67- 84% of *A. thaliana's* transcriptional responses to *P. rapae* were JA-mediated. Furthermore, mutants that are impaired in JA-signalling have been shown to be more susceptible to herbivory by *P. rapae* (Bodenhausen & Reymond, 2007; Verhage *et al*., 2011; Vos *et al*., 2013b). In this RNA-seq analysis, we observed an extensive transcriptome reprogramming (3548 DEGs) upon *P. rapae* herbivory in *A. thaliana* over a 24 h time period (Figure 3). This transcriptome reprogramming occurred very fast as indicated by the large number of DEGs (744 up- and 171 down-regulated) identified already at 3 HAI (Figures S3 and S4). Expression of several genes involved in JA biosynthesis (e.g. *DAD1*, *JMT*, *LOX2*, *LOX3*, *LOX4*, *OPR3*), signal-transduction (e.g. *JAZ1*, *JAZ2*, *JAZ3*, *JAZ5*, *JAZ6*, *JAZ7*, *JAZ8*, *JAZ9*, *JAZ10*) and response (e.g. *NSP4*, *TCH4*, *VSP2*, *TPS4*) were up-regulated in response to *P. rapae* herbivory (Supplementary Data 1). Several of these genes code for proteins involved in anti-insect defences. For example, *VSP2* encodes for a vegetative storage protein. Recombinant AtVSP2 included in diets increased mortality and delayed development in coleopteran and dipteran insects (Liu *et al*., 2005). Another example is *TPS4* which encodes a geranyllinalool synthase which is induced by JA application and feeding by *P. rapae* and *P. xylostella* larvae in *A. thaliana* and cabbage (*Brassica oleracea* L.) respectively (Broekgaarden *et al*., 2007; Huang *et al*., 2008). Geranyllinalool synthase is involved in the production of terpenes that function in indirect defence of *A. thaliana* (Herde *et al*., 2008; Markovic *et al*., 2014; Pangesti *et al*., 2015).

Several studies have investigated transcriptional responses of *A. thaliana* to herbivory by *P. rapae* using targeted or whole-genome arrays (Reymond *et al*., 2000; Reymond *et al*., 2004; De Vos *et al*., 2005; Bodenhausen & Reymond, 2007; Appel *et al*., 2014). Direct comparison across studies is challenging because research teams used different experimental conditions and protocols. For instance, in the studies mentioned above, the time of sample collection ranges from 15 min to 24 h after caterpillar feeding. The number of caterpillars, their developmental instar, plant age and tissue (local vs systemic) also differed between studies. To our knowledge, our study is the first using a platform independent from microarrays to address the

response of plants to chewing herbivores in *A. thaliana*. Thus, our data set can be used for comparing the results obtained with microarrays. Despite the limitations mentioned above, we attempted a non-exhaustive cross-experiment comparison. In our RNA-seq analysis we found higher numbers for up- and down-regulated genes respectively in comparison to Reymond *et al.* (2004) (140 up- and 3 down-regulated), De Vos *et al.* (2005) (128 up- and 58 down-regulated) and Appel *et al.* (2014) (480 up- and 295 down-regulated) (Figure S5). Although these latter three studies all used microarrays, large differences were observed among them. A total of 68% of the DEGs identified in the three studies described above were also identified in this study; in contrast, only 10% of DEGs identified in this study were also identified in the other studies. Thus, our approach clearly extends beyond what has been recorded with microarrays. The higher number of DEGs identified in the present study in comparison with Reymond *et al.* (2004) is not surprising because Reymond *et al.* (2004) used an array representing only 7200 unique genes of the *A. thaliana* genome. On the other hand, De Vos *et al.* (2005) and Appel *et al.* (2014) used an array representing almost the whole *A. thaliana* genome and we still observed striking differences in the number of DEGs. These differences may be due to inherent differences between RNA-seq and microarray analyses. For instance, microarrays have a lower dynamic range for quantitation than RNA-seq (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). Furthermore, the two platforms seem to correlate well for genes with intermediate expression levels but not for genes with high or low expression levels and RNA-seq analysis has been proven to outperform microarray analysis in the detection of low abundance transcripts (Wang *et al.*, 2009; Wang *et al.*, 2014). The large number of new DEGs identified with RNA-seq analysis underlines the potential of this technology for discovery of genes involved in plant-herbivore interactions. A logical follow-up of this study will be the functional characterization of new candidate genes identified in this study. In the long term these genes could be candidates for development of crops that are better defended against chewing herbivores.

Transcriptomic responses to combined stresses, imposed by drought and *P. rapae* or *B. cinerea* followed by *P. rapae*

In nature, plants are challenged by a diverse range of abiotic and biotic stresses that commonly occur simultaneously (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014). Whole transcriptome profiling using microarrays for plants exposed to multiple abiotic and biotic stresses has shed light onto plant responses to multiple stresses at the molecular level (Atkinson *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013). Here, we recorded considerable overlap between the transcriptional responses to combined stresses imposed by drought and *P. rapae* or *B. cinerea* and

P. rapae and the single stress imposed by *P. rapae* (Figure 3A). Despite this overlap, specific, time-dependent transcripts in response to combined stresses were identified, with early time points displaying the biggest difference between combined and single stress (Figures 2, S4). In one of the first studies addressing transcriptional responses to multiple stress exposure, Voelckel & Baldwin (2004) found that transcriptional changes in *Nicotiana attenuata* plants exposed to sequential or simultaneous attack by the sap-feeding insect *Tupiocorus notatus* and the chewing insect *Manduca sexta* were very similar. Furthermore, these transcriptional changes were different from the changes in response to the single stress situations. In tomato, simultaneous attack by the phloem feeder *Macrosiphum euphorbiae* and the chewing herbivore *Spodoptera exigua* induced a similar set of genes as in the single stress situation; however, the expression patterns were different (Rodriguez-Saona *et al.*, 2010).The specificity observed for the combined stress expression signature varies between studies; for example, Voelckel & Baldwin (2004) observed specificity only at early time points while Rodriguez-Saona *et al.* (2010) observed specificity also 5 d after the treatment had been applied. Here, we identified genes that were specifically differentially expressed in response to a combination of stresses as well as genes with altered expression patterns in the combined stresses compared to the single stress (Figures 3A and 4, Supplements 2 and 4). For plants exposed to combined stress imposed by drought and *P. rapae*, we observed a group of genes induced at higher levels at 3 HAI (Cluster 2) than in the single stress scenario (Figure 4C, Supplement 4). Cluster 2 contains two ABA receptors (*PYL4* and *PYL5*). These receptors belong to a family of 14 members in *A. thaliana;* they are involved in regulation of *ABI1* and *ABI2*, two genes that encode for negative regulators of the ABA signalling pathway (Ma *et al.*, 2009; Park *et al.*, 2009). Furthermore, PYL4 and PYL5 have been pinpointed as components of the crosstalk between the JA and ABA signalling pathways (Lackman *et al.*, 2011). For instance, expression of *PYL4* is regulated by JA in *Nicotiana tabacum* and *A. thaliana*. Loss-of-function mutants in *PYL4* and *PYL5* were hypersensitive to JA treatment, as reflected in reduced growth in comparison to wild-type Col-0 *A. thaliana* plants. On the other hand, both mutants *pyl4* and *pyl5* displayed reduced anthocyanin accumulation in response to JA compared to the wild-type (Col-0) (Lackman *et al.*, 2011). Another group of genes showing altered expression patterns upon combined stress imposed by drought and *P. rapae* compared to the single stress scenario was Cluster 4 (Figure 4C, Supplementary Data 4). Genes in Cluster 4 were down-regulated at all time points to a higher degree than in the caterpillar single-stress situation (Figure 4C). Cluster 4 contains genes involved in plant defences against biotrophic pathogens (e.g. *PR2*, *PR5*, *RLP39*, *RLP41*, *WAK3*). *PR-1* and *PR-2* encode pathogenesis-related proteins that are induced by a broad range of pathogens (Thomma *et al.*, 1998; De Vos *et al.*, 2005). *PR1* is often used as marker for SAR (Systemic Acquired Resistance) (Fu & Dong, 2013). We hypothesize that down-regulation of these *PR* genes may be an

effect of the drought stress experienced by the plants before caterpillar infestation. In support of this hypothesis, several studies have found that abiotic stresses have a negative impact on plant defence against pathogens (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). Tomato plants exposed to simultaneous stress imposed by salinity and a microbial pathogen were more susceptible to the pathogen than when exposed only to the pathogen (Kissoudis *et al.*, 2015). Application of ABA and drought stress made *A. thaliana* plants more susceptible to the pathogens *Pseudomonas syringae pv. tomato* and *Peronospora parasitica* (Mohr & Cahill, 2003; Goel *et al.*, 2008). For plants exposed to combined stress imposed by *B. cinerea* and *P. rapae*, we observed a group of genes being induced at higher levels at 3 HAI (Cluster 1) than in the single stress scenario; however, no differences were observed at subsequent time points (Figure 4C, Supplementary Data 4). Examples of genes in Cluster 1 are *ERF104* and *BAP1*. *ERF104* encodes a transcription factor that is involved in ET-mediated responses through interaction with *MPK6* (Bethke *et al.*, 2009). A homologue of *ERF104* (*ERF 106*) that also interacts with *MPK6* is involved in resistance against *B. cinerea* (Meng *et al.*, 2013), suggesting that *ERF104* may be involved in defence signaling in response to *B. cinerea* infection. *BAP1* encodes a negative regulator of plant defences and is required for growth homeostasis under normal conditions (Yang *et al.*, 2006; Yang *et al.*, 2007). Future efforts will focus on understanding the biological role of genes showing altered gene expression patterns under combined stresses.

Conclusion

Plants in natural and agricultural environments are subjected to multiple stresses. Here, we evaluated the transcriptomic changes to herbivory by *P. rapae* caterpillars alone, and to combinatorial stresses imposed by drought plus *P. rapae,* or *B. cinerea* plus *P. rapae*. The transcriptomic changes elicited by *P. rapae* herbivory alone are fast. Already at 3 HAI 915 genes are differentially expressed. Moderate differences were observed between the transcriptomic changes in response to the combined stresses compared to the single stress by *P. rapae* herbivory. Transcripts that were specifically differentially expressed in the combined stress treatments and transcripts that were expressed in both single and combined stresses but with altered expression pattern in the combined stress were identified. Differences observed in the transcriptomic response to single and combined stresses were larger at early time points and subsequently the responses converged. This indicates that the response to the more damaging stress imposed by feeding specialist caterpillars overrides the effects of previous exposure to drought or *B. cinerea*. This was also reflected at the phenotypic level because we did not observe a compromise in plant immunity against *P. rapae* by pre-treatment with drought or *B. cinerea* infection. This study highlights the importance of studying combinations of stresses. How these transcriptomic changes affect the plant phenotype needs further attention. For example, we observed a larger biomass reduction in plants exposed to the combined stress imposed by drought plus *P. rapae* than in the single stresses situation, but how these changes are related to the transcriptome changes remains to be investigated. Future experiments will be directed to mutant analysis of genes differentially expressed under combinatorial stresses compared to single stress.

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Supplementary Information (See Appendix)

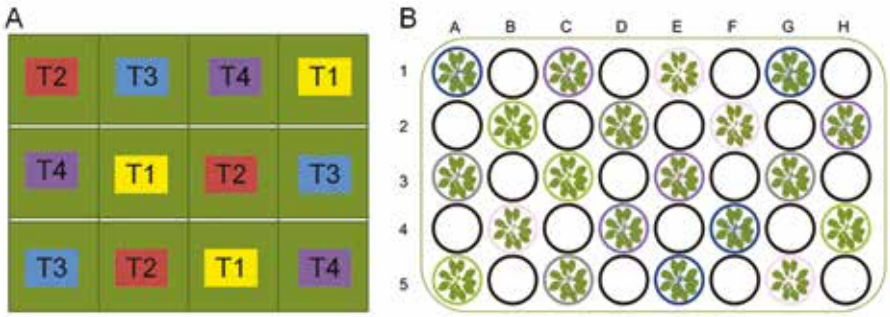

Figure S1. Experimental design. Samples were collected from five treatments at four time points. The experiment was carried out in a climatized growth chamber. (A) The experiments followed a flat table design with three blocks (rows in panel A) and plants to be sampled at different time points were randomized within every block (different colors in panel A) . (B) Within every time point, the five treatments were randomly allocated (indicated by the different colors in panel). To prevent caterpillars moving from one plant to another, plants were accomodated everyother spot within the trays (black circles). Three biological replicates were performed for every treatment and time point combination. Each biological replicate consisted of a pool of 4 plants. Samples were collected by 5 persons and each person was randomly assigned to a treatment.

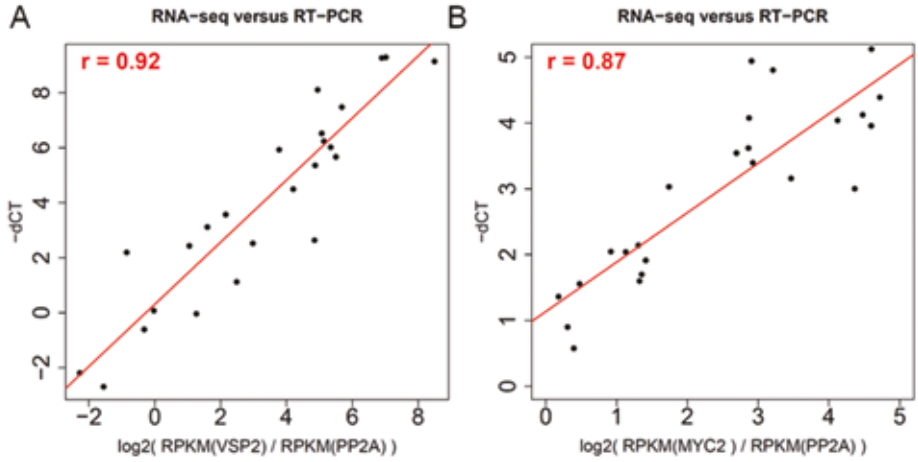

Figure S2. Validation of RNA-seq data with RT-PCR. We selected a subset of 24 samples to be validated by RT-PCR. These samples correspond to 12 samples from the control conditions and 12 samples from plants exposed to *P.rapae* as single stress. We performed RT-PCR for 2 genes that are known to be induced by *P. rapae* herbivory(Reymond *et al.*, 2004; De Vos *et al.*, 2005; Bodenhausen & Reymond, 2007) .These genes are (A) *Vegetative Storage Protein2* (*VSP2,* AT5G24770) and (B) MYC2 (AT1G32640). As housekeeping gene we used PP2A (AT1G13320). Primers are presented in Table S1. CT values for every sample are the average of 2 technical replicates. The delta CT values from RT-PCR were estimated by subtracting the CT values for the gene of interest from that of the housekeeping gene. CT values were compared to $\log_2$ RPKM (gene of interest) / RPKM (housekeeping gene) values for RNA-seq. Linear regression fit (red line) and correlation coefficient (r) are indicated.

Table S1. Primers for genes used for validation of the RNA-seq using RT-PCR

TAIR ID	Gene	Forward	Reverse
AT5G24770	VSP2	5'-TCAGTGACCGTTGGAAGTTGTG-3'	5'-CACGAGCTCCATTCCACTGAA-3'
AT1G32640	MYC2	5'-TCATACGACGGTTGCCAGAA-3'	5'-AGCAACGTTTACAAGCTTTGATTG-3'
AT1G13320	PP2A	5'-TAACGTGGCCAAAATGATGC-3'	5'-GTTCTCCACAAC^CGCTTGGT-3'

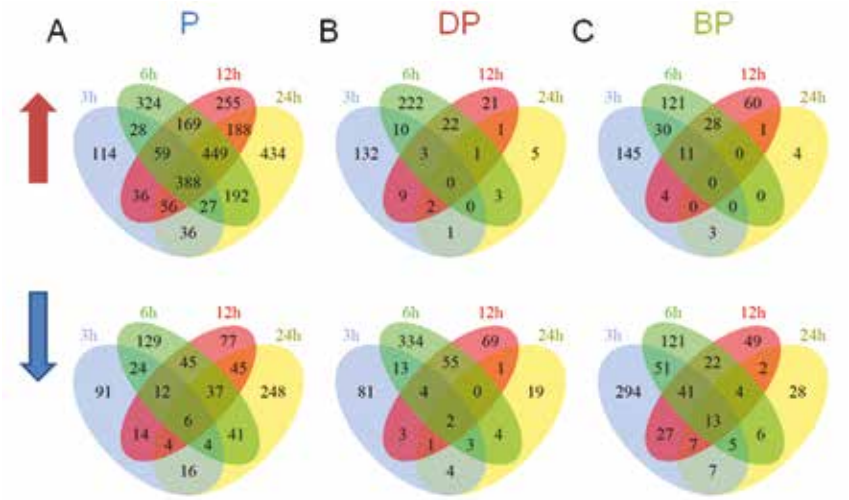

Figure S3. Dynamic transcriptional responses of *A. thaliana* plants exposed to *P. rapae* herbivory alone or to *P. rapae* herbivory preceded by drought or *B. cinerea* infection, over time. Blue = 3 HAI (hours after herbivore inoculation), Green = 6 HAI, Red= 12 h and Yellow= 24 h. (A) *Pieris rapae* as single stress, (B) drought plus *P. rapae* and (C) *B. cinerea* plus *P. rapae*.

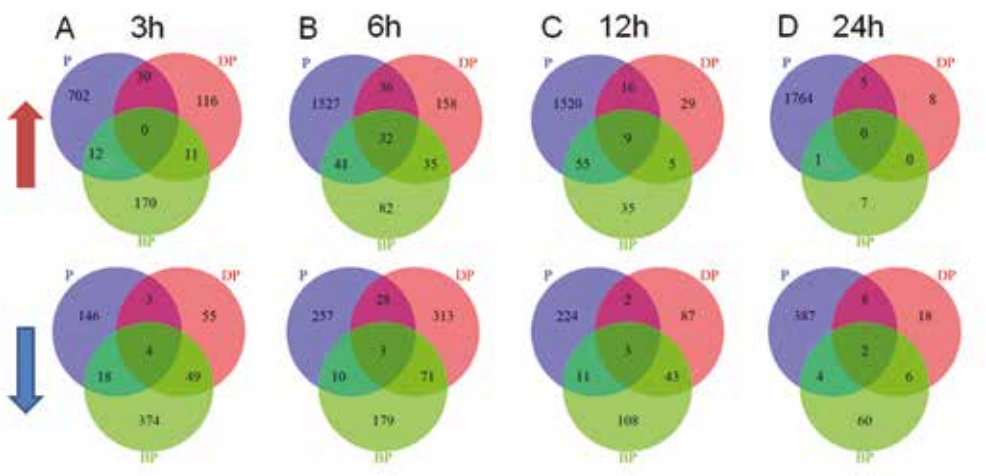

Figure S4. Dynamic transcriptional responses of *A. thaliana* plants to *Pieris rapae* feeding alone or to *P. rapae* feeding preceded by drought or *B. cinerea* infection over time. Blue = *P. rapae* as single stress, Orange = drought plus *P. rapae* and Green= *B. cinerea* plus *P. rapae*. (A) 3h, (B) 6h, (C) 12h and (D) 24h after herbivore inoculation.

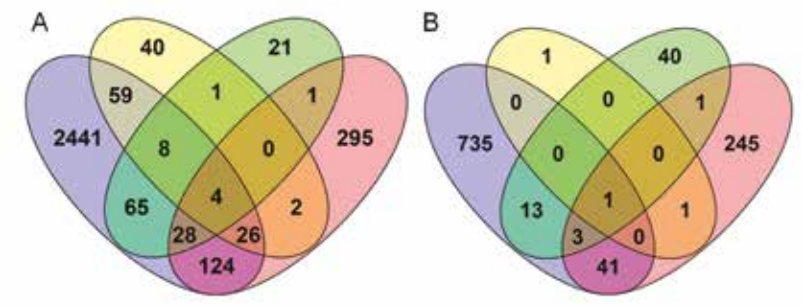

Figure S5. Venn diagrams comparing differentially expressed genes (DEGs) from this study to those from other relevant studies. A) up-regulated genes. B) down-regulated genes. Blue = data on *Pieris rapae* as single stress from the present study. Yellow = data from Reymond *et al.* (2004), that used a microarray representing 7200 genes from the *Arabidopsis* genome; genes were considered to be differentially regulated if they displayed a $\log_2$-fold change ≥1 for up-regulated genes or ≤ -1 for down-regulated genes compared to the control condition and a P-value ≤ 0.05 without correction for multiple testing. Green = transcriptome changes identified in De Vos *et al.* (2005), that used an Affymetrix ATH1 whole genome microarray representing 23,750 genes from the *Arabidopsis* genome; genes were considered to be differentially regulated if they displayed a $\log_2$-fold change ≥1 for up-regulated or ≤ -1 for down-regulated with respect to the control condition. Red = Data from Appel *et al.* (2014), that used microarray representing 26,090 genes from the *A. thaliana* genome. Genes were considered to be differentially regulated if they displayed a $\log_2$-fold change ≥1 for up-regulated genes or ≤ -1 for down-regulated genes compared to the control condition and a P-value ≤ 0.05.

Chapter 5

Genome Wide Association analysis reveals distinct genetic architectures for single and combined stress responses in *Arabidopsis thaliana*

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Abstract

In nature, plants are exposed to abiotic and biotic stresses that commonly occur simultaneously. Plants have evolved defences and adaptations to survive and reproduce despite these stresses. Most studies addressed adaptations to individual stresses. However, recent studies have emphasized the importance of studying stress combinations concluding that the phenotypic, transcriptomic and genetic responses to combined stresses can often not be extrapolated from the single stress situations. In this study, we used a panel of 350 *Arabidopsis thaliana* accessions and employed Genome Wide Association analysis to investigate the genetic architecture and the underlying loci involved in genetic variation in resistance to (1) specialist insect herbivores of brassicaceous plants, *Pieris rapae* and *Plutella xylostella*, and (2) combinations of stresses imposed by drought followed by *P. rapae,* as well as *B. cinerea* infestation followed by *P. rapae*. We found (1) variation in resistance to *P. rapae* and *P. xylostella* that is genetically determined; (2) genetic variation in resistance to combined stresses imposed by drought plus *P. rapae* was limited in comparison to *B. cinerea* plus *P. rapae* or *P. rapae* alone; (3) resistance to *P. rapae* and *P. xylostella* is controlled by different genetic components; (4) a limited overlap was observed in the QTLs identified for resistance to combined stresses imposed by drought plus *P. rapae* or *B. cinerea* and *P. rapae* and *P. rapae* alone; (5) several candidate genes involved in aliphatic glucosinolates and proteinase inhibitors were identified to be involved in resistance to *P. rapae* and *P. xylostella* respectively. This study underlines the importance of investigating plant responses to combinations of stresses. The value of this approach for breeding plants for resistance to combinations of stresses is discussed.

Keywords: Genome wide association, combined stresses, specialist, abiotic stress, biotic stress.

Introduction

During their life cycle, plants are exposed to diverse abiotic stresses such as drought, flooding, heat, cold, nutrient deficiency, or ozone (Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Roy *et al.*, 2011; Fahad *et al.*, 2015; Mickelbart *et al.*, 2015) and biotic stresses such as attack by bacteria, fungi, viruses, insects, or parasitic plants (Jones & Dangl, 2006; Howe & Jander, 2008; Dicke & Baldwin, 2010; Mithofer & Boland, 2012; Pieterse *et al.*, 2012; Dangl *et al.*, 2013; Pierik *et al.*, 2013). As a result, plants have evolved strategies that allow them to acclimate to abiotic stresses and defend themselves against biotic stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Mickelbart *et al.*, 2015). Substantial progress has been made in the identification of genes that provide resistance to individual stresses (Smith & Clement, 2012). However, in natural ecosystems plants suffer from combinations of stresses that occur simultaneously or sequentially. Recent studies have addressed this by investigating the phenotypic effect, transcriptomic changes and genetics underlying responses to combined stresses (De Vos *et al.*, 2006; Goel *et al.*, 2008; Xu *et al.*, 2008; Dicke *et al.*, 2009; Atkinson *et al.*, 2013; Makumburage *et al.*, 2013; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Zhang *et al.*, 2013; Kissoudis *et al.*, 2014). These studies have concluded that the effect of a combination of stresses can often not be predicted from the single stress effect at the phenotypic, transcriptome or genetic level.

Herbivory by insects is one of the major stresses that plants are exposed to: a quarter of all known eukaryote species are insect herbivores (Futuyma & Agrawal, 2009). As a result of the strong selection pressure imposed on plants by insects, plants have evolved mechanisms to protect them from insects (Gatehouse, 2002; Kessler & Baldwin, 2002; Schoonhoven *et al.*, 2005; Mithofer & Boland, 2012). Plant traits that influence the degree of damage caused by insects can be classified in resistance (traits that limit the damage by the insect) and tolerance (traits that allow plants to compensate for insect damage) (Strauss & Agrawal, 1999; Stout, 2013). Furthermore, resistance and tolerance are mediated by distinct genetic mechanisms (Strauss & Agrawal, 1999; Carmona *et al.*, 2011; Karinho-Betancourt & Nunez-Farfan, 2015). Resistance can be further divided in constitutive or induced defences (Schoonhoven *et al.*, 2005; Mithofer & Boland, 2012; Stout, 2013). One of the best studied defence mechanisms of plants against insects is the myrosinase-glucosinolate system in the Brassicaceae family (Hopkins *et al.*, 2009; Mithofer & Boland, 2012). Glucosinolates are hydrolysed by myrosinase enzymes upon insect herbivory and their breakdown products are toxic to generalist insect herbivores (Fahey *et al.*, 2001; Kliebenstein *et al.*, 2005; Brachi *et al.*, 2015). However, specialist insects such as *P. rapae* and *P. xylostella* have developed detoxification mechanisms (Brachi et al., 2015; Fahey et

al., 2001) and seem not to be affected by the myrosinase-glucosinolate defence of brassicaceous plants (Wheat *et al.*, 2007; De Vos *et al.*, 2008; Muller *et al.*, 2010). These two insect species are major pests in several crops from the *Brassica* genus (e.g. broccoli, cabbage, cauliflower) worldwide. For example, annual control costs of *P. xylostella* are estimated to be nearly US$ 4-5 billion (Zalucki *et al.*, 2012). A good understanding of the genetic architecture of plant resistance against these insects and the identification of molecular mechanisms behind resistance will provide breeders with better tools to develop crops that are more resistant to these insect species.

Arabidopsis thaliana, a member of the Brassicaceae, has been the model plant for genetic studies because of its small size, short life cycle, relatively small genome, vast collection of mutants, and publicly available genetic resources (Alonso-Blanco & Koornneef, 2000; Kaul *et al.*, 2000; Koornneef *et al.*, 2004; Koornneef & Meinke, 2010). *Arabidopsis thaliana* displays natural genetic variation in developmental and physiological traits, as well as in resistance to biotic and abiotic stresses (McKay *et al.*, 2003; Alonso-Blanco *et al.*, 2009; Baxter *et al.*, 2010; Juenger, 2013; Easlon *et al.*, 2014). In addition, natural genetic variation for resistance to specialist and generalist insects has been reported (Jander *et al.*, 2001; Kliebenstein *et al.*, 2002; Pfalz *et al.*, 2007). The causal genes for variation in resistance against generalist insects have been successfully identified (mostly glucosinolate biosynthesis-related genes) (Kliebenstein *et al.*, 2001; Lambrix *et al.*, 2001; Kliebenstein *et al.*, 2002; Zhang *et al.*, 2006). Less information is available on genes underlying variation in resistance to specialist insects (Kliebenstein *et al.*, 2002; Pfalz *et al.*, 2007; Kliebenstein, 2014).

QTL mapping using bi-parental or multi-parental populations has been traditionally used for the identification of genes responsible for natural genetic variation for a trait of interest (Alonso-Blanco & Koornneef, 2000; Koornneef *et al.*, 2004). However, QTL mapping has a low resolution and a lot of time and resources have to be invested in narrowing down to the causal gene (Doerge, 2002; Koornneef *et al.*, 2004; Kloth *et al.*, 2012; Weigel, 2012). In the last few years, large collections of *A. thaliana* natural accessions have been genotyped and re-sequenced enabling Genome Wide Association (GWA) studies in this model plant (Atwell *et al.*, 2010; Weigel, 2012). GWA makes use of linkage disequilibrium (LD), when two loci in the genome are statistically more or less often inherited together due to recombination history, to associate genotypes with phenotypes. GWA overcomes several of the drawbacks of QTL mapping; GWA (1) offers higher resolution (in some case down to the causal gene), (2) is less time consuming and requires fewer resources, and (3) considers more allelic diversity (Nordborg & Weigel, 2008; Zhu *et al.*, 2008; Korte & Farlow, 2013). Over the last years, several traits have been successfully dissected in *A. thaliana* using GWA such as glucosinolate content, shade avoidance, heavy metal tolerance, salt stress resistance, flowering time, and heat tolerance (Baxter *et al.*,

2010; Chan *et al.*, 2011; Chao *et al.*, 2012; Filiault & Maloof, 2012; Li, Y *et al.*, 2014; Bac-Molenaar *et al.*, 2015).

In this study, we used a collection of 350 *A. thaliana* accessions to explore the natural variation to a range of combinations of abiotic and biotic stresses. We chose the following stresses: drought, herbivory by caterpillars of *Pieris rapae* and *Plutella xylostella*, and infection by the necrotrophic fungal pathogen *Botrytis cinerea*. The rationale behind these choices is the fact that the response of *A. thaliana* to these three stresses is highly divergent but at the same time regulated by the plant hormones JA and/or ABA (Borel et al., 2001; Thomma et al., 2001; Reymond et al., 2004; Glazebrook, 2005). Hence, analysis of combinatorial stresses is likely to yield information on signaling nodes that are involved in tailoring the plant's adaptive response to combinations of these stresses. We have investigated the natural genetic variation in (1) resistance to two specialist insects, i.e. *P. rapae* and *P. xylostella*, (2) resistance to combined stresses imposed by drought plus *P. rapae,* and the plant pathogen *Botrytis cinerea* plus *P. rapae*. Furthermore, we used Genome Wide Association mapping to (1) gain insight into the genetic architecture of these traits and (2) to identify regions in the genome associated with variation in resistance.

Materials and Methods

Arabidopsis thaliana Hapmap population

We used a collection of 350 *A. thaliana* accessions from the Hapmap population (http://naturalvariation.org/hapmap). This population was developed from a global collection of 5,810 accessions with the purpose to minimize redundancy and relatedness, a common problem in Genome Wide Association studies (Atwell *et al.*, 2010; Platt *et al.*, 2010; Chao *et al.*, 2012). This population has been genotyped for 248,584 bi-allelic SNPs as described in Atwell *et al.* (2010). After quality control and imputation, this set of SNPs was reduced to a set of 214,051 SNPs. For GWA analysis we used only SNPs with minor allele frequency higher than 0.05, in order to prevent spurious associations, resulting in a total of 199,360 SNPs.

Plants, insects and pathogen

Plant growth conditions

Arabidopsis plants were grown under controlled conditions at 24 ± 1 °C, 70 ± 10 % relative humidity, 200 µmol m^{-2} s^{-1} photosynthetically active radiation and a diurnal cycle of 8:16 L:D. Seeds were vernalized at 4 °C for 5 d in order to induce even germination. Plants were individually grown in 0.08 L pots in a pasteurized (4 h, 80 °C) commercial potting soil (Lentse potgrond, Lent, The Netherlands), which was mixed 1:1 (v/v) with autoclaved sand in experiment (1) and in pasteurized (4 h, 80 °C) potting soil in experiment (2). Pots were accommodated in trays that were randomly distributed within a growth chamber. Plants were watered three times per week by adding water to the tray. Once per week the pots received entomopathogenic nematodes (Entonem; http://www.koppert.nl/) to prevent infestation by fungus gnats.

Insect rearing

Pieris rapae L. (Small Cabbage White butterfly; Lepidoptera; Pieridae) were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) in a growth chamber at 21 ± 1 °C, 50 - 70 % relative humidity and a diurnal cycle of 16:18 L:D.

Plutella xylostella L. (Diamondback moth; Lepidoptera; Plutellidae) were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) in a growth chamber at 22 ± 1 °C, 40-50% relative humidity and a diurnal cycle of 16:8 L:D.

Pathogen culture

The necrotrophic fungus *B. cinerea,* strain B0510 (Van der Ent *et al.*, 2008) was grown on half-strength PDA plates containing penicillin (100 μg mL^{-1}) and streptomycin (200 μg mL^{-1}), for 2 weeks at room temperature. Spores were collected and re-suspended in half-strength potato dextrose broth (Difco Laboratories) to a final density of 1.0 x

10^5 spores mL^{-1}. After a 3-h incubation period, the spores were used for inoculation (Thomma *et al*., 1998; Pre *et al*., 2008; Van der Ent *et al*., 2008).

Experimental design and treatments

Experimental design and treatments were described in detail in Davila Olivas *et al.* (2015a). Briefly, two experiments were conducted. In experiment (1), we evaluated the growth of *Arabidopsis* plants after exposure to drought, herbivory by *P. rapae*, herbivory by *P. rapae* preceded by drought and herbivory by *P. rapae* preceded by *B. cinerea* infestation. The experiment was performed in 10 temporal blocks. Each block consisted of approximately 37 randomly selected accessions. To correct for variation within temporal blocks in each block the same three accessions (CS28780;Tsu-0, CS76113;Col-0 and CS76129;Fei-0) were added. Within temporal blocks, plants were allocated in trays and the position of the tray in the rearing chamber recorded as its position in either of the six racks, each with four shelves. The spatial location of each plant within a tray was recorded in terms column C and row R. In each temporal block, accessions were exposed to the following five treatments: (a) no stress, (b) drought stress, (c) *P. rapae* herbivory, (d) drought and *P. rapae* or (e) *B. cinerea* and *P. rapae*. A total of 6 replicates were included per accession and treatment combination. Plants were grown under similar conditions during the first three weeks. Drought stress was imposed by withholding water for 7 d during the third week while the rest of the plants was watered every two days with 1 L of water per tray. *Botrytis cinerea* inoculation was carried out 24 h prior to *P. rapae* inoculation. Plants were inoculated with *B. cinerea* by pipetting 5 µl of spores suspended in half-strength PDB (Difco Laboratories) at a concentration of 1×10^5 spores mL^{-1} on two leaves of the rosette. Plants were kept at 100% RH for 24 h in order to ensure successful infection by *B. cinerea*. Four-week-old plants were exposed to herbivory by *P. rapae* as single or combined stress. Plants were inoculated with two newly hatched first instar (L1) caterpillars that were allowed to feed for 5 d. Rosette fresh weight was quantified for all treatments (Figure S1A).

In experiment (2) we evaluated the growth reduction in *Arabidopsis* after exposure to herbivory by *P. xylostella*. The experiment was performed in four temporal blocks. Within blocks, accessions were randomly distributed over 40 trays with nine accessions per tray. In this experiment, accession Col-0 was included to control for a positional effect within the chamber. Each tray contained both control and treatment for Col-0 and for nine other accessions. Plants were randomized within the trays. In each block, all accessions were screened; one replicate per accession was screened at a time. Within blocks, accessions were exposed simultaneously to either (a) no stress or (b) herbivory by *P. xylostella*. Plants were four weeks old when they were inoculated with two L2 larvae. Larvae were allowed to feed for 5 d. Rosette fresh weight was quantified for all treatments (Figure S1B).

Statistical analysis

Genotypic mean estimations

We obtained BLUEs (**b**est **l**inear **u**nbiased **e**stimator) for all genotype-treatment combinations as described in the literature (Jimenez-Gomez *et al.*, 2010; Filiault & Maloof, 2012; Riedelsheimer *et al.*, 2012). BLUEs were estimated by a linear mixed model using ASReml package in R (Butler, 2009).

Experiment 1: $Y = \mu + GEN + TRT + GEN \times TRT + \underline{BLOCK + RACK + SHELF + BLOCK \times RACK \times SHELF + BLOCK \times RACK \times SHELF \times TRAY + BLOCK \times RACK \times SHELF \times TRAY \times C + BLOCK \times RACK \times SHELF \times TRAY \times R + e}$,

Experiment 2: $Y = \mu + GEN + TRT + GEN \times TRT + \underline{BLOCK + BLOCK \times TRAY + BLOCK \times TRAY \times C + BLOCK \times TRAY \times R + e}$,

where Y represents the rosette fresh weight, *GEN* is genotype (accession), TRT is the treatment factor, BLOCK represents the temporal block, and RACK, SHELF, TRAY, C, and R are factors that represent the spatial location of the plants within the chamber and e is the residual error. *GEN + TRT + GEN×TRT* were fitted as a fixed effect while all other variables were fitted as random effects (underlined).

Using BLUEs, for each stress we estimated the percentage difference of rosette FW relative to control plants without stress. In the treatment where plants were exposed to both drought and herbivory by *P. rapae,* percentage difference of rosette FW was relative to plants exposed to drought. Hereafter, we will refer to the percentage of biomass reduction due to drought, *P. rapae* herbivory, *P. xylostella* herbivory, drought plus *P. rapae,* and *B. cinerea* plus *P. rapae* as Drought, *P. rapae*, *P. xylostella*, Drought&*Pieris*, and *Botrytis*&*Pieris* respectively (Supplementary Data 1).

Data inspection

We initially inspected the variation in response to each stress (Fig. 1). We observed that some accessions have larger biomass under treatment than under control conditions. We reasoned that these accessions displayed tolerance to the treatment. Because tolerance and resistance traits have a different genetic basis (Strauss & Agrawal, 1999; Carmona *et al.*, 2011; Karinho-Betancourt & Nunez-Farfan, 2015), we only included data for accessions displaying a reduction in biomass under the treatment compared to control conditions (Supplementary Data 1). This dataset was used for all downstream analyses.

Phenotypic and genotypic correlations

Phenotypic correlations were estimated by Spearman correlation of the genotypic mean BLUEs for every possible combination of two traits. Spearman correlation analyses were implemented in the package Hmisc in R (Butler, 2009). Genetic correlations

are a more accurate estimate of the shared genetic basis, and they represent an upper boundary for the joint genetic determination of pairs of traits. A perfect genetic correlation ($r_g = 1$) for any given combination of traits suggests that the same group of genes controls variation among them; a positive correlation reveals a consistent effect of the gene on the two traits and a negative correlation reveals opposing effects of the gene on the two traits. A non-perfect genetic correlation ($r_g<1$) reveals a mixture of unique and common genetic architecture among traits. The absence of genetic correlations ($r_g=0$) indicates that different groups of genes control variation among the traits (Juenger, 2013). Genetic correlations were estimated according to the multi-trait mixed model described in Korte *et al.* (2012).

Narrow sense heritability

Phenotypic variance can be decomposed into variance due to genetic and environmental factors. Broad sense heritability (H^2) estimates the proportion of phenotypic variance that is due to genetic factors. Genetic variance can be due to additive, dominant or epistatic effects. Narrow sense heritability (h^2) captures the proportion of genetic variance that is due to additive genetic effects. Narrow sense heritability is important because it is an indicator of how a population responds to artificial or natural selection (Wray, 2008). Narrow-sense heritability estimates for each response were estimated with the heritability package in R (Kruijer *et al.*, 2015).

Genome Wide Association Analysis

Variation in growth reduction under different stresses was linked to regions in the genome that explained the observed variation using a GWA analysis, carried out using the Fast-LMM software as described in Cao *et al.* (2011). Fast-LMM assumes for each SNP the following mixed model:

$$y = \mu + X\beta + g + e,$$

where *y* is a vector of n phenotype values. *X* is a design matrix where trait means are included with other fixed effects. In *X*, β is the effect of the Col-0 allele. $g \sim N\left(0\sigma_g^2 K\right)$ and $e \sim N\left(0\sigma_e^2 I\right)$ are random effects. We tested the hypothesis $\beta = 0$ using generalized least squares (GLS), conditional on residual maximum likelihood (REML) estimates σ_g^2 and σ_e^2 for the genetic- and environmental variance. The proportion of the genetic variance explained by each SNP was estimated using two methods; (1) The R^2_{LR} statistic proposed by Cox & Snell (1989), which is $1 - \exp(-(2/n)(L_1-L_0))$ and (2) $2\times(\beta^2 p(1-p)/\sigma^2)$, where β is the allele effect, *p* is the frequency and σ^2 is the sample variance. Fast-LMM corrects for population structure using a GRM (genetic relatedness matrix) instead of a kinship matrix like in the EMMAX software (Kang *et al.*, 2010). Fast-LMM is considered to be more powerful than EMMAX because (1) each SNP test is based on a local kinship matrix that consists of the GRM based on

all markers, except those that are in a window of 20 kb on each side of the tested SNP and (2) the genetic and residual variance components are estimated for each SNP, instead of assuming that these are constant across the genome. In order to reduce the amount of spurious associations due to rare variants, a MAF (Minor Allele Frequency) of 5% was used.

Candidate gene selection

For selection of candidate genes, an arbitrary threshold was considered. Regions containing SNPs with $-\log_{10}(P) \geq 4$ were considered for further analysis as described by El-Soda *et al.* (2015). A search window was defined by SNPs in linkage disequilibrium (LD ≥ 0.5, if no SNPs were found at 0.5 the threshold was lowered to 0.4) in a window ± 20 Kb with significant SNPs. SNPs in LD from the 250K array were enriched with SNPs in LD from 1001 genomes (http://1001genomes.org/) as described in Bac-Molenaar *et al.* (2015). Thus, a search window was defined by the first and last SNP in LD. All genes within a search window were considered to be potential candidate genes. To narrow down the list of candidate genes, further analyses were performed. First, gene annotation from candidate genes was obtained from TAIR 10. Furthermore, candidate genes were enriched with gene expression data from different sources. Data from tissue exposed to the phytohormones JA, ABA, or ET were obtained from a public database (http://bar.utoronto.ca/) (Toufighi *et al.*, 2005). Expression data for *A. thaliana* plants infested with *P. xylostella* were obtained from Ehlting *et al.* (2008). RNA-seq based expression data for *A. thaliana* plants infested with *P. rapae,* drought and *P. rapae* and *B. cinerea* and *P. rapae* were obtained from Davila Olivas *et al.* (2015b). Data are summarised in Supplementary Data Files 2-6.

Results

Variation within and between responses of *A. thaliana* to single or multiple stresses

We observed extensive variation among the accessions in the percentage of growth reduction for plants exposed to the different stresses addressed in this study (Figure 1, Table1). The variation within treatment was larger than the variation between treatments (Table 1). For instance, the mean percentage of growth reduction was smallest for the response to *Botrytis*&*Pieris* (18.9%) and largest for the response to Drought&*Pieris* (48.9%) (Table 1). The mean percentage of growth reduction for the population in response to *P. rapae* alone was 32.6%, which is higher than in response to *Botrytis*&*Pieris* and lower than in response to Drought&*Pieris*. The largest variation was observed for the response to *P. xylostella* (CV = 78%) while the lowest variation was observed for the response to Drought&*Pieris* (CV = 31%)(Table 1). Narrow sense heritability estimates ranged from 0.17 to 0.52 (Table 1). For instance, the largest narrow sense heritability was observed for the response to *Botrytis*&*Pieris* ($h^2 = 0.52$), followed by the response to *P. rapae* ($h^2=0.51$). The lowest heritability ($h^2 = 0.17$) was observed for the response to combined stress Drought&*Pieris*.

Table 1. Summary of variation in the percentage of biomass reduction of 350 *Arabidopsis thaliana* accessions upon exposure to drought, herbivory by *Plutella xylostella,* and herbivory by *Pieris rapae* alone or preceded by drought or infection with the necrotrophic fungus *Botrytis cinerea*. Traits are ordered by narrow sense heritability.

Trait	Min.	Mean	Max.	N	CV	h^2	va	ve
Botrytis&Pieris	0.08	18.88	90.13	285	74	0.52	103.80	94.77
P. rapae	2.67	32.62	87.80	345	42	0.51	96.96	93.20
P. xylostella	0.03	21.66	82.22	234	78	0.42	121.71	166.41
Drought	0.29	22.67	50.07	307	48	0.42	49.99	68.16
Drought*&Pieris*	3.62	48.29	89.06	344	31	0.17	36.08	181.48

Min. = Lowest value, Max. = Highest values, N= Number of accessions analysed, h^2 = Narrow sense heritability, va = additive genetic variance, ve = residual variance. CV = coefficient of variation (%).

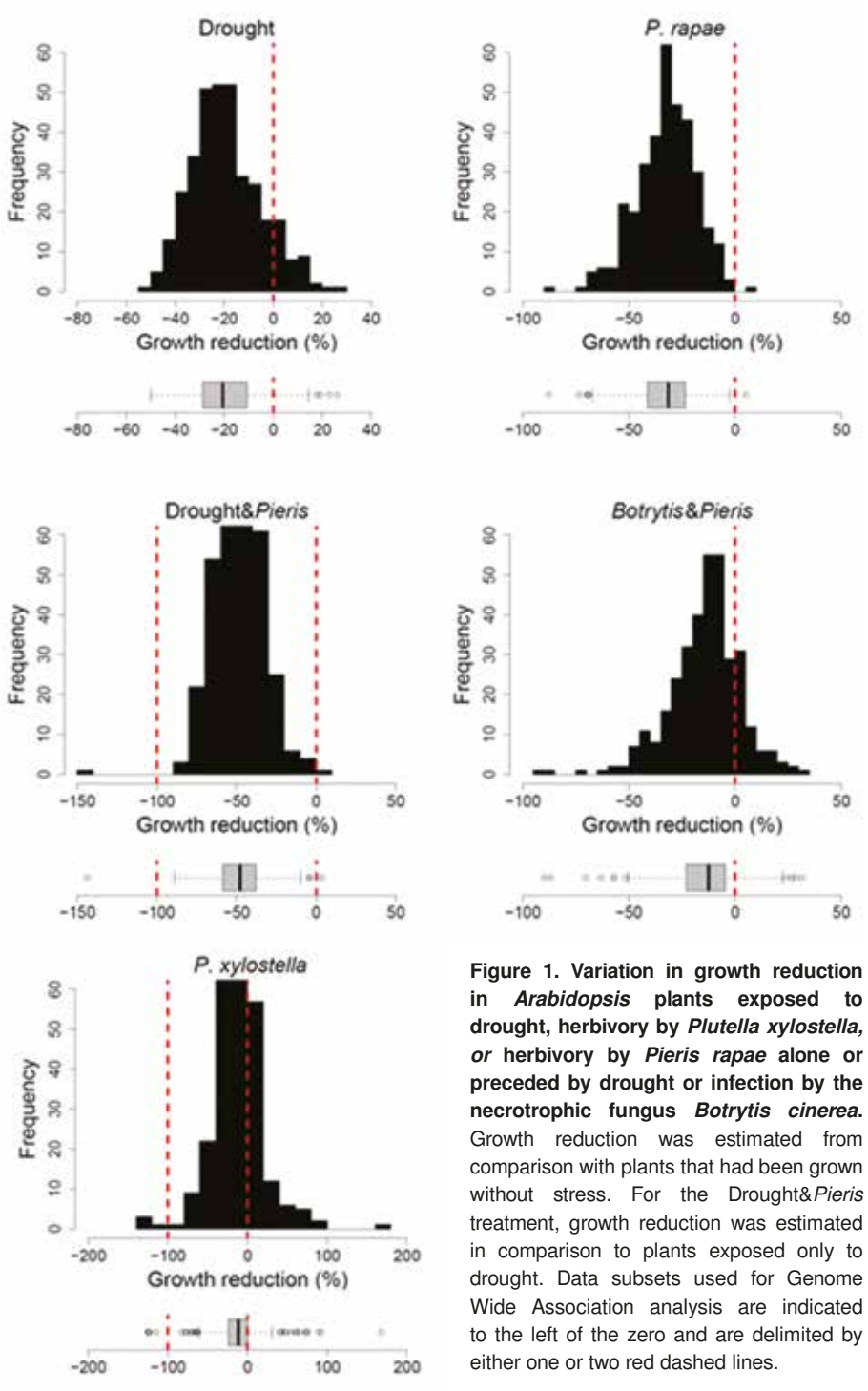

Figure 1. Variation in growth reduction in *Arabidopsis* plants exposed to drought, herbivory by *Plutella xylostella, or* herbivory by *Pieris rapae* alone or preceded by drought or infection by the necrotrophic fungus *Botrytis cinerea*. Growth reduction was estimated from comparison with plants that had been grown without stress. For the Drought&*Pieris* treatment, growth reduction was estimated in comparison to plants exposed only to drought. Data subsets used for Genome Wide Association analysis are indicated to the left of the zero and are delimited by either one or two red dashed lines.

Genetic and phenotypic correlations among traits

To explore the relationship among different traits, we performed Spearman correlation analysis on the phenotypic values (Table 2). The response to drought displayed a negative correlation with the other traits. Furthermore, the largest phenotypic correlation was observed between the responses to *Botrytis&Pieris* and *P. rapae* (rho = 0.52). A low phenotypic correlation was observed between the responses to *P. xylostella* and *P. rapae* (rho = 0.15). Because phenotypic correlations may arise due to genetic and environmental factors, a better estimate of shared genetic basis between traits are genetic correlations The largest genetic correlation was between the responses to *Botrytis&Pieris* and to *P. rapae* (r_g = 0.98), followed by the responses to drought and to *Botrytis&Pieris* (r_g = -0.81) .

Table 2. Phenotypic and genetic correlations among the percentage of biomass reduction in plants that had been exposed to drought, herbivory by *Plutella xylostella,* and herbivory by *Pieris rapae* alone or preceded by drought or infection with the necrotrophic fungus *Botrytis cinerea*.

Trait	Drought	*P. rapae*	Drought&*Pieris*	*Botrytis&Pieris*	*P. xylostella*
Drought		-0.65	NC	-0.89	-0.42
P. rapae	-0.25		NC	0.98	0.20
Drought&*Pieris*	-0.38	0.48		NC	0.64
Botrytis&Pieris	-0.29	0.53	0.40		0.33
P. xylostella	-0.12	0.15	0.16	0.14	

NC= residual maximum likelihood did not converge. Phenotypic correlations (Spearman correlation coefficients) are indicated below the diagonal. Genetic correlations were estimated by residual maximum likelihood as in Korte *et al.* (2012) Genetic correlation estimates (r_g) are indicated above the diagonal. Values below the diagonal that were not significant ($P > 0.05$ after Bonferroni correction) are indicated in red font.

Genetic architecture underlying variation in responses to single and multiple stresses

To get insight into the genetic architecture underlying the variation in responses to single stress imposed by drought or *P. rapae* feeding and the two multiple stress situations, Drought&*Pieris* and *Botrytis&Pieris*, we performed a Genome Wide Association analysis. We used a threshold of $-\log_{10}(P) \geq 4$ to declare a SNP being associated with a trait. SNPs in LD were considered in a region of ± 20 Kb from

a significant SNP (Supplementary Data 2-5). A summary of the Genome Wide Association analysis for each trait is presented in Figure 2 and Table 3. For the responses to single stresses, the numbers of significant SNPs amounted to a total of 20 (64 SNPs in LD) for the response to drought and 34 (78 SNPs in LD) for the response to *P. rapae*. For the responses to combined stresses, the numbers of significant SNPs were greater than in the response to single stress situations, 38 (106 SNPs in LD) for the response to Drought&*Pieris* and 40 (106 SNPs in LD) for the response to *Botrytis*&*Pieris*. Effect sizes for the Col-0 allele were estimated for each trait. For most of the traits the significant SNPs displayed low effect sizes, except for the response to *Botrytis*&*Pieris* (Figure 2B, Supplementary Data files 2-5). The response to drought displayed the lowest effect sizes ranging from -4 (meaning that accessions with the Col-0 allele have 4% less biomass reduction than accessions carrying the alternative allele) to 4. The response to *Botrytis*&*Pieris* displayed the highest effect sizes ranging from -16 to 23. For most of the traits, the significant SNPs explained a low percentage of the genetic variance (Supplementary Data 2-5). The maximum percentage of genetic variance explained by a SNP for the response to each stress was 7% for drought, 7% for *P. rapae*, 5% for Drought&*Pieris* and 12% for *Botrytis*&*Pieris*. Despite the moderate to high genetic correlations among traits, little overlap was observed between the significant SNPs, regions delimited by SNPs in LD (QTLs) and, therefore, also between the genes contained within QTLs little overlap was found (Figure 2C).

Table 3. Summary of Genome Wide Association analysis per trait.

Trait	SNPs	SNPs in LD[a]	Strings	Singletons	QTLs	Genes[b]
Drought	20	64	12	6	18	119
P. rapae	34	78	13	5	18	102
Drought&*Pieris*	38	106	13	6	19	110
Botrytis*&*Pieris	40	106	16	9	25	109
P. xylostella	57	238	22	10	32	141

[a] SNPs in LD ≥ 0.5 were considered in a region of ± 20 Kb from a significant SNP. Number of SNPs in LD are based on the 250 K SNPs array.

[b] A search window was defined taking in consideration additional SNPs in LD from 1001 genomes project (See M&M section). All genes within a search window were considered as candidate genes.

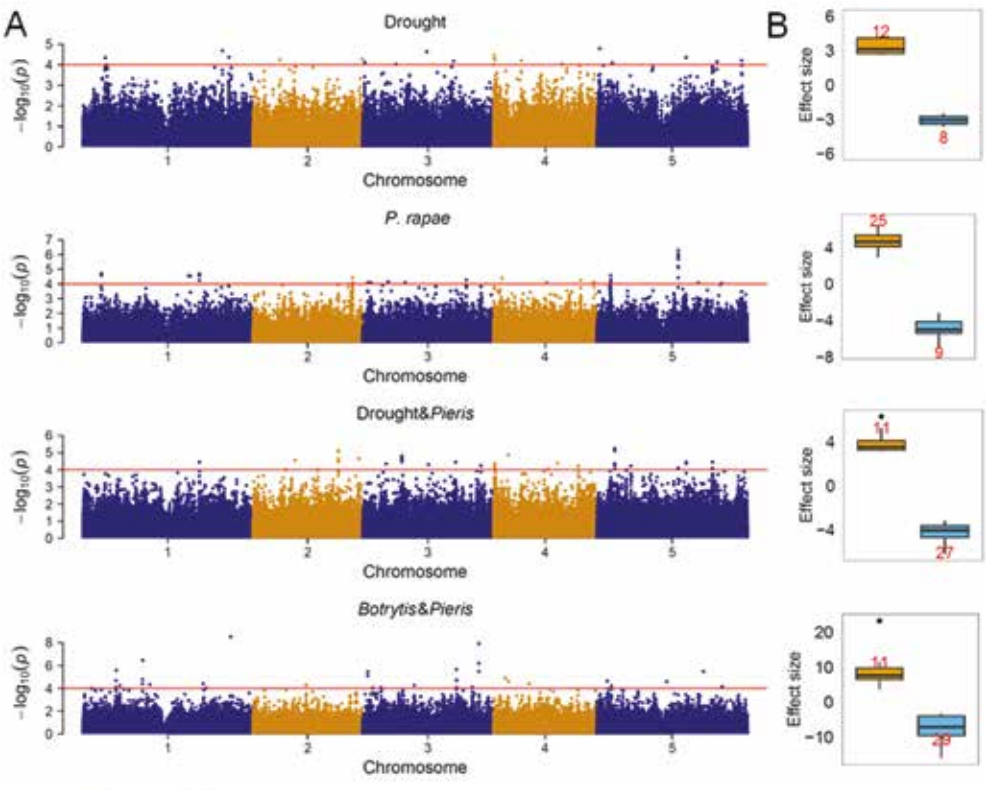

Figure 2. Genome wide association analysis of growth reduction in plants exposed to single stress imposed by drought or herbivory by *Pieris rapae* or multiple stresses imposed by *P. rapae* preceded by drought or infection by the necrotrophic fungus *Botrytis cinerea*. A) Manhattan plots. Red line indicates an arbitrary threshold set at $-\log_{10}(P) \geq 4$ as described in the Methods section for detecting significant associations. B) Effect sizes for significant SNPs. Effect sizes are indicated for the Col-0 allele. Yellow and blue indicate a higher and lower reduction in biomass associated with the Col-0 allele, respectively. Number of significant SNPs are indicated in red. C) Candidate genes. Genes in a 20 kb window of a significant SNP were considered as candidates.

Differences in genetic architecture underlying responses to two specialist insect herbivores

We also investigated the genetic architecture of *A. thaliana's* response to *P. xylostella* and compared it to the genetic architecture of the response to *P. rapae*. We identified a larger number of significant SNPs for the response to *P. xylostella* (57 SNPs plus 238 SNPs in LD) than for the response to *P. rapae* (34 SNPs plus 78 SNPs in LD) (Figure 3, Table 3). Furthermore, the effect size of SNPs associated with the response to *P. xylostella* (from -20 to 22) was larger than for the response to *P. rapae* (from -7 to 7)

(Figure 3B). The maximum percentage of genetic variance explained by the SNPs associated with the response to *P. xylostella* was 10%, while for the response to *P. rapae* this was 7% (Supplementary Data 6). No common significant SNPs, regions delimited by SNPs in LD (QTLs) and therefore also between the genes contained within QTLs were observed between *P. xylostella* and *P. rapae* (Figure 3C).

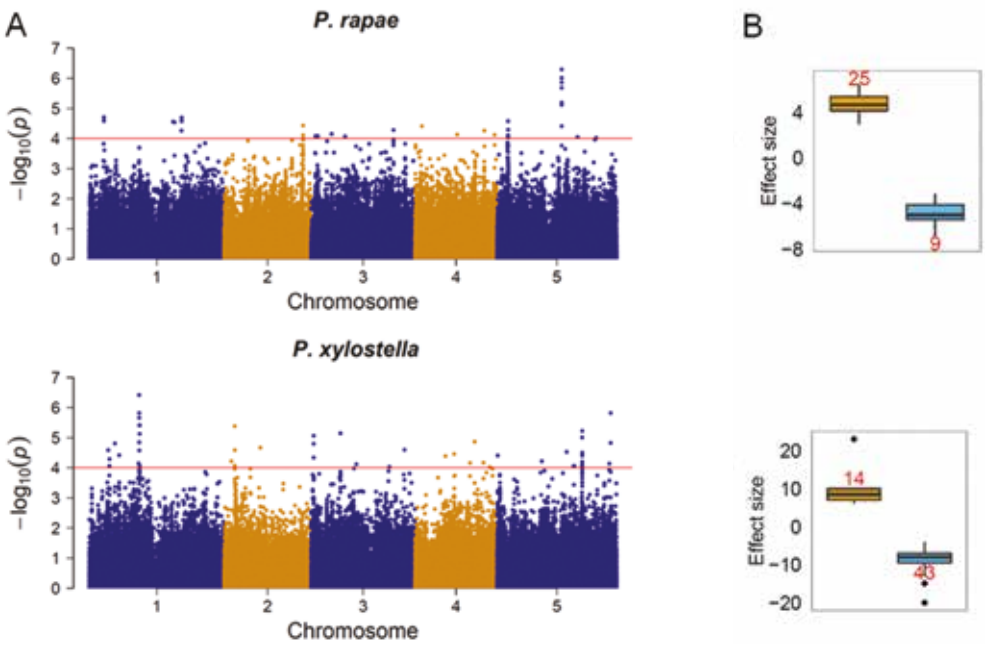

Figure 3. Genome wide association analysis of growth reduction in plants exposed to herbivory by the specialist insects *Pieris rapae* or *Plutella xylostella*. A) Manhattan plots. Red line indicates an arbitrary threshold set at $-\log_{10}(P) \geq 4$ as described in the Methods section for detecting significant associations. B) Effect sizes for significant SNPs. Effect sizes are indicated for the Col-0 allele. Yellow and blue indicate a higher and lower reduction in biomass associated with the Col-0 allele, respectively. Numbers of significant SNPs are indicated in red. C) Candidate genes. Genes in a 20 kb window of a significant SNP were considered as candidates.

Candidate genes for drought resistance

A total of 18 QTLs were identified for biomass reduction in response to drought. Within those regions, several genes that are known to play a role in drought acclimation were identified. For example, QTL 7 on chromosome 3 contained only one gene, *AT3G17520,* which encodes a late embryogenesis abundant protein (LEA protein). In general, LEA proteins have been suggested to play a protective role for other proteins under conditions of water stress in vegetative tissues (Battaglia *et al.*, 2008). The closest significant SNP

(Chr3: 5997119) explained 5 % of the phenotypic variance; the Col-0 allele was rare (86 accessions including Col-0) and was associated with a higher reduction in *A. thaliana* fresh weight (Supplementary Data 2, Figure S2). This gene was induced upon drought stress and ABA application (Supplementary Data 2). This suggests that this may be the causal gene for QTL 7.

Candidate genes involved in plant-insect interactions

We analysed the variation in growth reduction of *Arabidopsis* in response to two specialist insect herbivores, *P. rapae* and *P. xylostella*. GWA allowed linking this variation to several regions in the plant genome. We identified a total of 18 and 32 QTLs for the responses to *P. rapae* and *P. xylostella* respectively (Table 3). Within those regions several candidate genes with a known function in plant resistance against insect herbivores were identified.

For *P. rapae*, QTL 15 on chromosome 5 contained *AT5G07690* (*MYB29*) and *AT5G0700* (*MYB76*) (Supplementary Data 3). The closest significant SNP (Chr5: 2454480) explained 4% of the phenotypic variance. The Col-0 allele was rare (55 accessions including Col-0) and was associated with higher reduction in *A. thaliana* fresh weight (Supplementary Data 3, Figure S3). Both genes were induced in response to *P. rapae* infestation. Furthermore, *MYB76* was induced by JA and ET treatment (Supplementary Data 3).

Another interesting QTL for the response to *P. rapae* was QTL 1 on chromosome 1, which contained *AT1G10060* (*BCAT-1)* and *AT1G10070* (*BCAT-2)* (Supplementary Data 3). The closest significant SNP (Chr1: 3294935) explained 5% of the phenotypic variance; the Col-0 allele was rare (89 accessions including Col-0) and was associated with higher reduction in *A. thaliana* fresh weight (Supplementary Data 3, Figure S3). Furthermore, both genes were induced by *P. rapae* infestation and application of the phytohormones JA and ABA (Supplementary Data 3).

For the response to *P. xylostella,* more QTLs were identified than for the response to *P. rapae* (Table 3). QTL 18 on chromosome 4 contained only two genes *AT4G11310* (*CP1*) and *AT4G11320* (*CP2*). The closest significant SNP (Chr4: 3294935) explained 7% of the phenotypic variance; the Col-0 allele was common (159 accessions including Col-0) and was associated with lower reduction in *A. thaliana* fresh weight (Supplementary Data 6, Figure S6). *CP1* and *CP2* were induced by both *P. rapae* and *P. xylostella* infestation. In addition, it was also induced by JA application (Supplementary Data 6). Both, *CP1 and CP2* encode CYSTEINE PROTEASE enzymes (TAIR10). CP2 has been implied in increasing resistance of cotton against *Helicoverpa armigera* (Mao *et al*., 2013).

Another example is QTL 32 on chromosome 5 that contains *AT5G64080* (*XYP1*). The closest significant SNP (Chr5: 25640504) explained 9% of the phenotypic variance; the Col-0 allele was common (178 accessions including Col-0) and was associated with

lower reduction in *A. thaliana* fresh weight (Supplementary Data 6, Figure S5). *XYP1* was induced by *P. rapae* and *P. xylostella* infestation (Supplementary Data 6) and encodes a proteinase inhibitor/seed storage/lipid transfer protein. This kind of proteins has been implied in anti-nutritional defences against insect herbivores (Heidel-Fischer *et al.*, 2014).

Candidate genes for combined stresses

A total of 19 and 25 QTLs were identified for the responses to the combined stresses Drought&*Pieris* and *Botrytis*&*Pieris*, respectively (Table 3). QTL1 for Drought&*Pieris* and QTL 3 for *P. rapae* on chromosome 1 overlapped to some extent. The significant SNPs associated with each QTL were different, but the QTLs overlapped by SNPs in LD. The Col-0 allele for significant SNPs was rare and was associated with higher reduction in *A. thaliana* fresh weight (Supplementary Data 3 and 4, Figures S3 and S4). *AT1G55740* (*SIP1*) and *AT1G55760* within that QTL displayed interesting expression patterns. *SIP1* was induced by *P. rapae* infestation, drought, and ABA application. *AT1G55760* was induced by drought and ABA, while it was repressed by JA application (Supplementary Data 3).

For the response to Drought&*Pieris*, QTL 10 on chromosome 4 and QTL 19 on chromosome 5 contained the bHLH transcription factors *AT4G00480* (*MYC1*) and *AT5G50915*. The Col-0 allele in both QTLs was rare and was associated with lower reduction in *A. thaliana* fresh weight. Both genes were induced by *P. rapae* infestation and slightly induced by drought (Supplementary Data 4). Natural variation in trichome density in *A. thaliana* has been associated with genetic variation in *MYC1* (Symonds *et al.*, 2011). Several other bHLH transcription factors (e.g. *MYC2*, *MYC3*, *MYC4*, *MYC5*) are well established in the literature as major regulators of JA- and ABA-mediated responses, insect resistance and drought responses (Dombrecht *et al.*, 2007; Shinozaki & Yamaguchi-Shinozaki, 2007; Schweizer *et al.*, 2013; Li, R *et al.*, 2014; Qi *et al.*, 2015). QTLs containing bHLH transcription factors were also identified for the responses to *P. rapae* (*AT1G51140*) and *P. xylostella* (*AT1G12540*) (Supplementary Data 3, 5 and 6).

For the response to *Botrytis*&*Pieris* no bHLH transcription factors were identified. On the other hand, QTL 3 on chromosome 1 contained *AT1G19210*, an ERF/AP2 transcription factor (Supplementary Data 5). The significant SNP with the highest effect within this QTL (Chr1: 6627245) explained 6% of the phenotypic variance; the Col-0 allele was common (232 accessions including Col-0) and was associated with lower reduction in *A. thaliana* fresh weight (Supplementary Data 5, Figure S5). *AT1G19210* was induced upon *P. rapae* infection, drought, JA, ABA and ET application (Supplementary Data 5). Several homologues of *AT1G19210* (e.g. *RAP2.1*, *RAP2.9*, *RAP2.10*) have been implied in tolerance to drought and freezing and resistance to necrotrophic fungi (Tsutsui *et al.*, 2009; Dong & Liu, 2010).

Discussion

Genetic architecture of *A. thaliana* resistance to specialist insects

In this study, we have analysed the genetic architecture of *A. thaliana* responses to *P. xylostella* and *P. rapae*, two insect species specialised on the mustard family (Brassicaceae). We identified variation in resistance to both insect herbivores among *A. thaliana* accessions that is genetically determined as indicated by the moderate narrow-sense heritability estimates for the responses to both species (Table 1). Heritability estimates reported for resistance to generalist insects are higher than for specialist insects (Jander *et al*., 2001; Kliebenstein *et al*., 2002). For example, in the latter study using two RIL populations, broad-sense heritability estimates for resistance to the generalist *Trichoplusia ni* ranged from 0.26 to 0.31 while for resistance to the specialist insect *P. xylostella* it ranged from 0.12 to 0.18) (Kliebenstein *et al*., 2002). Furthermore, differences in genetic architecture of resistance against different generalist insect herbivores have also been reported. Whereas resistance to generalists is dominated by QTLs of large effect, resistance to specialists seems to be dominated by QTLs of small effect (Jander *et al*., 2001; Kliebenstein *et al*., 2002; Pfalz *et al*., 2007). For example, QTLs for resistance to *T. ni* explained up 20% of the phenotypic variance, while QTLs for *P. xylostella* explained up to 2% of the genetic variance (Kliebenstein *et al*., 2002). Similarly, small-effect QTLs have been identified for resistance to *P. xylostella* in *Brassica oleracea* (Ramchiary *et al*., 2015).

Several studies have reported QTLs associated with insect resistance but few of them have identified the causal loci (Jander *et al*., 2001; Pfalz *et al*., 2007; Ordas *et al*., 2009; Schranz *et al*., 2009; Prasad *et al*., 2012). QTLs that we identified in this study for both insect species had small effects on plant phenotype (Supplementary Data 3 and Supplementary Data 6). However, none of the QTLs that we identified were shared for resistance to the two specialist insect herbivores (Figure 3, Table 3), suggesting that the resistance mechanisms are species specific. Similar results were obtained in a QTL study using *P. brassicae* and *P. xylostella* and *A. thaliana*, where no common QTLs were identified (Pfalz *et al*., 2007). Furthermore, microarray analyses have revealed that *P. rapae* and *P. xylostella* elicit different transcriptomic responses in *A. thaliana,* supporting the notion of species-specific mechanisms of resistance (Ehlting *et al*., 2008; Bidart-Bouzat & Kliebenstein, 2011).

QTL analyses in *A. thaliana* and other species in the Brassicaceae have identified several genes involved in the metabolism of glucosinolates as source of resistance to generalist insects (Jander *et al*., 2001; Kliebenstein *et al*., 2002; Schranz *et al*., 2009). However, specialist insects such as *P. rapae and P. xylostella* have developed distinct detoxification mechanisms rendering glucosinolates ineffective (Schoonhoven

et al., 2005; Wheat *et al.*, 2007; De Vos *et al.*, 2008; Muller *et al.*, 2010; Agrawal *et al.*, 2012). Interestingly, one of the QTLs that we identified for resistance to *P. rapae* contained as most likely candidates *MYB29 and MYB76,* encoding for two transcription factors involved in the induced production of aliphatic glucosinolates (Hirai *et al.*, 2007). In fact, the double mutant *myb29myb28* that lacks aliphatic glucosinolates is less preferred for feeding by *P. rapae* than Col-0 (Muller *et al.*, 2010). Another QTL, identified for the response to *P. rapae* contained as most likely candidates *BCAT-1 and BCAT-2* which are enzymes involved in branched amino acid (Leu, Val and Ile) metabolism (BCAA) (Diebold *et al.*, 2002). Interestingly, homologues of these genes *(BCAT-3, BCAT-4, BCAT-6)* have been implied in the production of aliphatic glucosinolates (Schuster *et al.*, 2006; Lachler *et al.*, 2015). Furthermore, co-expression networks have revealed that *BCAT-4* is co-expressed with *MYB29, MYB28* and several putative genes involved in Leu metabolism (Hirai *et al.*, 2007). Interestingly, an evolutionary link has been suggested between aliphatic glucosinolates and BCAA metabolism (Schuster *et al.*, 2006). In *Boechera stricta*, a species related to *A. thaliana*, QTL analysis identified a QTL that controls variation in allocation between methionine and BCAA derived glucosinolates and resistance to the generalist caterpillar *T. ni* (Schranz *et al.*, 2009).

For *P. xylostella,* a small-effect QTL near *ERECTA* on chromosome 2 has been reported in *Arabidopsis* and *B. oleracea* (Kliebenstein *et al.*, 2002; Ramchiary *et al.*, 2015). We identified 2 QTLs on chromosome 2. However, neither of these was in the vicinity of *ERECTA*.

Genetic architecture of resistance against multiple stresses

In complex environments such as natural and agricultural ecosystems, plants experience several stresses that co-occur (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014). Here, we compared the genetic architecture of the combined stresses imposed by drought plus *P. rapae* or *B. cinerea* plus *P. rapae* to the single stress imposed by *P. rapae* alone. We observed genetically determined variation for both combined stresses as indicated by their narrow sense heritability values (Table 3). However, while the total phenotypic variance for resistance to drought plus *P. rapae* was larger than for the single stress situation, the proportion of it that was explained by genetic factors was dramatically lower (Table 3). This implies that there is little genetic variation for this trait and this may have implications for the power of GWA analysis to identify true associations with this trait. Only few studies have conducted QTL analysis on plant responses to combined stresses and some of them have identified similar caveats. For example, in a study conducted in a maize population, a lower genetic variance was

observed under combination of drought plus heat than in the single-stress situations (Cairns *et al.*, 2013). Furthermore, in a tomato population exposed to a combination of salt and powdery mildew a reduction in phenotypic variation in disease resistance was observed under combined stress in comparison to the single-stress situation (Kissoudis *et al.*, 2015). The low heritability and phenotypic variation under combined stresses may represent a pitfall for QTL identification and breeding for combined stresses.

On the other hand, for the combined stress *B. cinerea* plus *P. rapae* no difference in narrow sense heritability was observed compared to the single stress imposed by *P. rapae* (Table 1). Furthermore, both traits displayed a high level of genetic correlation, suggesting that common genes influence both traits (Table 3). Despite the high genetic correlation between the response to *B. cinerea* plus *P. rapae* versus the response to single stress *P. rapae,* no common QTLs were identified (Table 3, Figure 2). It may be that the QTLs that underlie the similarity of both traits are QTLs of small effect that were not identified at the threshold used in this study. An alternative tool that may help to unravel the genetic commonality between these two traits is a multi-trait GWA that allows for the identification of SNPs with common and opposite effects among highly correlated traits (Korte *et al.*, 2012). This may increase the power of univariate GWAs for highly correlated traits (Korte *et al.*, 2012).

Contrary to the limited overlap that we found between QTLs identified for combined stresses (Table 3, Figure 2), other studies have identified a mixture of novel QTLs and QTLs that were present in the single stress (Cairns *et al.*, 2013; Makumburage *et al.*, 2013). However, the effect of the QTLs under stress combinations was never observed to be in the same direction as in the single stress (Makumburage *et al.*, 2013). Thus, the genetic architecture underlying single and combined stresses appears to different. In addition to the few studies addressing QTL identification, several papers have addressed whole transcriptome changes in response to combinations of stresses (Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Sewelam *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015; Sham *et al.*, 2015). These studies concluded that transcriptional response to combined stresses was different from the single stress situation. Furthermore, up 60 % of the transcriptional changes in response to combined stresses could not be predicted from the response to each individual stress (Rasmussen *et al.*, 2013).

Despite their co-occurrence being the rule rather than the exception under natural conditions, the importance of studying stress combinations has only just started to be acknowledged by the scientific community. Maybe the complexity of the experimental design, the number of possible stress combinations and the complex logistics have set back the adoption of this kind of experiments. The present study together with several studies on QTL mapping and transcriptomic changes under combinations of

stresses have concluded that responses to combined stresses could not be predicted from the responses to individual stresses (Voelckel & Baldwin, 2004). This further underlines the complexity of the events that take place when plants are challenged by combinations of stresses and highlights the importance of studying combinations of stresses in addition to studies of single stresses.

Finally, *P. rapae* and *P. xylostella* are major pests on *Brassica* crops such as cabbage and broccoli (Agrawal & Kurashige, 2003; Zalucki *et al.*, 2012). A good understanding of genetic architecture and the unequivocal identification of genes underlying variation in resistance will benefit the breeding process of cultivars that are more resistant to these insect pests. In this study we identified several candidate genes that upon functional validation may constitute a valuable source enhancing resistance to these insect herbivores. Future efforts will be devoted to validation of candidate genes by mutant analysis and/or allelic complementation.

Conclusions

In this study we have used a large collection of *A. thaliana* accessions and explored their genetic variation in resistance (1) to two species of specialist insects *P. rapae* and *P. xylostella* and (2) to two combined stresses, drought plus *P. rapae* and *B. cinerea* plus *P. rapae*. We used Genome Wide Association analysis with two purposes: (1) to understand the genetic architecture of resistance to the different stresses and (2) to identify regions of the genome and possible candidate genes associated with variation in resistance to those stresses. We have identified distinct differences in genetic architecture and QTLs underlying variation between resistance to *P. rapae* and to *P. xylostella* and between resistance to single and combined stresses. Some most likely candidate genes for the different stresses were highlighted and their mode of action discussed. Future efforts will be devoted to the validation of candidate genes through mutant analysis. Finally, this study highlights the importance as well as the complexity of studying combinations of stresses.

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Supplementary Information (See Appendix)

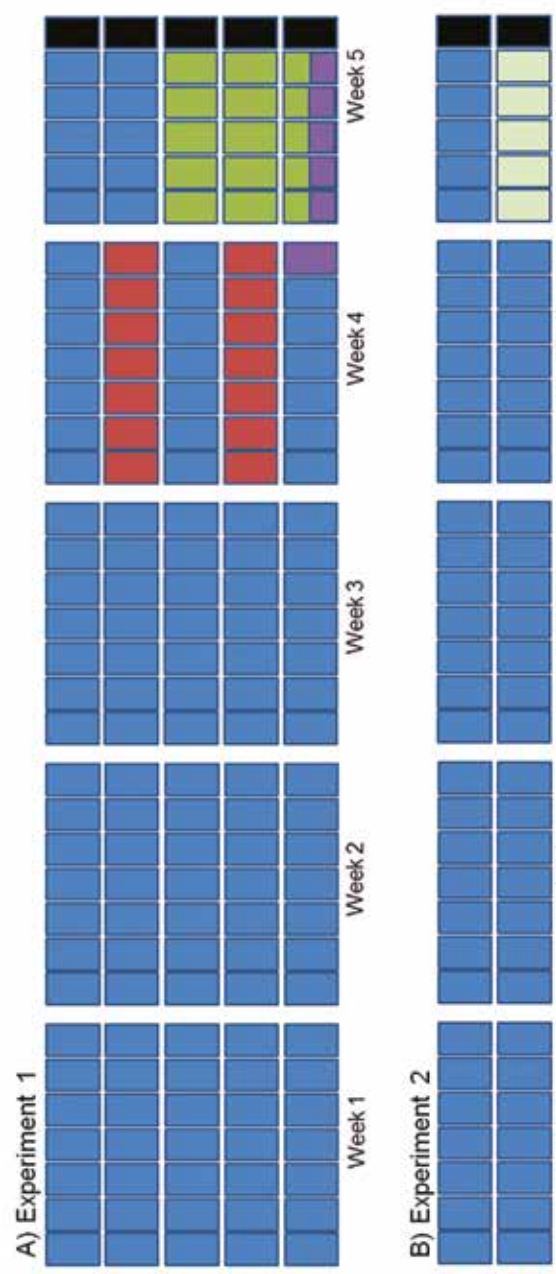

Figure S1. Treatments time scheme. A) In experiment (1) the effects of drought and herbivory by *P. rapae* caterpillars either as single treatment or preceded by drought stress or *B. cinerea* infestation were evaluated. Blue indicates that the plants were growing without stress. Drought stress is indicated in red. Time of inoculation of *B. cinerea* is indicated in purple. Time of infestation with *P. rapae* is indicated in dark green. Measurement time is indicated in black. B) In experiment (2) the effect of herbivory by *P. xylostella* caterpillars was evaluated. Blue indicates that the plants were growing without stress. Time of infestation with *P. xylostella* is indicated in light green. Measurement time is indicated in black.

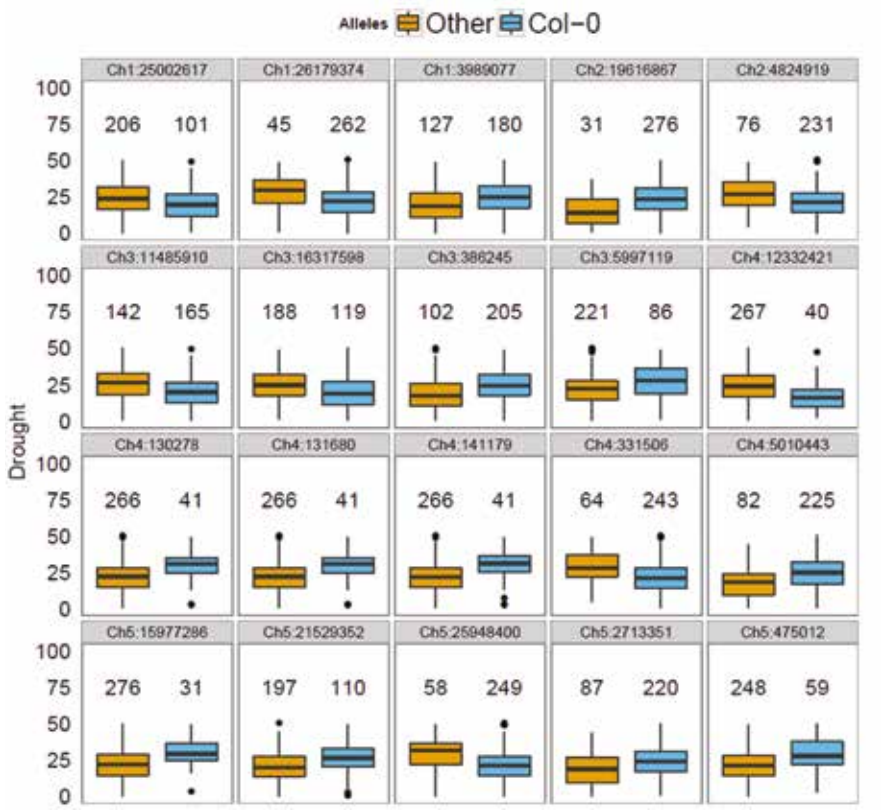

Figure S2. Allele effects of significant SNPs associated with *Arabidopsis thaliana* rosette fresh weight reduction in response to drought. Chromosome and SNP position are indicated. Number of accessions having the Col-0 allele or another allele are indicated in above the boxes. Y-axis represents the percentage of biomass reduction compared to the control situation.

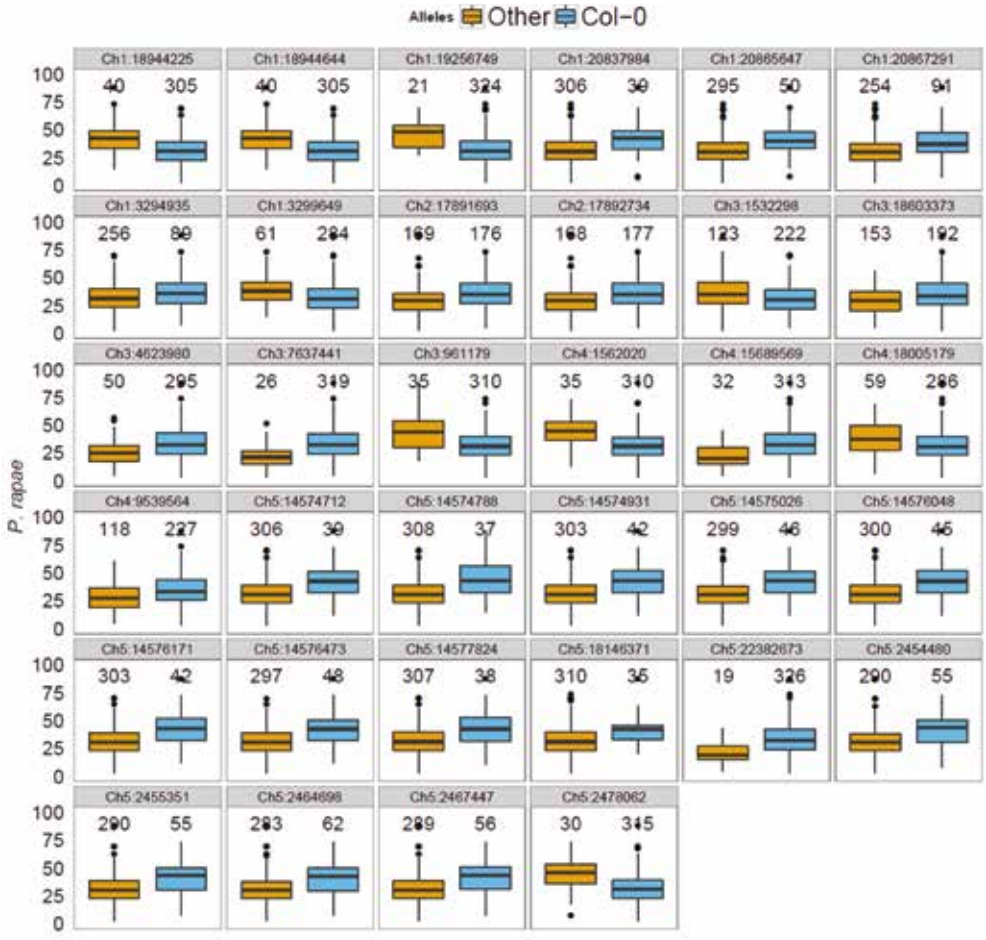

Figure S3. Allele effects of significant SNPs associated with *Arabidopsis thaliana* rosette fresh weight reduction in response to *P. rapae*. Chromosome and SNP position are indicated. Number of accessions having the Col-0 allele or another allele (Other) are indicated above the boxes. Y-axis represents the percentage of biomass reduction compared to the control situation.

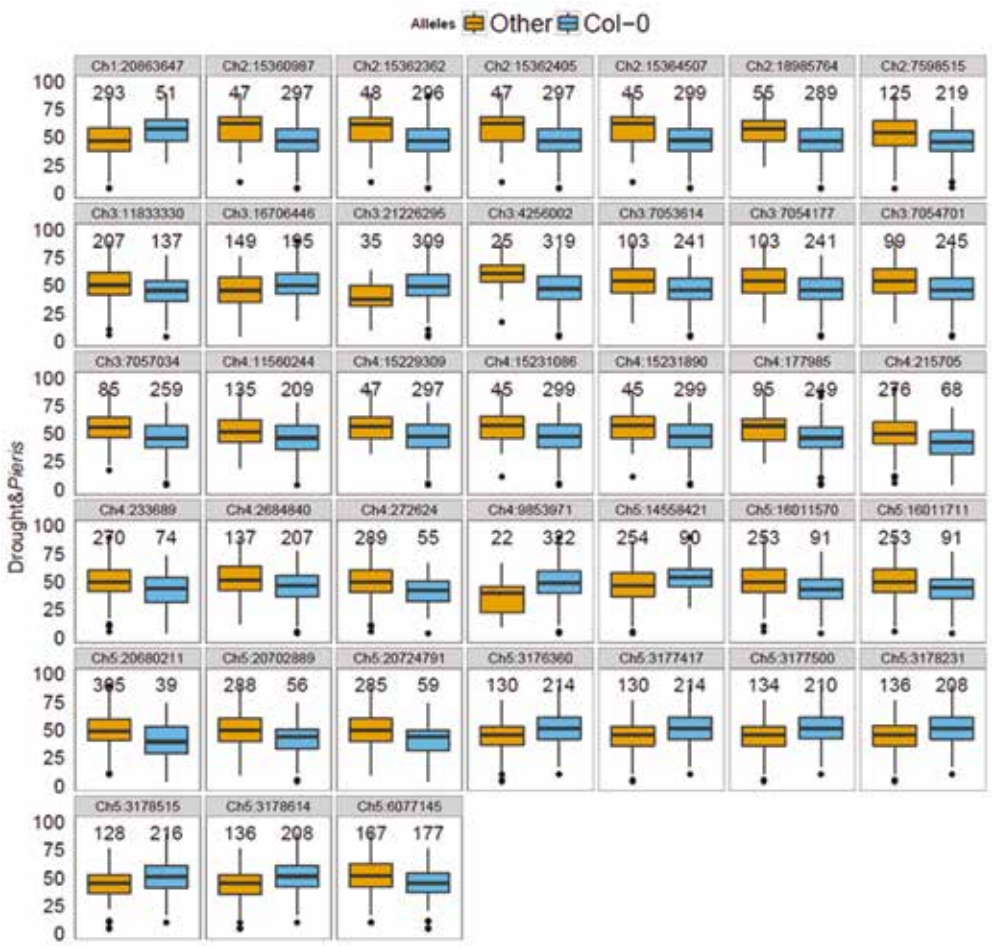

Figure S4. Allele effects of significant SNPs associated with *Arabidopsis thaliana* rosette fresh weight reduction in response to drought and *P. rapae*. Chromosome and SNP position are indicated. Number of accessions having the Col-0 allele or another allele (Other) are indicated above the boxes. Y-axis represent the percentage of biomass reduction with respect to the control situation.

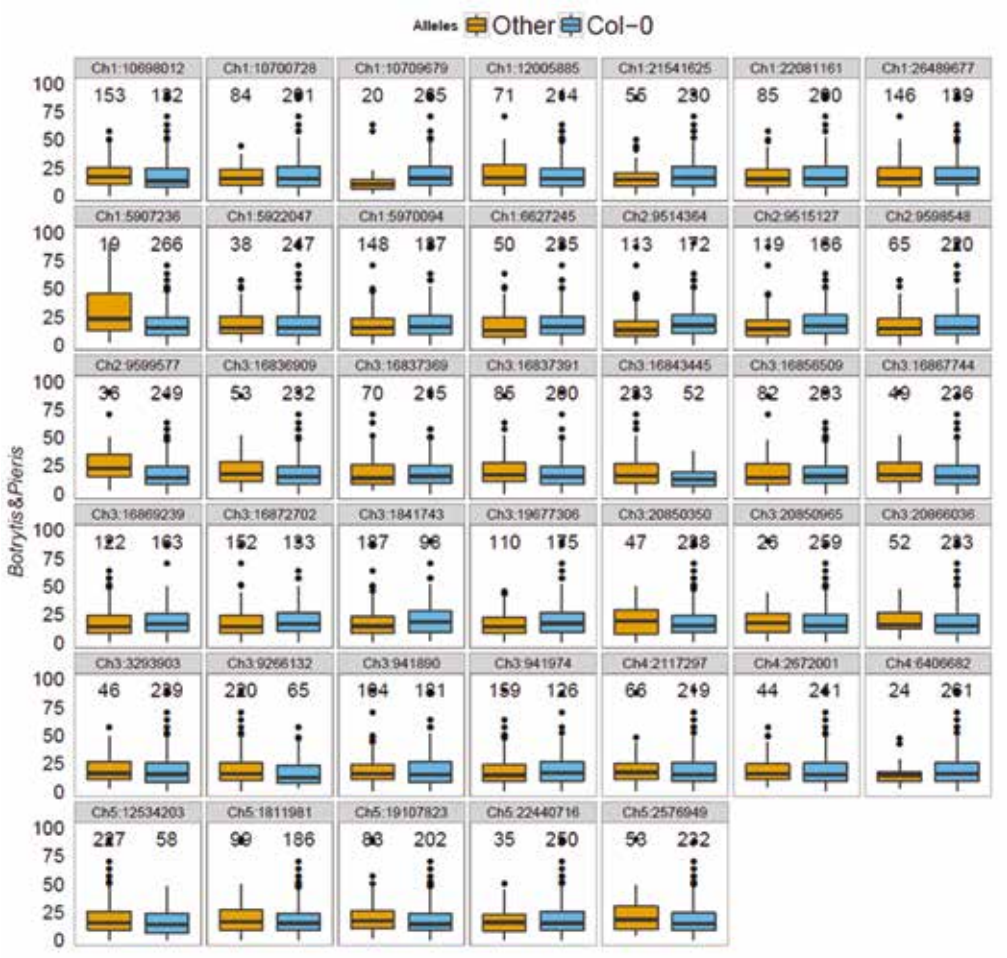

Figure S5. Allele effects of significant SNPs associated with *Arabidopsis thaliana* rosette fresh weight reduction in response to *B. cinerea* and *P. rapae*. Chromosome and SNP position are indicated. Number of accessions having the Col-0 allele or another allele (Other) are indicated above the boxes. Y-axis represent the percentage of biomass reduction with respect to the control situation.

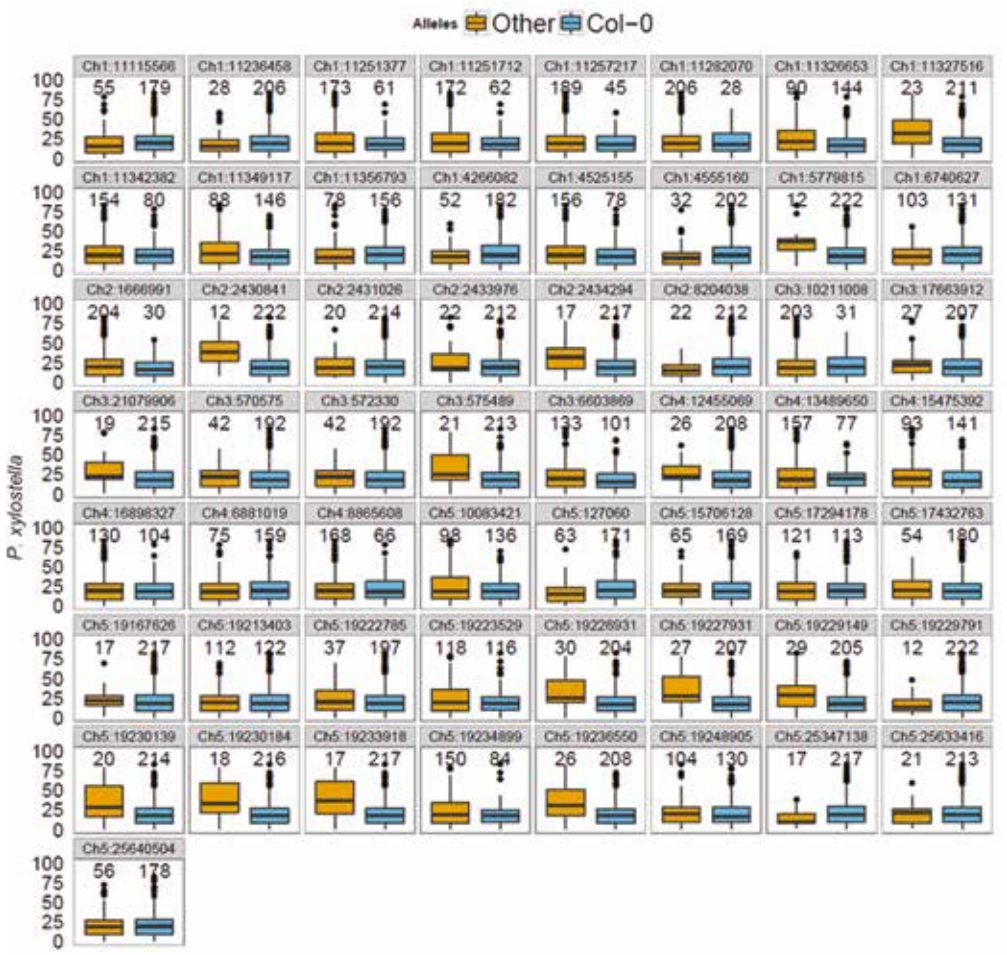

Figure S6. Allele effects of significant SNPs associated with *Arabidopsis thaliana* rosette fresh weight reduction in response to *P. xylostella*. Chromosome and SNP position are indicated. Number of accessions having the Col-0 allele or another allele (Other) are indicated above the boxes. Y-axis represent the percentage of biomass reduction with respect to the control situation.

Chapter 6

Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping

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Abstract

Plants are exposed to combinations of various biotic and abiotic stresses. Here we investigated the genetic architecture underlying plant responses to 11 single stresses and several of their combinations by phenotyping 350 *Arabidopsis thaliana* accessions. A set of 214k SNPs was screened for marker-trait associations in Genome-Wide Association analyses using tailored multi-trait mixed models. Stress responses that share phytohormonal signaling pathways also share genetic architecture underlying these responses. For the 30 most significant SNPs, average QTL-effect-sizes were stronger for dual stresses than single stresses. Plants appear to deploy broad-spectrum defensive mechanisms influencing multiple traits in response to combined stresses. Association analyses identified QTLs with contrasting and with similar responses to (a) biotic versus abiotic stresses and (b) belowground versus aboveground stresses. Extensive phenotyping combined with tailored multi-trait mixed model association analyses allowed for an unprecedented comprehensive genetic analysis of how plants deal with a wide spectrum of stress conditions.

Keywords: Genome wide association, multi-trait, multi-enviroment, complex traits, cross-phenotype associations, pleiotropy.

Introduction

In nature, plants face variable environments that impose a wide range of biotic and abiotic stresses. These include e.g. belowground and aboveground stresses, stresses imposed by unicellular and multicellular organisms, short and long-lasting stresses. Thus, plants are under strong selection to adapt to local conditions and have evolved sophisticated mechanisms to withstand multiple adverse environmental conditions (Howe & Jander, 2008; Pieterse *et al.*, 2012; Stam *et al.*, 2014; Brachi *et al.*, 2015; Julkowska & Testerink, 2015; Kerwin *et al.*, 2015). Yet, investigating this experimentally is a major challenge due to the complexity of multiple stress exposure. To gain insight into the adaptation of plants to the wide variety of stress-inducing conditions they face, genetic variation and mechanisms underlying stress resistance should be studied (Alonso-Blanco *et al.*, 2009; Brachi *et al.*, 2015; Kerwin *et al.*, 2015).

The responses of plants to stresses have traditionally been investigated for individual stresses (Howe & Jander, 2008), but research focus is currently shifting towards plant responses to combinations of stresses (Holopainen & Gershenzon, 2010; Pierik & Testerink, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014; Kissoudis *et al.*, 2015). The emerging picture is that responses to stress combinations cannot be predicted reliably from the responses to individual stresses (Makumburage *et al.*, 2013). For instance, the majority of transcriptional responses of *Arabidopsis* to combinations of two abiotic stresses could not be predicted from responses to the individual stresses (Rasmussen *et al.*, 2013). Moreover, phenotype expression in response to two biotic stresses could not be predicted on the basis of existing information on interactions between underlying signaling pathways (De Vos *et al.*, 2006). Phytohormones are major players in a signaling network, mediating responses to both biotic and abiotic stresses (Pieterse *et al.*, 2009). For instance, chewing insect herbivores elicit especially the jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) signaling pathways, phloem-sucking insects and biotrophic microbial pathogens elicit especially the salicylic acid (SA) pathway, and drought elicits the abscisic acid (ABA) pathway (Pieterse *et al.*, 2009). The phytohormonal responses exhibit extensive crosstalk, resulting in specific changes in plant phenotype in response to individual stresses (De Vos *et al.*, 2005; Pieterse *et al.*, 2012).

Most studies that examined plant responses to multiple stresses included only one or a few genotypes (Holopainen & Gershenzon, 2010; Rasmussen *et al.*, 2013; Pierik & Testerink, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014; Kissoudis *et al.*, 2015). To obtain a further understanding of the genetic architecture of complex traits such as plant adaptation to a diversity of stresses, extensive study of the natural genetic variation within a species is instrumental. Genome-wide association (GWA)

analysis is an important tool for this, requiring a large number of well-genotyped plant accessions. Yet, although the interest in natural variation and GWA mapping is rapidly increasing (Wijnen & Keurentjes, 2014; Ogura & Busch, 2015), a large-scale evaluation of natural genetic variation for resistance of plants to the diversity of stresses that they are exposed to, including pathogens, herbivores and abiotic stresses and their interactions, has not been made to date. To elucidate the genetic architecture of plant stress resistance, an integrated approach is needed that models the genetics of responses to a range of single and combined stresses, including the interaction between those responses.

Here, we have taken a comprehensive and integrated approach to investigate the genetics underlying plant responses to 15 single stresses or stress combinations (Table 1), making use of a global population of 350 *Arabidopsis* accessions that have been genotyped for 214k SNPs (Baxter *et al.*, 2010; Li *et al.*, 2010). We developed a tailored multi-trait GWA analysis that allowed the identification of candidate genes associated with adaptive plant responses to multiple stresses that were validated by gene expression and mutant analyses.

Methods

Phenotyping

Detailed information on experimental protocols and observation and assessment of phenotypes, including T-DNA insertion mutants is presented in the Supplementary Methods, section 2.

Statistics: Genetic correlation networks

Pairwise genetic correlations between traits were estimated using a multi-trait mixed model (MTMM) (Korte *et al.*, 2012). Residuals were assumed uncorrelated for traits that were measured on different plants. For some pairs of traits the likelihood was monotone, which can also occur in single-trait mixed models (Kruijer *et al.*, 2015). In this case, the genetic correlation was estimated by the (Pearson) correlation between the univariate G-BLUPs (De los Campos *et al.*, 2013) estimated for these traits. A network between predefined groups of traits was constructed by connecting groups whose average genetic correlation across pairs of traits was above 0.2.

Statistics: Multi-trait mixed models

Following (Zhou & Stephens, 2014), we assume the MTMM , $Y = XB + G + E$ with Y being the genotypes by traits ($n \times p$) matrix of phenotypic observations. The terms XB, G and E stand for respectively the fixed effects (including trait specific intercepts and SNP-effects) and the random genetic and environmental effects. G follows a zero mean matrix-variate normal distribution with row-covariance (marker-based kinship) matrix K and column (trait) covariance matrix V_g. V_g is a $p \times p$ matrix modeling the genetic correlations between traits. This is equivalent with $g = vec(G)$ (the vector containing the columns of G being multivariate normal with a covariance matrix defined by the Kronecker product $V_g \otimes K$ (Zhou & Stephens, 2014). Similarly, follows a zero mean normal distribution with covariance , $V_e \otimes I_n$ where V_e accounts for the non-genetic correlations between traits.

Statistics: Factor-analytic models

Since V_g and V_e contain a total of $p(p + 1)$ parameters, the MTMM above becomes difficult to fit for more than 10 traits (Zhou & Stephens, 2014). For V_g we therefore assumed a factor analytic model, which is well known in the context of QTL-mapping for experimental populations with limited numbers of markers (Boer et al., 2007), but has not been used in the context of multivariate GWAS. As almost all traits were derived from measurements on different plants, a diagonal model $V_e = diag(\sigma^2_{e,1},\ldots,\sigma^2_{e,p})$ was chosen for the environmental covariances. For V_g a first order factor analytic structure

was chosen $V_g = \sigma_g^2(\lambda\lambda^t + diag(\tau_1^2,\ldots,\tau_p^2))$, where σ_g^2 represents a scale parameter, the magnitude of genetic effects, the vector $\lambda = (\lambda_1,\ldots,\lambda_p)^t$ contains the trait specific scores belonging to the factor analytic part of the model that provides a rank one variance-covariance structure between traits, and $diag(\tau_1^2,\ldots,\tau_p^2)$ provides trait specific residual genetic variances (Piepho, 1997; Meijer, 2009). The model was fitted with the R-package ASRreml (Butler *et al.*, 2009).

Statistics: Compressed kinship

Factor analytic models have been successfully applied to experimental populations with a simple genetic relatedness structure (Boer *et al.*, 2007; Malosetti *et al.*, 2008; Alimi *et al.*, 2013), but currently available software could not perform REML-estimation for the hapmap-population. The kinship matrix was therefore replaced by a compressed kinship matrix (Bradbury *et al.*, 2007; Zhang *et al.*, 2010), modeling the genetic relatedness between a number of internally homogeneous groups. Assuming there are *m* such groups, containing n_1, ..., n_m accessions each, the original kinship matrix K is replaced by ZK_CZ^t, where K_C is the kinship matrix for the groups, and Z is the $n \times m$ incidence matrix assigning each of the *n* accessions to one of the *m* groups. The groups were created by a procedure that restricted the marker data to be linear combinations of environmental covariates representing the conditions at the place of origin of the accessions (Supplementary Methods, section 3.1).

Statistics: Multi-trait GWAS

Traits (columns of Y) were standardized. Along the genome, MTMMs of the type $Y = XB + G + E$ were fitted with initially for each marker trait-specific QTL $\beta_1, \ldots, \beta_p$ effects (contained in *B*). To identify general QTLs with trait-specific effects, for individual markers, the null hypothesis $\beta_1 = \beta_2 = \ldots \beta_p = 0$ was tested by a Wald test against the alternative hypothesis that at least one of the trait specific effects was nonzero (Zhou & Stephens, 2014). To identify consistent QTLs, the null hypothesis $\beta_1 = \beta_2 = \ldots \beta_p = \beta \neq 0$ was tested. To identify adaptive QTLs, contrasts defined on the trait specific QTL effects were tested. For example, suppose the first *p1* of the full set of *p* traits represent responses measured under abiotic stresses, while the second *p2* traits represent responses under biotic stresses. A contrast can now be defined to test the hypothesis whether the QTL effect for abiotic stresses differs from that for biotic stresses: $\beta_1 = \beta_2 = \ldots \beta_{p1} = a_{abiotic}$; $\beta_{p1+1} = \beta_{p1+2} = \ldots \beta_p = a_{biotic}$ and H_0: $a_{abiotic} = a_{biotic}$ versus H_a: $a_{abiotic} \neq a_{biotic}$ (see also Supplementary Methods, section 3.2).

Selecting candidate genes

A significance threshold of P<0.0001 was chosen after implementation of genomic control (see Supplementary Methods 3.3). For MTMM this resulted in 43 SNPs meeting this criterion. The surrounding region of interest was set to a maximum of 40kb window (20kb on both sides), where the final boundaries were determined by SNPs in LD (threshold 0.5) the furthest away of the significant SNP. This resulted in 30 genome regions, for which in each region the SNP with the lowest P value was selected as representative for the LD block. For Figures 3-5, however, we selected the SNP with the highest absolute effect size to maximize visual contrasts in the figures. Contrast analyses followed the same selection procedure as MTMM.

Results

The phenotypic response of a population of 350 *Arabidopsis* accessions to an extensive set of stress-inducing conditions was quantified relative to the respective control treatments. Thirty traits, including e.g. root length, number of damaged leaves, or number of pathogen-inflicted spreading lesions (Table 1) were quantified when the plants were exposed to 15 different stresses, i.e. four abiotic stresses (drought, salt stress, osmotic stress and heat), seven biotic stresses (parasitic plant, phloem-feeding aphid, phloem-feeding whitefly, cell-content feeding thrips, leaf-chewing caterpillar, root-feeding nematode, and necrotrophic fungus) and four stress combinations (fungus and caterpillar, drought and fungus, drought and caterpillar, caterpillar and osmotic stress). For detailed information on the stress treatments and the trait definitions see Supplementary Methods (Sections 1 and 2).

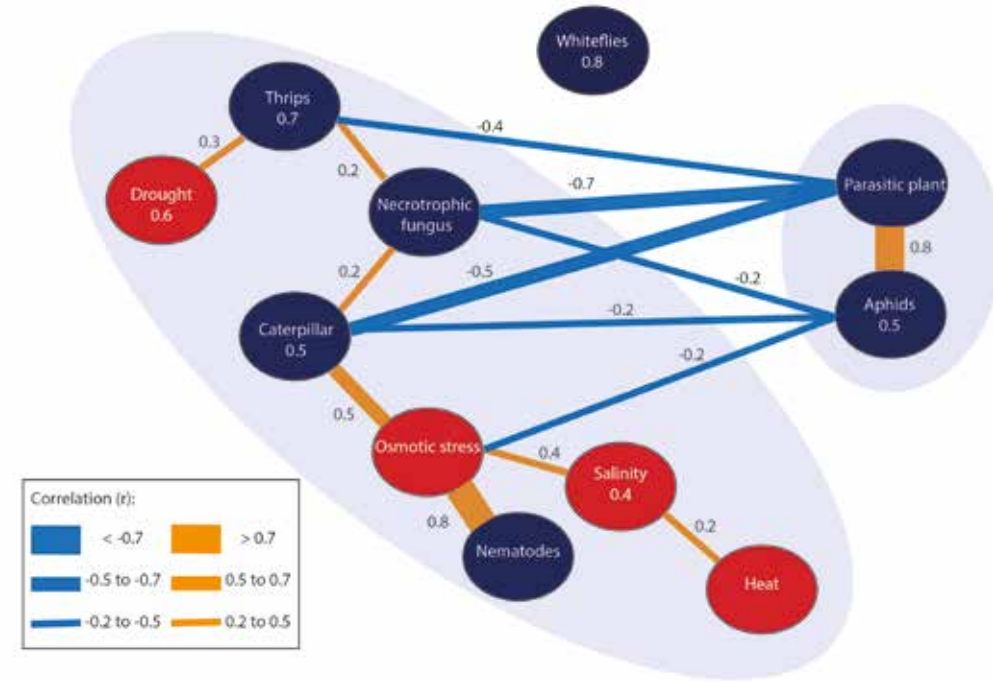

Figure 1. Mean genetic correlations between responses to abiotic (red) and biotic (dark blue) plant stresses. Thickness of lines represents the strength of mean genome-wide correlations, annotated with r values (orange=positive, blue=negative correlation). The more shared genetic associations between stresses, the higher the absolute genetic correlation. Correlations are negative when alleles have opposite effects, i.e. resulting in increased resistance to one stress, but decreased resistance to the other stress. Values in balloons represent mean within-group correlation (not shown for groups consisting of a single trait). Mean between-group correlations are not shown if they are below an absolute value of r=0.2. Two clusters can be distinguished: (1) parasitic plants and aphids and (2) the other stresses, except whiteflies.

Table 1. Phenotypes assessed. The dataset contains three plant stress categories; abiotic stress, biotic stress and combinations of both abiotic and biotic stress. Phenotype assessments that were performed under similar environmental conditions have similar background shading (light and dark grey). 'Phenotype' refers to different phenotypic assessments (in some cases the first principal component of a group of phenotypes). 'Treatment' refers to the sort of stress that was applied. Additional information on traits can be found in Supplementary methods (section2).

	Stress	Trait name	Trait phenotype	Treatment
Abiotic stresses	Salt	Salt_1	Main root length, number of lateral roots and straightness	75 mM NaCl
		Salt_2	Main root length	125 mM NaCl
		Salt_3	Number of lateral roots	125 mM NaCl
		Salt_4	Main root angle	125 mM NaCl
		Salt_5	Biomass	25 mM NaCl
	Drought	Drought_1	Biomass	Drought
		Drought_2	Biomass	Drought
	Osmotic	Osmotic	Biomass	PEG8000
	Heat	Heat	Number of siliques	35 °C
Biotic stresses	Parasitic plant	Parasitic plant	Attachments	*Phelipanche ramosa*
	Nematode	Nematode	Offspring, eggmass	*Meloidogyne incognita*
	Whitefly	Whitefly_1	Survival, whiteflies	*Aleyrodes proletella*
		Whitefly_2	Reproduction, eggs	*A. proletella*
	Aphid	Aphid_1	Behavior T1, probing	*Myzus persicae*
		Aphid_2	Behavior T2, probing	*M. persicae*
		Aphid_3	Offspring, aphids	*M. persicae*
	Thrips	Thrips_1	Feeding damage	*Frankliniella occidentalis*
		Thrips_2	Behavior T1	*F. occidentalis*
		Thrips_3	Behavior T2	*F. occidentalis*
	Caterpillar	Caterpillar_1	Leaf area consumed	*Pieris rapae*
		Caterpillar_2	Biomass	*P. rapae*
		Caterpillar_3	Number of damaged leaves and feeding sites	*P. rapae*
	Fungus	Fungus	Number of spreading lesions	*Botrytis cinerea*
	Double stress	Fungus and caterpillar_1	Biomass	*B. cinerea* and *P. rapae*
		Fungus and caterpillar_2	Number of damaged leaves and feeding sites	*B. cinerea* and *P. rapae*
		Caterpillar and fungus	Number of spreading lesions	*P. rapae* and *B. cinerea*
Abiotic and biotic stress	Double stress	Drought and fungus	Number of spreading lesions	Drought and *B. cinerea*
		Drought and caterpillar	Number of damaged leaves and feeding sites	Drought and *P. rapae*
		Caterpillar and osmotic_1	Projected leaf area	*P. rapae* and PEG8000
		Caterpillar and osmotic_2	Biomass	*P. rapae and* PEG8000

Heritability of responses to biotic and abiotic stresses

The phenotypic analysis resulted in a wide range of marker-based narrow sense heritability (Kruijer *et al.*, 2015) estimates with 15 traits of low (h^2<0.2), 10 of moderate (0.2<h^2<0.5) and 5 of high (h^2>0.5) heritability (Supplementary Figure S1). The number of abiotic stress traits per heritability category was similar, while the number of traits related to biotic and combined stresses decreased with increasing heritability class. The most heritable traits were responses to feeding damage by thrips (Thrips_1; h^2=0.8), and nematodes (h^2=0.7), and responses to salt (Salt_1 and Salt_3; resp. h^2=0.6 and h^2=0.7) and heat (Heat; h^2=0.6) (Supplementary Table S1). The traits related to combined stresses have predominantly low heritabilities.

Genetic commonality underlying responses to different stresses

To analyze the phenotypic variation between *Arabidopsis* accessions as a function of molecular marker variation, we used various mixed model approaches (see Methods section and Supplementary Methods, section 3). We estimated marker-based genetic correlations, i.e. correlations based on the genome-wide commonality of SNP effects underlying pairs of traits (see Methods), to investigate the magnitude of genetic commonality underlying resistance mechanisms in response to a range of biotic and abiotic stresses. For brevity, we will refer to these marker-based genetic correlations as genetic correlations. Such genetic correlations can be interpreted as upper boundaries to the joint determination of pairs of traits by genetic factors. Genetic correlation analysis revealed a strong connection between the responses to parasitic plants and to aphids (r=0.8), which were both negatively associated with other stress responses (Figure 1). Parasitic plants and aphids have in common that they target phloem and xylem tissue (Tjallingii & Hogen Esch, 1993; Dorr & Kollmann, 1995), and induce the SA phytohormonal pathway (De Vos *et al.*, 2005; Runyon *et al.*, 2008). In contrast, the biotic stress responses that were negatively associated with the responses to parasitic plants and aphids, i.e. responses to necrotrophic fungi, caterpillars, and thrips, represent JA-inducing stresses (De Vos *et al.*, 2005; Pieterse *et al.*, 2009; Pieterse *et al.*, 2012). Because the SA and JA pathways predominantly interact through negative crosstalk (Pieterse *et al.*, 2009), the two main clusters resulting from the genetic correlation analysis represent different phytohormonal signaling response mechanisms. We also observed a strong genetic correlation between plant responses to osmotic stress and root-feeding nematodes. This supports the notion that root-knot nematodes trigger a differentiation of root cells to multinucleate giant cells with severely altered water potential and osmotic pressure (Baldacci-Cresp *et al.*, 2015). While the correlations between traits at the phenotypic level were generally rather low, the genetic correlation analysis revealed a common genetic architecture underlying the responses to sets of single and combined stresses (Supplementary Figure S2).

Candidate genes underlying responses to stresses

To identify individual candidate genes that contributed most to the pattern of genetic correlations, we fitted multi-trait QTL mixed models (MTMMs) to the total set of 30 traits, using a 214k SNP set that is commonly used for GWA studies in Arabidopsis (Kim *et al.*, 2007; Atwell *et al.*, 2010; Li *et al.*, 2010; Horton *et al.*, 2012; Bac-Molenaar *et al.*, 2015). Our multi-trait GWA approach closely follows the modeling framework developed by Zhou & Stephens (2014) and generalizes the use of MTMMs as described previously (Boer *et al.*, 2007; Malosetti *et al.*, 2008; Alimi *et al.*, 2013) for classical biparental offspring populations to association panels. This GWA analysis identified 30 chromosome regions with multiple, significant SNP-trait associations. From each of those regions, the most significant SNP was chosen to represent the locus (Figure 2; Supplementary Table S2). Clustering of stresses by estimated SNP-effect profiles (Figure 2) revealed that multiple SNPs were associated with response to more than one stress. Stress combinations induced large QTL allele substitution effects in the MTMM mapping (Figure 2 and Supplementary Table S2), indicating that combinations of stresses trigger broad-spectrum defensive mechanisms. A total of 125 genes were in linkage disequilibrium (LD) with the 30 most significant SNPs from the GWA analysis. Twenty of these genes were stress-related according to gene ontology (GO) annotation data (Supplementary Table S3). Of these 20 genes, six have been functionally characterized by at least one study (Table 2a). For these six genes, we explored expression data to evaluate the biological relevance of these genes in stress-responsive mechanisms of *Arabidopsis* (Supplementary Figure S3). Of special interest were SNPs chr5.7493620, chr5.22041081 and chr4.6805259, that were in LD with *WRKY38* (encoding a WRKY transcription factor involved in SA-dependent disease resistance)(Kim *et al.*, 2008), *AtCNGC4* (involved in pathogen resistance)(Chin *et al.*, 2013) and *RMG1* (coding for disease resistance protein)(Yu *et al.*, 2013) respectively.

Phytohormonal signaling underlying contrasts in stress responses

The MTMM framework allowed imposing constraints on the values of the estimated QTL effects (see Methods). In this way specific hypotheses can be tested about stresses sharing a common QTL effect or having opposite QTL effects.

We investigated whether polymorphisms for genes involved in SA and JA biosynthesis or genes responsive to signals from these pathways were the cause of the negative genetic correlations between the groups of traits sharing one or the other phytohormonal signaling pathway. To this end, we performed a multi-trait GWA mapping to test the contrast between: (1) parasitic plant and aphid response, versus (2) the most negatively correlated traits, i.e. fungus, caterpillar, thrips and drought response (Figure 1). Fifteen SNPs were significantly associated with contrasting effects between the two trait clusters (Supplementary Figure S4).

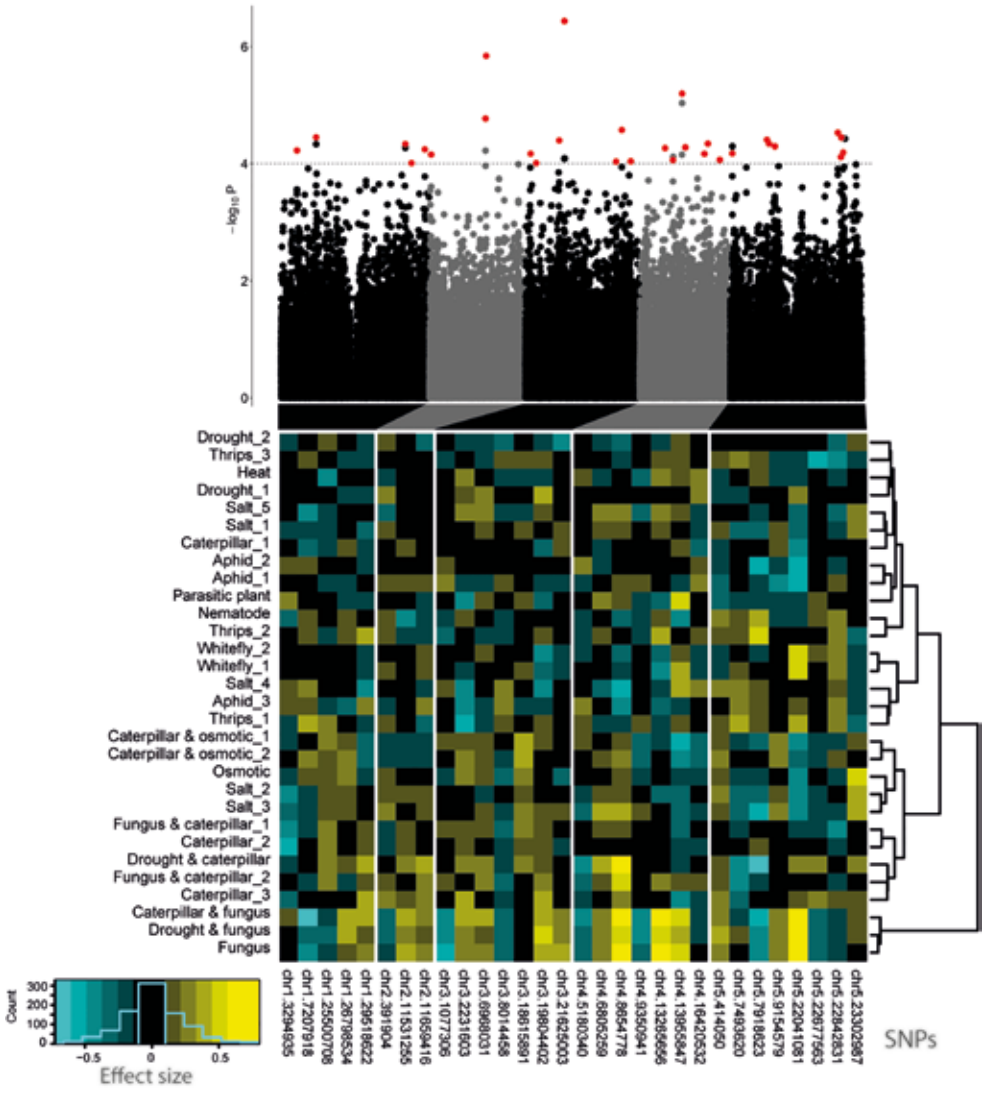

Figure 2. Multi-trait mixed-model (MTMM) GWA mapping with 30 different stress responses of *Arabidopsis*. The top panel shows the 214k SNPs with their corresponding -$\log_{10}(P)$ values for the five chromosomes. The lower panel depicts the trait-specific effect size of the rare allele for SNPs with a LOD score higher than 4 (effects were estimated from the full MTMM). When several SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included (red-flagged in the Manhattan plot). SNPs are named by chromosome number and position on the chromosome. Negative effect sizes (blue) correspond to reduced plant resistance due to the rare allele, positive effect sizes (yellow) to increased resistance due to the rare allele. Stress responses were clustered hierarchically according to their effect size, using Ward's minimum variance method. The key shows the frequency distribution of SNPs across effect sizes.

Seven of these SNPs, were in LD with one or more genes known to be involved in JA-, SA- or resistance-related signal transduction (Supplementary Table S4). Among these genes are *LOX5*, whose product is involved in facilitating aphid feeding, *MYB107* encoding a transcription factor responsive to SA, the JA-inducible genes *TPS02* and *TPS03* encoding terpene synthases and *MES16*, encoding a methyl jasmonate esterase. In addition to screening for SNPs with contrasting effects, we screened for SNPs with a similar effect across the above-mentioned trait clusters (Supplementary Figure S5) and found candidate genes involved in oxidative stress and plant responses to salinity and pathogens (Supplementary Table S5).

Table 2. Candidate genes resulting from (a) MTMM analysis of all 30 stress responses as presented in Figure 2 and (b) contrast-specific analysis with MTMM for contrasting effects of biotic and abiotic stresses as presented in Figure 3.

Table 2a:

Marker*	Gene in LD	Gene name	Gene description**	References
chr2.11659416	*AT2G27250*	*CLV3*	One of the three *CLAVATA* genes controlling the size of the shoot apical meristem (SAM) in *Arabidopsis*	(Clark *et al*., 1996; Fletcher *et al*., 1999; Shinohara & Matsubayashi, 2010)
chr3.19804402	*AT3G53420*	*PIP2*	A member of the plasma membrane intrinsic protein subfamily PIP2.	(Martiniere *et al*., 2012; Peret *et al*., 2012; Sanchez-Romera *et al*., 2014)
chr4.6805259	*AT4G11170*	*RMG1*	Encodes RMG1 (Resistance Methylated Gene 1), an NB-LRR disease resistance protein with a Toll/interleukin-1 receptor (TIR) domain at its N terminus.	(Yu *et al*., 2013)
chr5.7493620	*AT5G22570*	*WRKY38*	Member of WRKY Transcription Factor; Group III	(Mare *et al*., 2004; Kim *et al*., 2008)
chr5.22041081	*AT5G54250*	*CNGC4*	Member of Cyclic Nucleotide Gated Channel family, a downstream component of the signaling pathways leading to hypersensitive response (HR) resistance. Mutant plants exhibit gene-for-gene disease resistance against avirulent *Pseudomonas syringae* despite the near-complete absence of the HR. Salicylic acid accumulation in *dnd2* mutants is completely *PAD4*-independent.	(Jurkowski *et al*., 2004; Keisa *et al*., 2011; Chin *et al*., 2013)
chr5.23302987	*AT5G57560*	*TCH4*	Encodes a cell wall modifying enzyme, rapidly upregulated in response to environmental stimuli	(Braam & Davis, 1990; Xu *et al*., 1996; Purugganan *et al*., 1997; Iliev *et al*., 2002)

* markers derived from MTMM analysis (see Figure 2)

** based on information on http://www.arabidopsis.org/tools/bulk/go/index.jsp

Table 2b:

Marker	Gene in LD	Gene name	Gene description*	References
chr1.30381439	*AT1G80820*	*CCR2*	*CINNAMOYL COA REDUCTASE.* Encodes a cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis.	(Luderitz & Grisebach, 1981; Lauvergeat *et al*., 2001; Zhou *et al*., 2010)
chr1.30381439	*AT1G80840*	*WRKY40*	Pathogen-induced transcription factor. Binds W-box sequences in vitro. Forms protein complexes with itself and with WRKY60. Co-expression with *WRKY18* or *WRKY60* made plants more susceptible to both *P. syringae* and *Botrytis*.	(Chen *et al*., 2010a; Pandey *et al*., 2010; Liu *et al*., 2012)
chr1.6038270	*AT1G17610*	*CHS1*	*CHILLING SENSITIVE 1,* mutant accumulates steryl-esters at low temperature.	(Wang *et al*., 2013; Zbierzak *et al*., 2013)
chr5.171177	*AT5G17640*	*ASG1*	*ABIOTIC STRESS GENE 1*; Expression of this gene is induced by abscisic acid and salt stress.	(Coste *et al*., 2008; Batelli *et al*., 2012)
chr5.23247572	*AT5G57380*	*VIN3*	Encodes a plant homeodomain protein VERNALIZATION INSENSITIVE 3 (VIN3). In planta VIN3 and VRN2, VERNALIZATION 2, are part of a large protein complex that can include the polycomb group (PcG) proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE), CURLY LEAF (CLF), and SWINGER (SWN or EZA1). The complex has a role in establishing FLC (FLOWERING LOCUS C) repression during vernalization.	(Sung *et al*., 2007; Bond *et al*., 2009; Finnegan *et al*., 2011)
chr5.23293119	*AT5G57560*	*TCH 4*	Encodes a cell wall-modifying enzyme	(Braam & Davis, 1990; Xu *et al*., 1996; Purugganan *et al*., 1997; Iliev *et al*., 2002)
chr5.23293870	*AT5G57490*	*VDAC4*	Encodes a voltage-dependent anion channel (VDAC: AT3G01280/VDAC1)	(Lee *et al*., 2009; Tateda *et al*., 2011)
chr5.23366252	*AT5G57685*	*GDU3*	Encodes a member of the GDU (glutamine dumper) family proteins involved in amino acid export: At4g31730 (GDU1)	(Chen *et al*., 2010b)

* based on information on http://www.arabidopsis.org/tools/bulk/go/index.jsp

QTLs underlying contrasts in responses to biotic and abiotic stresses

We expected a negative correlation between the responses to biotic and abiotic stresses. Testing for this contrast within the GWA analysis using our MTMM approach significantly identified 43 SNPs with a QTL effect size that changed sign between biotic and abiotic conditions. Traits were then ordered by a cluster analysis on estimated SNP effects across significant SNPs, while SNPs were ordered by clustering their effects across traits. Figure 3 shows the SNPs with the strongest overall effects, identified in 18 LD intervals. The minor alleles of nine of these SNPs displayed a positive effect on biotic stress response traits and a negative effect on abiotic response traits. The remaining nine SNPs displayed the opposite effect (Figure 3).

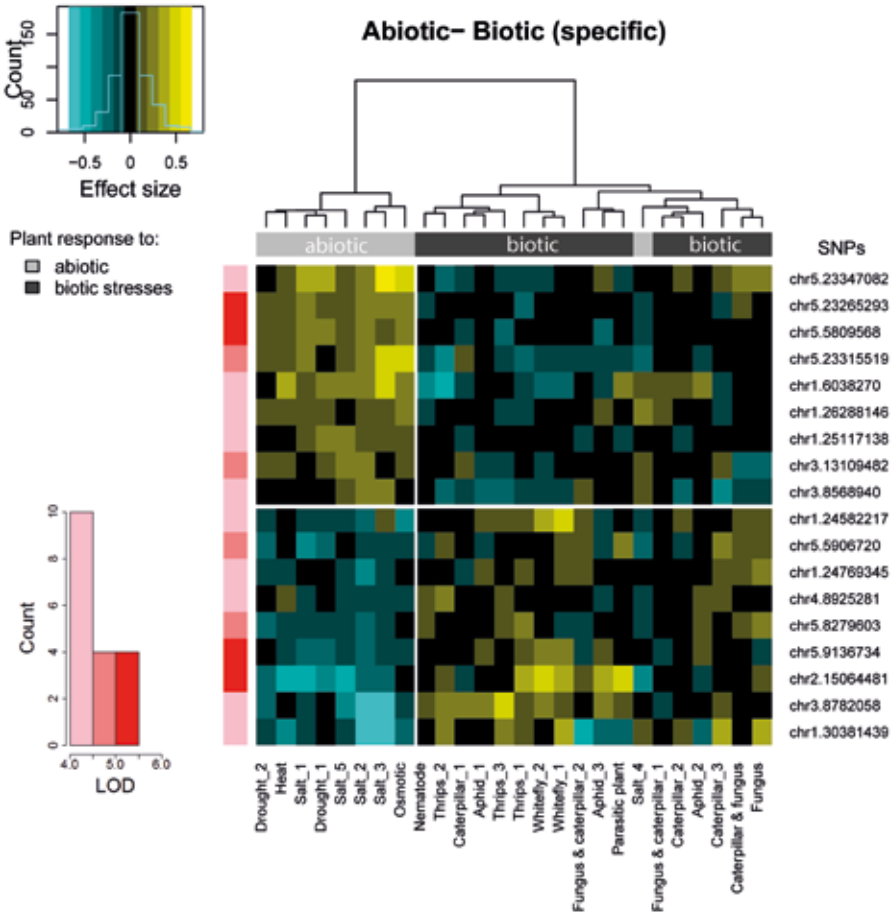

Figure 3. Genetic associations specific for contrasting plant responses to either abiotic or biotic stress. Genetic associations were estimated with a contrast-specific analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the biotic-abiotic contrast are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used a representative for the LD block. Negative effect sizes (blue) were cases

where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 9 SNPs are associated with enhanced resistance to abiotic stresses and reduced resistance to biotic stresses; the bottom 9 SNPs show the inverse. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

Several candidate genes were identified in LD with the SNPs that are specific for plant responses to either abiotic or biotic stresses (Table 2b), such as *TCH4* (encoding a cell-wall modifying enzyme), *AtCCR2* (involvement in lignin biosynthesis) and *ASG1* (a gene induced by ABA and salt stress), were identified. Transcription data (Supplementary Figure S6) support the notion that these genes play a contrasting role in responses to abiotic and biotic stresses. A screen for QTLs with similar effects on resistance to biotic and abiotic stress (Supplementary Figure S7) identified three genes annotated to be responsive to stress stimuli. Transcriptional data show that these genes respond differentially to different (a) biotic stresses and phytohormones (Supplementary Figure S8). Genes like *ARGAH2* (involved in JA-mediated resistance to necrotrophic fungus) and *PKS1* (involved in light responses) are promising candidates for consistent effects across biotic and abiotic stresses (Supplementary Table S6).

QTLs underlying contrasts in responses to below- and aboveground stresses

We expected a negative correlation between responses to below- and aboveground stresses. A strong QTL signal was found on chromosome 1 for this contrasting response (Supplementary Figure S9). The associated marker (chr1. 13729757) had 12 genes in LD with it, of which 11 are annotated as pseudogenes. Transcriptional data on abiotic stresses for the only protein coding gene (*AT1G36510*) shows an upregulation in above tissues, yet a downregulation in the root tissues. Marker chr5.16012837 showed the strongest signal for similar effects on responses to below- and aboveground stresses (Supplementary Figure S10) for which the *pathogenesis-related thaumatin superfamily protein* (*AT5G40020*) is the most promising candidate gene.

Validation of identified QTLs

To obtain experimental support for the most interesting QTLs resulting from the MTMM, we tested homozygous T-DNA insertion lines for candidate genes *RMG1* and *WRKY38* (both resulting from the MTMM analysis), and *TCH4* (from MTMM analysis on biotic versus abiotic contrast) for several of the stresses addressed in this study. Two independent *rmg1* T-DNA insertion lines showed a phenotype that was different from the wild type (Col-0) for some of the stress conditions (Figure 4, Supplementary Methods Section 2.11), being more resistant to caterpillar feeding and osmotic stress (Figure 4).

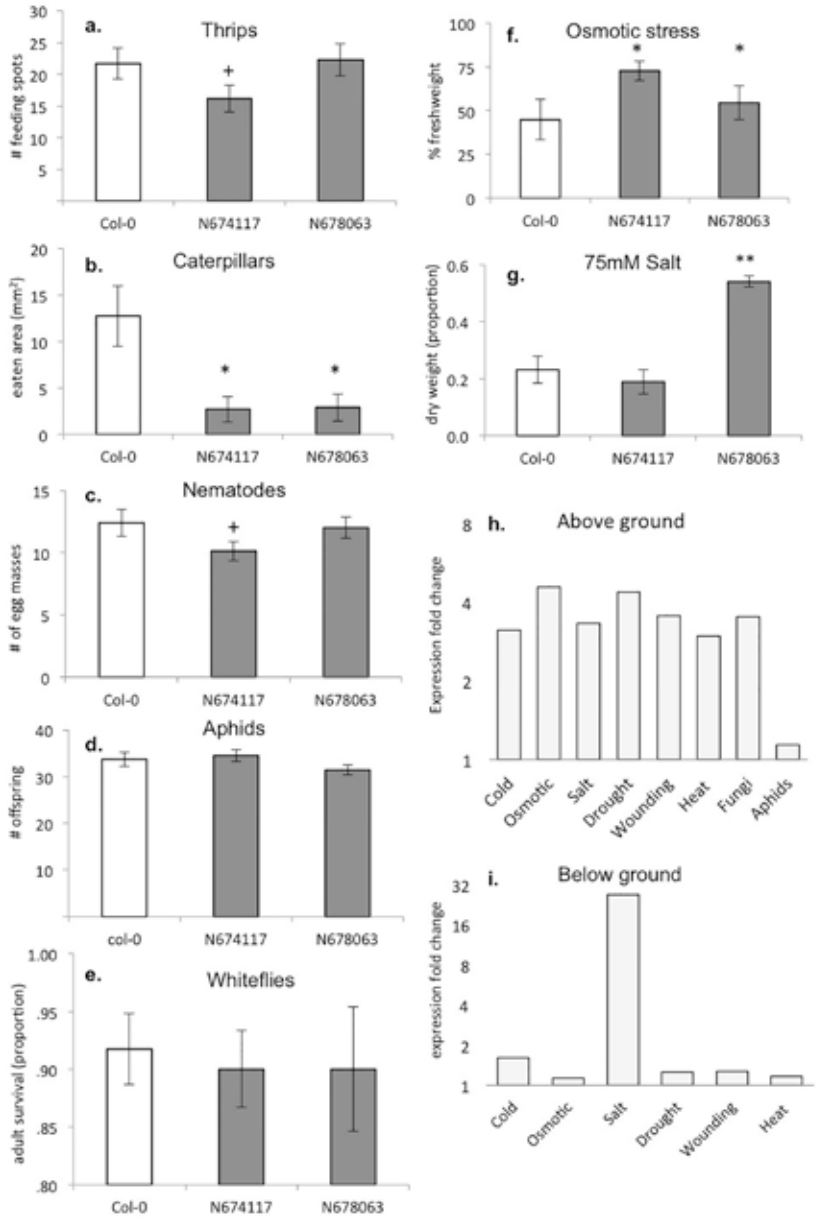

Figure 4. Phenotypes of *RMG1* T-DNA mutant screenings. Coloured boxes indicate the effect size of the rare allele (non Col-0 in this case) for marker chr4.6805259. Phenotypes are given for two T-DNA lines in the *RMG1* gene and for Col-0 as control. a. Number of thrips feeding spots on a detached leaf, 6 days post infestation (N=24); b. Leaf area consumed by *P. rapae* caterpillars (N=6); c. Number of nematode egg masses (N=23); d. Number of *M. persicae* aphid offspring (N=10-17); e. Percent survival of adult whiteflies (*A. proletella*) (N=10); f. Plant fresh weight after osmotic treatment in comparison to control (% relative to control) (N=4); g. Plant dry weight after 75mM salt treatment in comparison to control (ratio)(N=7-10); Mean ± SE, +: P < 0.01, *: P < 0.05, **: P < 0.01, difference in comparison to Col-0. Relative expression fold change for *RMG1* compared to untreated control plants in aboveground (h) and belowground (i) tissue. Expression data from *Arabidopsis* eFP browser (http://bbc.botany.utoronto.ca).

RMG1 (*AT4G11170*) encodes an NB-LRR disease resistance protein, and transcription is highly induced by the bacterial peptide flg22 (Yu *et al.*, 2013). The rare allele of the corresponding marker chr4.6805259 is associated with enhanced resistance to salt stress and the combined stresses 'caterpillar and drought' and 'caterpillar and fungus' and with enhanced susceptibility to drought and thrips stress. Gene expression data show that *RMG1* is upregulated by several abiotic and biotic stresses (Figure 4). T-DNA insertion lines for *TCH4* and *WRKY38* did not show a phenotype different from the wild type (Col-0) for any of the tested stress conditions. Whether this is dependent on the genetic background used, remains to be investigated.

Summarizing, our multi-trait GWA methodology facilitated a detailed analysis of the genetic architecture of resistance in *Arabidopsis* to a wide diversity of biotic and abiotic stresses. Application of this methodology revealed novel candidate genes associated with multiple stress responses, where specific contrasts were identified with some genes positively associated with the resistance to one set of stresses while being negatively associated with another set of stresses. In plant breeding (Brady *et al.*, 2005; Ballesteros *et al.*, 2015), such genes are classified as adaptive. Alternatively, other genes were identified with consistent effects across a wide spectrum of stress conditions. Such genes are labelled as constitutive in the plant breeding literature (Brady *et al.*, 2005; Ballesteros *et al.*, 2015). Both adaptive and constitutive QTLs are important factors to contribute to improved stress resistance and tolerance in commercial crop species (Brady *et al.*, 2005; Ballesteros *et al.*, 2015).

Discussion

Using a novel mixed-model approach to multi-trait GWA mapping, the genetic architecture of Arabidopsis underlying a total of 30 stress response traits was analyzed. A special feature of our statistical approach is that the GWA analysis accounted simultaneously for dependencies between genotypes and between traits. Through this approach, candidate genes for adaptive stress responses were identified that are involved in contrasting responses when comparing biotic and abiotic stresses, above- and belowground stresses, and attack by phloem feeders compared with other biotic stresses. Among these genes many are involved in phytohormone-mediated processes, supporting the notion that the phytohormonal regulatory network plays an important role in plant stress responses (Pieterse *et al.*, 2012). The MTMM approach further showed that certain SNPs were associated to multiple stress responses and that transcriptional patterns of genes to which the SNPs were linked, as well as the phenotype expressed upon knocking out one of these genes, matched with the observed stress responses of the plants. The *RMG1* gene that was identified through this procedure has relevant effects on plant phenotype in the context of responses to individual stresses. *RMG1* is a bacterium-inducible resistance gene whose activity is modulated by the plant through RNA-directed DNA methylation (RdDM) (Yu *et al.*, 2013). *RMG1* expression activates the SA pathway (Yu *et al.*, 2013). Thus, the increased resistance against caterpillars in *rmg1* mutants may be the result of elimination of SA-mediated interference with JA-induced resistance to caterpillars (Pieterse *et al.*, 2012). *RMG1* appears to be inducible by several stresses and deserves further in-depth analysis for its role in plant response to multiple stresses.

Our data show that for the 30 most significant SNPs resulting from the MTMM analysis, the average absolute effect size for double stresses is on average higher than that for single stresses ($P < 0.007$, Supplementary Table S2). This indicates that resistance mechanisms involved in countering dual stresses are of a more general nature, in contrast to the rather specific resistance mechanisms involved in single stress responses.

The MTMM framework that we used for GWA mapping provides unbiased estimates for QTL allele substitution effects together with correct standard errors for these effects. Within the same framework we developed unique facilities to test hypotheses on QTL-by-stress interactions in multi-trait models, which are not available in competing meta-analysis approaches (Zhu *et al.*, 2015). The variance-covariance structure that we used for the polygenic term protects against inflated type I error, i.e. too many false positive SNP-trait associations, as a consequence of population structure and kinship on the genotypic side and genetic correlations between traits on the trait side. The inclusion of trait correlations will, for most QTLs, improve the power of detection

in comparison to single-trait GWA mapping. Our choice for the variance-covariance structure of the polygenic term as a Kronecker product of a compressed kinship on the genotypes with an approximated unstructured variance-covariance model on the environments is sometimes used in plant breeding for genomic prediction models (Burgueno *et al*., 2012). However, implementation of such models in GWA mapping and especially on the scale that we present here, with 30 traits, is unprecedented and is practically far from straightforward. It required substantial work on preparatory phenotypic analyses as well as fine-tuning of the genotypic and trait variance-covariance structures to achieve convergence of the mixed models.

The MTMM analyses identified candidate genes associated with contrasting responses to biotic and abiotic stresses. Stress combinations appeared to have a strong influence on the MTMM outcome, indicative for significant interactions between different stresses when occurring simultaneously, and underlining the importance of studying the resistance of plants to combinations of stress. Transcriptional data and phenotyping of mutants provide initial support for the role of several of the candidate genes identified Studies of plant responses to a diverse set of biotic stresses show that the transcriptional pattern is stress-specific and that phytohormonal signaling pathways can explain up to 70% of the induced gene regulation (De Vos *et al*., 2005). Taking the outcome of the MTMM analyses to investigate the involvement of identified candidate genes in the resistance of plants to several stresses, not only in Arabidopsis but also in related crop species such as e.g. *Brassica* species will be valuable in the breeding by design of future crops to protect them against combinations of stresses, including biotic and abiotic stresses. This will be of great value for next generation crops.

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Supplementary Information (See Appendix)

Table S1: Data overview on phenotyping the 350 *Arabidopsis thaliana* accessions of the HapMap collection.

Trait	Section[a]	Min.	Mean	Max.	Variance	h^2 [b]	L 95% CI h^2	R 95% CI h^2	NA[c]
Salt_1	2.1	-3.62	0.00	3.06	1.31	0.60	0.22	0.89	328
Salt_2	2.1	-1.34	0.00	1.29	0.20	0.43	0.15	0.77	323
Salt_3	2.1	-7.70	-0.01	9.73	8.91	0.64	0.27	0.89	323
Salt_4	2.1	-0.14	0.00	0.09	0.00	0.30	0.08	0.68	322
Fungus	2.10	-0.58	0.01	0.58	0.05	0.40	0.13	0.74	336
Drought&fungus	2.10	-0.62	0.01	0.50	0.05	0.31	0.08	0.68	336
Caterpillar&fungus	2.10	-0.72	0.00	0.45	0.04	0.17	0.03	0.55	336
Heat	2.2	-6.98	0.00	5.44	6.31	0.62	0.25	0.89	275
Osmotic	2.2	-4.35	0.00	10.69	3.62	0.10	0.004	0.75	346
Drought_1	2.2	-7.05	0.00	4.64	2.69	0.39	0.12	0.75	323
Salt_5	2.2	-4.47	0.00	6.37	2.38	0.15	0.01	0.76	334
Whitefly_1	2.5	-1.60	-1.31	-0.13	0.09	0.01	0.00	1.00	339
Whitefly_2	2.5	-0.71	-0.24	1.29	0.05	0.01	0.00	1.00	339
Aphid_1	2.6	-5.11	0.00	4.14	2.38	0.10	0.004	0.76	341
Aphid_2	2.6	-4.74	0.00	4.73	1.94	0.36	0.08	0.79	341
Aphid_3	2.6	-46.28	-27.97	-13.12	31.63	0.19	0.03	0.66	337
Thrips_1	2.7	-56.51	-22.29	-2.95	97.60	0.80	0.37	0.96	346
Thrips_2	2.7	0.14	0.49	0.83	0.01	0.14	0.01	0.66	347
Thrips_3	2.7	0.03	0.47	1.00	0.03	0.29	0.06	0.73	436
Caterpillar_1	2.8	-4.75	0.00	6.60	2.53	0.15	0.01	0.78	328
Caterpillar&osmotic_1	2.8	-3.36	0.00	6.71	2.37	0.08	0.003	0.72	326
Caterpillar&osmotic_2	2.8	-0.15	0.00	0.45	0.01	0.08	0.002	0.82	324
Drought_2	2.9	-0.12	0.00	0.21	0.00	0.06	0.002	0.66	346
Caterpillar_2	2.9	-0.15	0.00	0.16	0.00	0.23	0.04	0.68	346
Fungus&caterpillar_1	2.9	-0.16	0.00	0.19	0.00	0.20	0.03	0.64	346
Caterpillar_3	2.9	-3.92	0.00	3.69	1.43	0.27	0.06	0.69	346
Drought&caterpillar	2.9	-5.07	0.00	3.99	1.45	0.28	0.07	0.67	346
Fungus&caterpillar_2	2.9	-4.06	0.00	3.99	1.30	0.10	0.005	0.72	346
Nematode	2.3	-0.50	-0.30	-0.15	0.00	0.72	0.35	0.93	313
Parasitic_plant	2.4	-1.65	0.01	3.23	0.56	0.03	0.00	1.00	238

[a] Section in Supplementary methods where additional information on phenotyping can be found

[b] Narrow sense heritability estimated using the 'heritability' R package

[c] Number of accessions included in the analyses

Table S2. Summed effect sizes of 30 most significant SNPs in MTMM per trait

Trait	Stress	Summed absolute effect size
Caterpillar_2	Single	3.42
Drought_1	Single	3.59
Caterpillar_1	Single	3.81
Aphid_2	Single	3.99
Salt_1	Single	4.13
Drought_2	Single	4.25
Whitefly_2	Single	4.29
Heat	Single	4.37
Thrips_3	Single	4.42
Whitefly_1	Single	4.51
Aphid_1	Single	4.54
Fungus and Caterpillar_1	Double	4.67
Salt_5	Single	4.99
Nematode	Single	5.09
Parasitic plant	Single	5.11
Salt_2	Single	5.11
Thrips_2	Single	5.19
Fungus and Caterpillar_2	Double	5.21
Osmotic	Single	5.30
Aphid_3	Single	5.33
Caterpillar_3	Single	5.44
Caterpillar and osmotic_2	Double	5.69
Thrips_1	Single	6.03
Salt_4	Single	6.06
Caterpillar and osmotic_1	Double	6.17
Salt_3	Single	6.77
Drought and Caterpillar	Double	7.42
Drought and fungus	Double	10.06
Fungus	Single	10.09
Caterpillar and fungus	Double	11.93

Table S3: 125 candidate genes derived from the Multitrait Mixed Model analysis. Stress-responsive genes are highlighted in yellow.

Significant SNP or gene in LD	Associated marker	Gene	Gene name	Gene description
Significant SNP	Ch1: 25500708	AT1G68030		RING/FYVE/PHD zinc finger superfamily protein
Significant SNP	Ch1: 26798534	AT1G71040	Low Phosphate Root2 (*LPR2*)	Encodes LPR2. Function together with LPR1 (AT1G23010) and a P5-type ATPase (At5g23630/PDR2) in a common pathway that adjusts root meristem activity to inorganic phosphate availability
Significant SNP	Ch1: 29518622	AT1G78460		SOUL heme-binding family protein
Significant SNP	Ch1: 3294935	AT1G10090		Early-responsive to dehydration stress protein (ERD4)
Significant SNP	Ch1: 7207918	AT1G20750		RAD3-like DNA-binding helicase protein
Significant SNP	Ch2: 11531255	AT2G27020	20S proteasome alpha subunit G1 (*PAG1*)	Encodes 20S proteasome alpha 7 subunit PAG1
Significant SNP	Ch2: 11659416	AT2G27240		Aluminium-activated malate transporter family protein
Significant SNP	Ch2: 391904	AT2G01880		Purple acid phosphatase 7 (PAP7)
Significant SNP	Ch3: 1077306	AT3G04110	glutamate receptor 1.1 (*GLR1.1*)	Putative glutamate receptor (GLR1.1). Contains a functional cation - permeable pore domain. Involved in cellular cation homeostasis.
Significant SNP	Ch3: 18615891	AT3G50210		2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Significant SNP	Ch3: 19804402	AT3G53420	plasma membrane intrinsic protein 2A (*PIP2A*)	Member of the plasma membrane intrinsic protein subfamily PIP2. Localizes to the plasma membrane and exhibits water transport activity in *Xenopus* oocyte. Expressed specifically in the vascular bundles and protein level increases slightly during leaf development. When expressed in yeast cells can conduct hydrogen peroxide into those cells
Significant SNP	Ch3: 21625003	AT3G58460		RHOMBOID-like protein 15 (RBL15)
Significant SNP	Ch3: 2231603	AT3G07050		GTP-binding family protein
Significant SNP	Ch3: 6968031	AT3G20000	translocase of the outer mitochondrial membrane 40 (*TOM40*)	Encodes a component of the TOM receptor complex responsible for the recognition and translocation of cytosolically synthesized mitochondrial preproteins. With TOM22, functions as the transit peptide receptor at the surface of the mitochondrial outer membrane and facilitates the movement of preproteins into the translocation pore.

Significant SNP	Ch3: 8014458	AT3G22640		PAP85
Significant SNP	Ch4: 5180340	AT4G08200		Similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G43722.1)
Significant SNP	Ch4: 6805259	AT4G11160		Translation initiation factor 2, small GTP-binding protein
Significant SNP	Ch4: 8654778	AT4G15180		SET domain protein 2 (SDG2)
Significant SNP	Ch4: 9350941	AT4G16600		Nucleotide-diphospho-sugar transferases superfamily protein
Significant SNP	Ch4:13265656	AT4G26190		Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
Significant SNP	Ch4:13955847	AT4G28080		Tetratricopeptide repeat (TPR)-like superfamily protein
Significant SNP	Ch4:16420532	AT4G34320		Protein of unknown function (DUF677)
Significant SNP	Ch5: 22041081	AT5G54280	myosin 2 (*ATM2*)	Type VII myosin gene
Significant SNP	Ch5: 22677563	AT5G56000		HEAT SHOCK PROTEIN 81.4 (Hsp81.4)
Significant SNP	Ch5: 22842831	AT5G56390		F-box/RNI-like/FBD-like domains-containing protein
Significant SNP	Ch5: 23302987	AT5G57535		unknown protein
Significant SNP	Ch5: 414050	AT5G02100	Unfertilized embryo sac 18 (*UNE18*)	Encodes a protein that binds to beta-sitosterol and localizes to the ER. The WFDE motif in ORP3a appears to be important for a direct interaction with PVA12 [Plant VAMP-Associated protein 12]. Mutation of this motif causes ORP3a to relocalize to the Golgi and cytosol. The interaction between PVA12 and ORP3a does not appear to be sterol-dependent
Significant SNP	Ch5: 7493620	AT5G22560		Plant protein of unknown function (DUF247)
Significant SNP	Ch5: 7493623	AT5G23480		SWIB/MDM2 domain
Significant SNP	Ch5: 9154579	AT5G26190		Cysteine/Histidine-rich C1 domain family protein
in_LD_with	Ch1: 25500708	AT1G67990	*TSM1*	Encodes a tapetum-specific O-methyltransferase. In vitro enzyme assay indicated activity with caffeoyl-CoA, caffeoyl glucose, chlorogenic acid and polyamine conjugates. RNAi mutants had impaired silique development and seed setting.
in_LD_with	Ch1: 25500708	AT1G68010	hydroxypyruvate reductase (*HPR*)	Encodes hydroxypyruvate reductase.
in_LD_with	Ch1: 25500708	AT1G67980	caffeoyl-CoA 3-O-methyltransferase (*CCOAMT*)	Encodes S-adenosyl-L-methionine: transcaffeoyl Coenzyme A 3-O-methyltransferase.

in_LD_with	Ch1: 25500708	AT1G67960		CONTAINS InterPro DOMAIN/s: Membrane protein,Tapt1/CMV receptor (InterPro:IPR008010)
in_LD_with	Ch1: 25500708	AT1G68000	phosphatidylinositol synthase 1 (*PIS1*)	phosphatidylinositol synthase 1
in_LD_with	Ch1: 25500708	AT1G68020	*ATTPS6*	Encodes an enzyme putatively involved in trehalose biosynthesis. The protein has a trehalose synthase (TPS)-like domain and a trehalose phosphatase (TPP)-like domain. It can complement a yeast mutant lacking both of these activities suggesting that this is a bifunctional enzyme.
in_LD_with	Ch1: 25500708	AT1G67970	heat shock transcription factor A8 (*HSFA8*)	member of Heat Stress Transcription Factor (Hsf) family
in_ID_with	Ch1: 29518622	AT1G78440	*Arabidopsis thaliana* gibberellin 2-oxidase 1 (*ATGA2OX1*)	Encodes a gibberellin 2-oxidase that acts on C19 gibberellins.
in_ID_with	Ch1: 29518622	AT1G78430		ROP interactive partner 2 (RIP2)
in_ID_with	Ch1: 29518622	AT1G78450		SOUL heme-binding family protein
in_ID_with	Ch1: 29518622	AT1G78470		BEST Arabidopsis thaliana protein match is: F-box family protein (TAIR:AT1G67390.1)
in_LD_with	Ch1: 7207918	AT1G20740		Protein of unknown function (DUF833)
in_LD_with	Ch1: 7207918	AT1G20760		Calcium-binding EF hand family protein
in_LD_with	Ch1: 7207918	AT1G20780	senescence-associated E3 ubiquitin ligase 1 (*SAUL1*)	Encodes a protein containing a U-box and an ARM domain.
in_LD_with	Ch1: 7207918	AT1G20790		F-box family protein
in_LD_with	Ch1: 7207918	AT1G20770		Unknown protein

in_LD_with	Ch2: 11659416	AT2G27250	*AtCLV3*	One of the three CLAVATA genes controlling the size of the shoot apical meristem (SAM) in *Arabidopsis*. Belongs to a large gene family called CLE for CLAVATA3/ESR-related. Encodes a stem cell-specific protein CLV3 presumed to be a precursor of a secreted peptide hormone. The deduced ORF encodes a 96-amino acid protein with an 18-amino acid N-terminal signal peptide. The functional form of CLV3 (MCLV3) was first reported to be a posttranscriptionally modified 12-amino acid peptide, in which two of the three prolines were modified to hydroxyproline
in_LD_with	Ch3: 19804402	AT3G53400		BEST Arabidopsis thaliana protein match is: conserved peptide upstream open reading frame 47 (TAIR:AT5G03190.1)
in_LD_with	Ch3: 21625003	AT3G53410		RING/U-box superfamily protein
in_LD_with	Ch3: 21625003	AT3G58490		Phosphatidic acid phosphatase (PAP2) family protein
in_LD_with	Ch3: 21625003	AT3G58450		Adenine nucleotide alpha hydrolases-like superfamily protein
in_LD_with	Ch3: 21625003	AT3G58510		DEA(D/H)-box RNA helicase family protein
in_LD_with	Ch3: 21625003	AT3G58440		TRAF-like superfamily protein
in_LD_with	Ch3: 21625003	AT3G58520		Ubiquitin carboxyl-terminal hydrolase family protein
in_LD_with	Ch3: 21625003	AT3G58480		Calmodulin-binding family protein
in_LD_with	Ch3: 21625003	AT3G58470		Nucleic acid binding
in_LD_with	Ch3: 21625003	AT3G58500		Encodes one of the isoforms of the catalytic subunit of protein phosphatase 2A: AT1G59830/PP2A-1, AT1G10430/PP2A-2, At2g42500/PP2A-3, At3g58500/PP2A-4 [Plant Molecular Biology (1993) 21:475-485 and (1994) 26:523-528
in_LD_with	Ch3: 6968031	AT3G20010		SNF2 domain-containing protein / helicase domain-containing protein / zinc finger protein-related
in_LD_with	Ch3: 6968031	AT3G19990		Unknown protein
in_LD_with	Ch3: 6968031	AT3G19980		Encodes catalytic subunit of serine/threonine protein phosphatase 2A. It can associate with phytochromes A and B in vitro. Mutant plants display an accelerated flowering phenotype.

in_LD_with	Ch3: 8014458	AT3G22670		Pentatricopeptide repeat (PPR) superfamily protein
in_LD_with	Ch3: 8014458	AT3G22680	RNA-directed DNA methylation 1 (RDM1)	Encodes RNA-DIRECTED DNA METHYLATION 1 (RDM1), forming a complex with DMS3 (AT3G49250) and DRD1 (AT2G16390). This complex is termed DDR. The DDR complex is required for polymerase V transcripts and RNA-directed DNA methylation.
in_LD_with	Ch3: 8014458	AT3G22650		CEGENDUO (CEG)
in_LD_with	Ch3: 8014458	AT3G22690		Involved in: photosystem II assembly, regulation of chlorophyll biosynthetic process, photosystem I assembly, thylakoid membrane organization, RNA modification
in_LD_with	Ch3: 8014458	AT3G22700		F-box and associated interaction domains-containing protein
in_LD_with	Ch3: 8014458	AT3G22710		F-box family protein
in_LD_with	Ch3: 8014458	AT3G22720		F-box and associated interaction domains-containing protein
in_LD_with	Ch3: 8014458	AT3G22730		F-box and associated interaction domains-containing protein
in_LD_with	Ch3: 8014458	AT3G22740	homocysteine S-methyltransferase 3 (*HMT3*)	Homocysteine S-methyltransferase (HMT3)
in_LD_with	Ch3: 8014458	AT3G22750		Protein kinase superfamily protein
in_LD_with	Ch3: 8014458	AT3G22760	*SOL1*	CXC domain containing TSO1-like protein 1. The gene is expressed in stamens, pollen mother cells, and immature ovules.
in_LD_with	Ch4: 5180340	AT4G08190		P-loop containing nucleoside triphosphate hydrolases superfamily protein
in_LD_with	Ch4: 5180340	AT4G08180		OSBP(oxysterol binding protein)-related protein 1C (ORP1C)
in_LD_with	Ch4: 5180340	AT4G08230		Glycine-rich protein
in_LD_with	Ch4: 5180340	AT4G08210		Pentatricopeptide repeat (PPR-like) superfamily protein
in_LD_with	Ch4: 5180340	AT4G08220		Mutator-like transposase family, has a $5.3*10^{-67}$ P-value blast match to Q9SUF8 /145-308 Pfam PF03108 MuDR family transposase (MuDr-element domain)
in_LD_with	Ch4: 6805259	AT4G11140	cytokinin response factor 1 (*CRF1*)	Encodes a member of the ERF (ethylene response factor) subfamily B-5 of the ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily. Also named as CRF1 (cytokinin response factor 1).

in_LD_with	Ch4: 6805259	AT4G11150	vacuolar ATP synthase subunit E1 (*TUF*)	Encodes a vacuolar H^+-ATPase subunit E isoform 1 which is required for Golgi organization and vacuole function in embryogenesis.
in_LD_with	Ch4: 6805259	AT4G11170	*RMG1*	Disease resistance protein (TIR-NB-LRR class) family
in_LD_with	Ch4: 8654778	AT4G15210	*Arabidopsis thaliana* BETA-AMYLASE (*ATBETA-AMY*)	Cytosolic beta-amylase expressed in rosette leaves and inducible by sugar. RAM1 mutants have reduced beta amylase in leaves and stems.
in_LD_with	Ch4:13265656	AT4G26180		Mitochondrial substrate carrier family protein
in_LD_with	Ch4:13265656	AT4G26150	cytokinin-responsive gata factor 1 (*CGA1*)	Encodes a member of the GATA factor family of zinc finger transcription factors.
in_LD_with	Ch4:13265656	AT4G26170		Molecular_function unknown
in_LD_with	Ch4:13265656	AT4G26220		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
in_LD_with	Ch4:13265656	AT4G26140	beta-galactosidase 12 (BGAL12)	Putative beta-galactosidase
in_LD_with	Ch4:13265656	AT4G26160	atypical CYS HIS rich thioredoxin 1 (*ACHT1*)	Encodes a member of the thioredoxin family protein. Located in the chloroplast. Shows high activity towards the chloroplast 2-Cys peroxiredoxin A, and poor activity towards the chloroplast NADP-malate dehydrogenase
in_LD_with	Ch4:13265656	AT4G26210		Mitochondrial ATP synthase subunit G protein
in_LD_with	Ch4:13955847	AT4G26200	1-amino-cyclopropane-1-carboxylate synthase 7 (*ACS7*)	Member of a family of proteins in *Arabidopsis* that encode 1-Amino-cyclopropane-1-carboxylate synthase, an enzyme involved in ethylene biosynthesis. Not expressed in response to IAA
in_LD_with	Ch4:13955847	AT4G28100		Unknown protein
in_LD_with	Ch4:13955847	AT4G28060		Cytochrome c oxidase, subunit Vib family protein
in_LD_with	Ch4:13955847	AT4G28070		AFG1-like ATPase family protein
in_LD_with	Ch4:13955847	AT4G28090		SKU5 similar 10 (sks10)
in_LD_with	Ch4:13955847	AT4G28085		Unknown protein
in_LD_with	Ch4:13955847	AT4G28088		Low temperature and salt responsive protein family
in_LD_with	Ch4:13955847	AT4G34310		alpha/beta-Hydrolases superfamily protein

in_LD_with	Ch5: 22041081	AT5G54250	cyclic nucleotide-gated cation channel 4 (*CNGC4*)	Member of Cyclic nucleotide gated channel family, downstream component of the signaling pathways leading to HR resistance. Mutant plants exhibit gene-for-gene disease resistance against avirulent *Pseudomonas syringae* despite the near-complete absence of the hypersensitive response (HR). Salicylic acid accumulation in dnd2 mutants is completely PAD4-independent.
in_LD_with	Ch5: 22041081	AT5G54260	Meiotic recombination 11 (*MRE11*)	DNA repair and meiotic recombination protein, component of MRE11 complex with RAD50 and NBS1
in_LD_with	Ch5: 22041081	AT5G54270	light-harvesting chlorophyll B-binding protein 3 (*LHCB3*)	Lhcb3 protein is a component of the main light harvesting chlorophyll a/b-protein complex of Photosystem II (LHC II).
in_LD_with	Ch5: 22041081	AT5G54240		Protein of unknown function (DUF1223)
in_LD_with	Ch5: 22677563	AT5G55990	calcineurin B-like protein 2 (*CBL2*)	Encodes a member of the *Arabidopsis* CBL (Calcineurin B-like Calcium Sensor) protein family
in_LD_with	Ch5: 22677563	AT5G55980		Serine-rich protein-related
in_LD_with	Ch5: 22677563	AT5G55970		RING/U-box superfamily protein
in_LD_with	Ch5: 22842831	AT5G56380		F-box/RNI-like/FBD-like domains-containing protein
in_LD_with	Ch5: 22842831	AT5G56370		F-box/RNI-like/FBD-like domains-containing protein
in_LD_with	Ch5: 22842831	AT5G56368		Encodes a defensin-like (DEFL) family protein.
in_LD_with	Ch5: 23302987	AT5G57520	zinc finger protein 2 (*ZFP2*)	Encodes a zinc finger protein containing only a single zinc finger.
in_LD_with	Ch5: 23302987	AT5G57560	Touch 4 (*TCH4*)	Encodes a cell wall-modifying enzyme, rapidly upregulated in response to environmental stimuli
in_LD_with	Ch5: 23302987	AT5G57490	voltage dependent anion channel 4 (*VDAC4*)	Encodes a voltage-dependent anion channel (VDAC: AT3G01280/VDAC1, AT5G67500/VDAC2, AT5G15090/VDAC3, AT5G57490/VDAC4, AT5G15090/VDAC5). VDACs are reported to be porin-type, beta-barrel diffusion pores. They are prominently localized in the outer mitochondrial membrane and are involved in metabolite exchange between the organelle and the cytosol.

in_LD_with	Ch5: 23302987	AT5G57565		Protein kinase superfamily protein
in_LD_with	Ch5: 23302987	AT5G57540		Encodes a xyloglucan endotransglucosylase/hydrolase with only only the endotransglucosylase (XET
in_LD_with	Ch5: 23302987	AT5G57550	xyloglucan endotransglucosylase/ hydrolase 25 (*XTH25*)	Xyloglucan endotransglycosylase-related protein (XTR3)
in_LD_with	Ch5: 23302987	AT5G57500		Galactosyltransferase family protein
in_LD_with	Ch5: 23302987	AT5G57530		Xyloglucan endotransglucosylase/hydrolase 12 (XTH12)
in_LD_with	Ch5: 23302987	AT5G57510		Unknown protein
in_LD_with	Ch5: 23302987	AT5G57570		GCK domain-containing protein
in_LD_with	Ch5: 23302987	AT5G57590	biotin auxotroph 1 (*BIO1*)	Mutant complemented by E coli Bio A gene encoding 7,8-diaminopelargonic acid aminotransferase.
in_LD_with	Ch5: 23302987	AT5G57580		Calmodulin-binding protein
in_LD_with	Ch5: 414050	AT5G02110		CYCLIN D7
in_LD_with	Ch5: 7493620	AT5G22550		Plant protein of unknown function (DUF247)
in_LD_with	Ch5: 7493620	AT5G22570	*WRKY38*	member of WRKY Transcription Factor
in_LD_with	Ch5: 7493620	AT5G22545		Unknown protein
in_LD_with	Ch5: 7493620	AT5G22555		Unknown protein
in_LD_with	Ch5: 7493623	AT5G23510		Unknown protein
in_LD_with	Ch5: 7493623	AT5G23490		Unknown protein

Table S4. Genes in linkage with SNPs with $-\log_{10}$ (*P*) score above 4 (20 kb half-window size) in the contrast-specific GWA mapping of parasitic plants and aphids on the one hand versus fungus, caterpillar, thrips and drought on the other hand.

Marker	Gene in LD	Gene name	Gene description	Reference
chr1.19711816	*AT1G52900*	-	Toll-Interleukin-Resistance (TIR) domain family protein, signal transduction, defense response	(Cartieaux *et al.*, 2008)
chr1.24785939	*AT1G66410*	*CAM4*	Calmodulin 4, calcium-binding EF-hand site, calcium-mediated signalling	(Zhao *et al.*, 2013)
chr3.672138	*AT3G02940*	*MYB107*	Transcription factor, responsive to salicylic acid	(Stracke *et al.*, 2001)
chr3.7945317	*AT3G22400*	*LOX5*	Oxidoreductase activity (9-LOX pathway), facilitates *M. persicae* aphid feeding	(Nalam *et al.*, 2012a; Nalam *et al.*, 2012b)
chr3.23145919	*AT3G62610*	*MYB11*	Transcription factor, involved in production of flavonol glycosides	(Stracke *et al.*, 2007)
chr4.9390514	*AT4G16730, AT4G16740,*	*TPS02, TPS03*	Terpene synthases, (*E,E*)-alpha-farnesene synthase	(Chuang *et al.*, 2010)
	AT4G16690	*MES16*	Methyl jasmonate esterase	(Christ *et al.*, 2012)
chr5.22829754	*AT5G56360*	*PSL4*	Calmodulin binding protein, involved in MAMP-triggered defense to bacteria	(Lu *et al.*, 2009)

Table S5. Candidate genes in linkage with SNPs with $-\log_{10}$(P) score above 4 (20 kb half-window size) that have common effects on plant response to parasitic plants and aphids on the one hand versus fungus, caterpillar, thrips and drought on the other hand.

Marker	Gene	Gene name	Description
chr2.15762021	*AT2G37570*	*SLT1*	Encodes a protein that can complement the salt-sensitive phenotype of a calcineurin (CaN)-deficient yeast mutant.
in_LD_with_ chr2.15762021	*AT2G37630*	*MYB91*	Encodes a MYB-domain protein involved in specification of the leaf proximodistal axis. Also functions as a regulator of the plant immune response.
in_LD_with_ chr3.22345759	*AT3G60490*	-	Encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family. Pathogenesis-related.
chr4.9598560	*AT4G17070*	-	Encodes a peptidyl-prolyl cis-trans isomerase. Involved in response to oxidative stress.

Table S6. Candidate genes in linkage with SNPs with $-\log_{10}$(P) score above 4 (20 kb half-window size) that have common effects on biotic and abiotic stress responses

Marker	Gene	Gene name	Description
in_LD_with_ chr4.5651749	*AT4G08870*	*ARGAH2*	Encodes one of the two arginases in the genome. Gene expression is enhanced by methyl jasmonate treatment. It is involved in the defence response to *B. cinerea*.
chr4. 8057710	*AT4G13940*	*AtSAHH1*	Encodes an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing.
chr2.856085	*AT2G02950*	*PKS1*	Encodes a basic soluble protein which can independently bind to either PHYA or PHYB, regardless of whether the phytochromes are in the Pr or Pfr state. PKS1 can be phosphorylated by oat phyA *in vitro* in a light-regulated manner. It is postulated to be a negative regulator of phyB signalling.

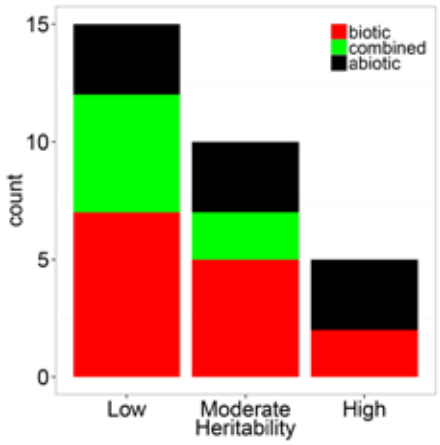


Figure S1. Narrow sense heritability for *Arabidopsis thaliana* resistance to abiotic and biotic stresses. Narrow sense heritability values were estimated using the 'heritability' R package. Traits were classified in three biological categories: resistance to abiotic, biotic and double stresses. These biological categories were grouped based on their heritability in low (h^2<0.2), moderate (0.2<h^2<0.5) and high (h^2>0.5) heritability classes.

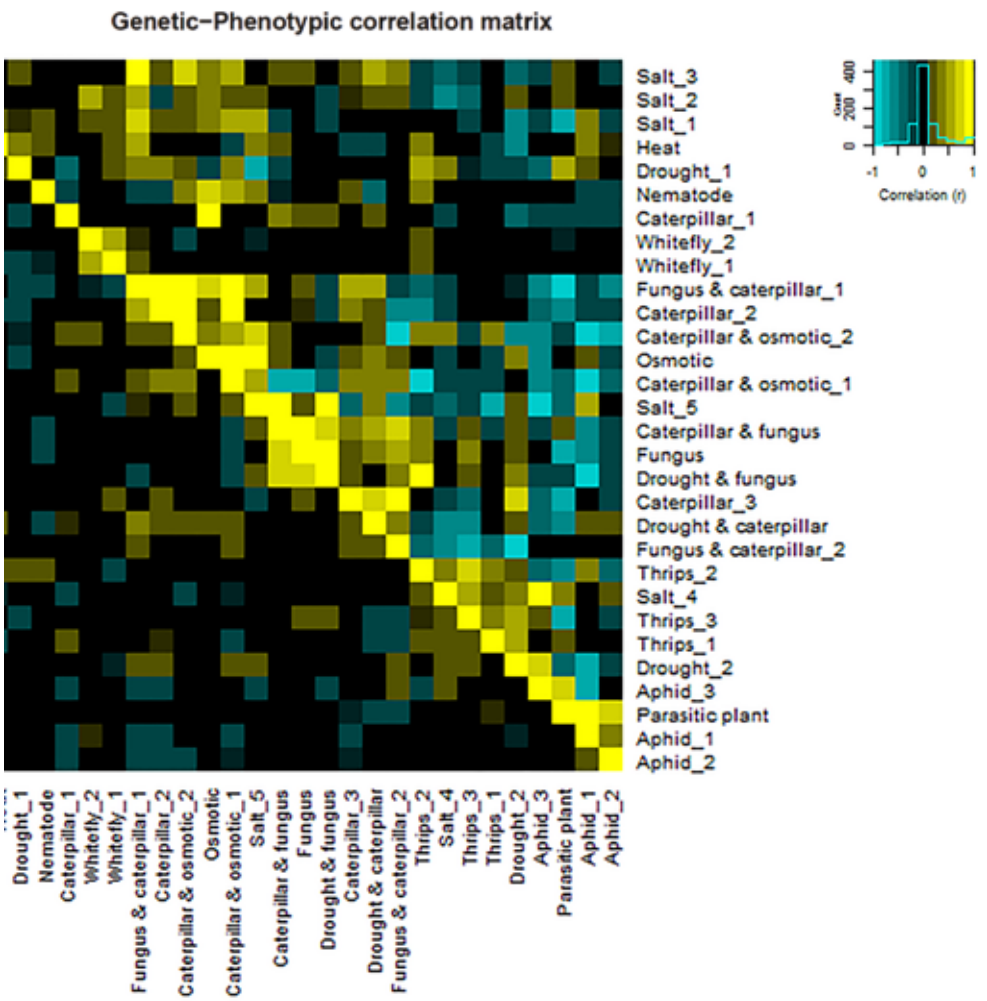
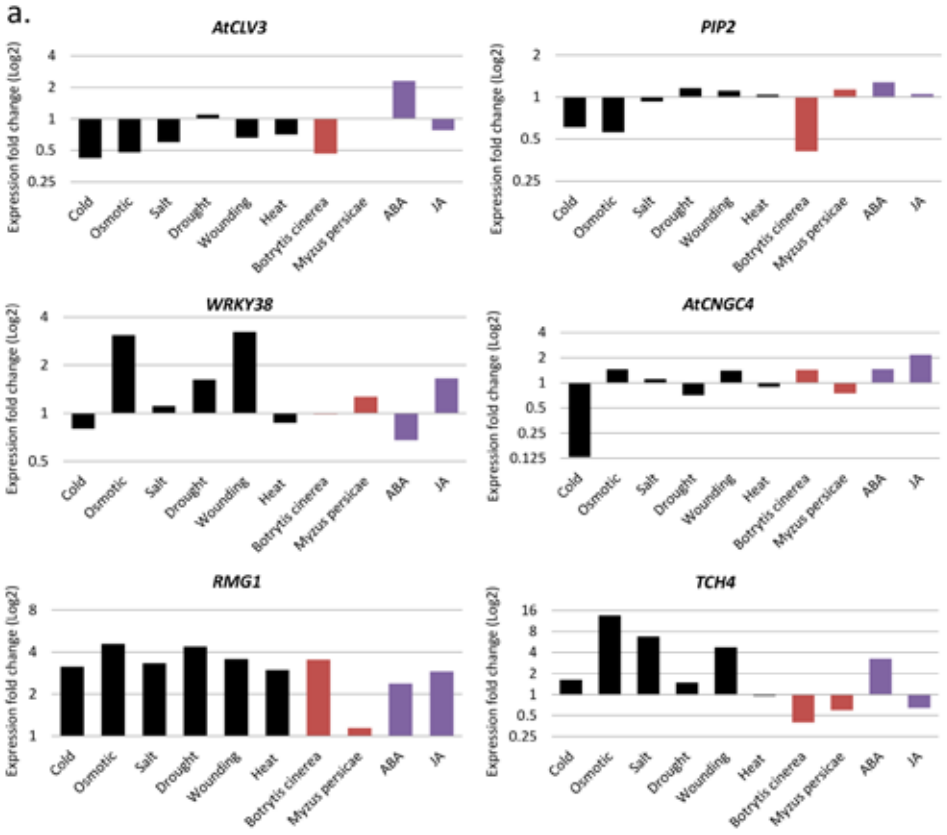


Figure S2. Genetic and phenotypic correlation matrix. Heatmap displaying phenotypic correlations below the diagonal and genetic correlations above the diagonal. Phenotypic correlations were calcluated using Spearman's correlation coefficient *rho*, whereas the genome-wide genetic correlations were estimated bivariately and with correction for population structure (on full kinship matrix). For Whitefly_1 and Whitefly_2 the maximum likelihood estimates were not available so genetic correlations were estimated using G-BLUP. Traits were clustered according to Ward's minimum variance method for the genetic correlation coefficient values.


a.
AtCLV3
PIP2
WRKY38
AtCNGC4
RMG1
TCH4
Expression fold change (Log2)
Cold
Osmotic
Salt
Drought
Wounding
Heat
Botrytis cinerea
Myzus persicae
ABA
JA

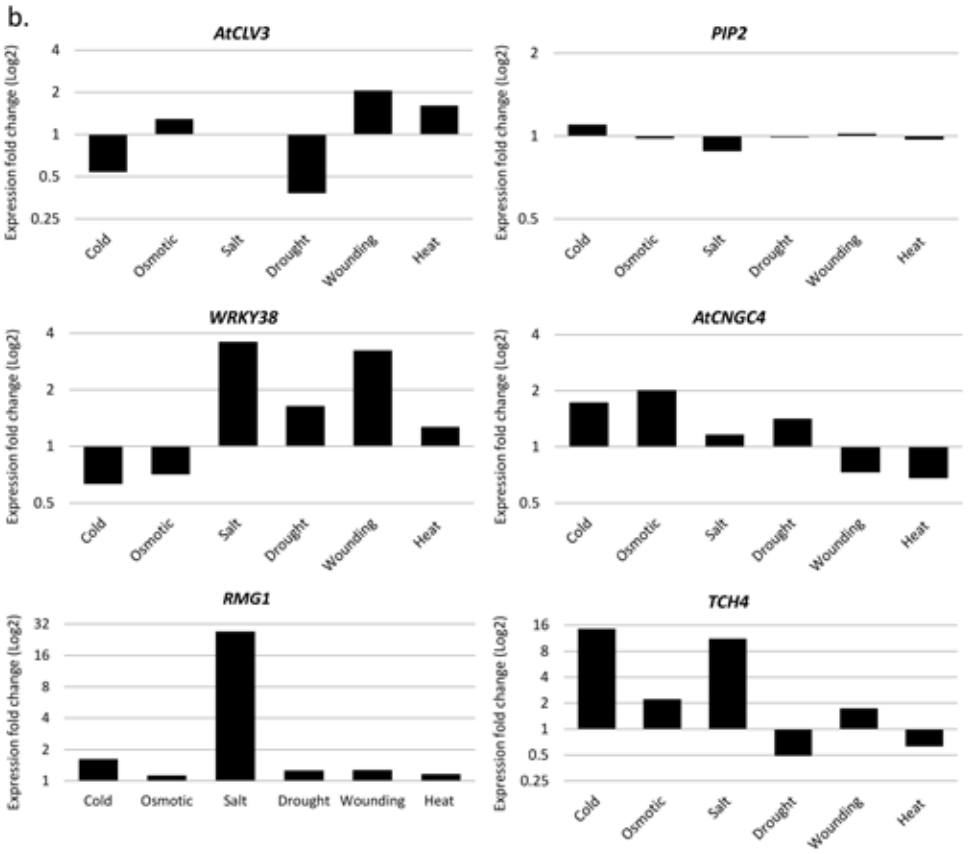


Figure S3. Expression data of 6 candidate genes (resulting from MTMM, see Table 2a) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

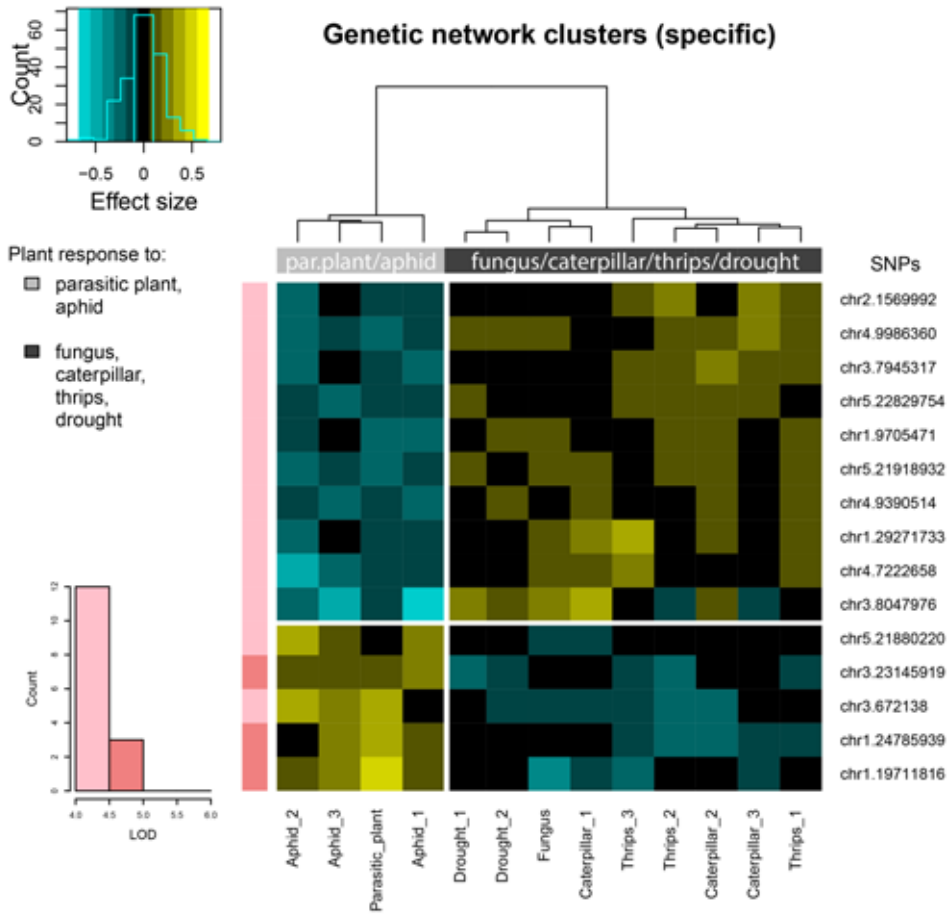


Figure S4. Genetic associations specific for plant responses to the main clusters of the genetic correlation network (see Figure 1): parasitic plant and aphid versus fungus, caterpillar, thrips and drought. Genetic associations were estimated with a contrast-specific analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the contrast are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 10 SNPs are associated with enhanced resistance to **fungus, caterpillar, thrips and drought** stresses and reduced resistance to stresses inflicted by **parasitic plants and aphids**; the bottom 5 SNPs show the inverse. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

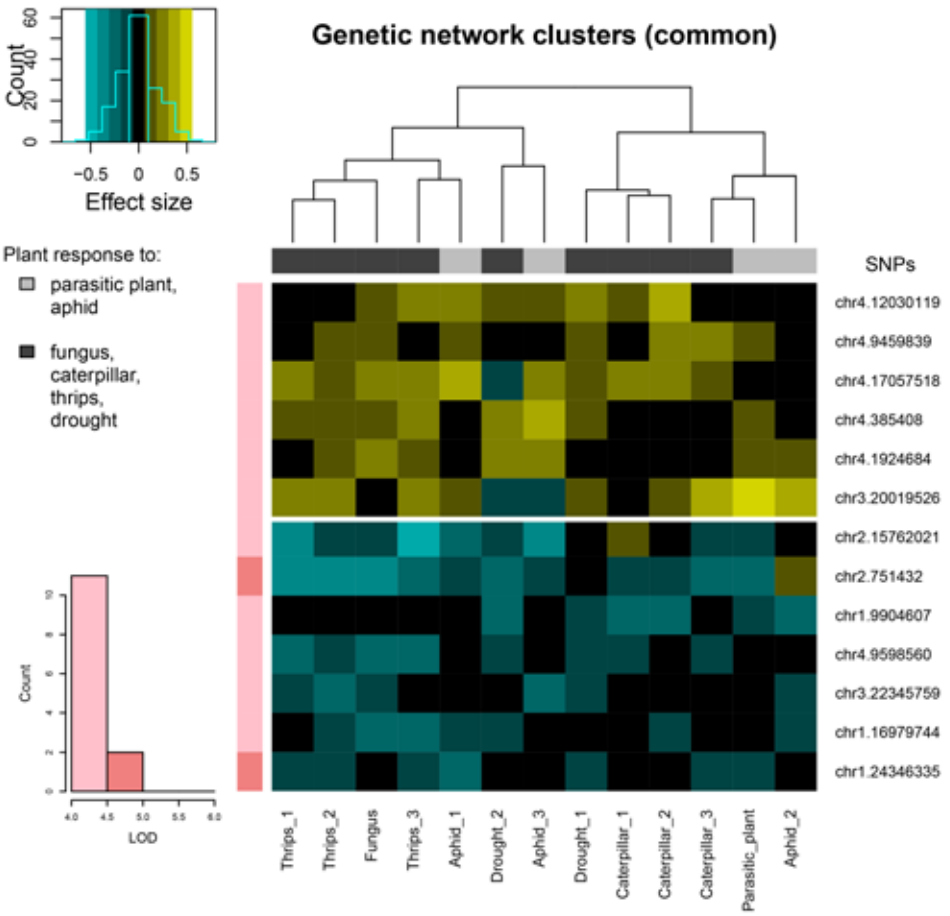
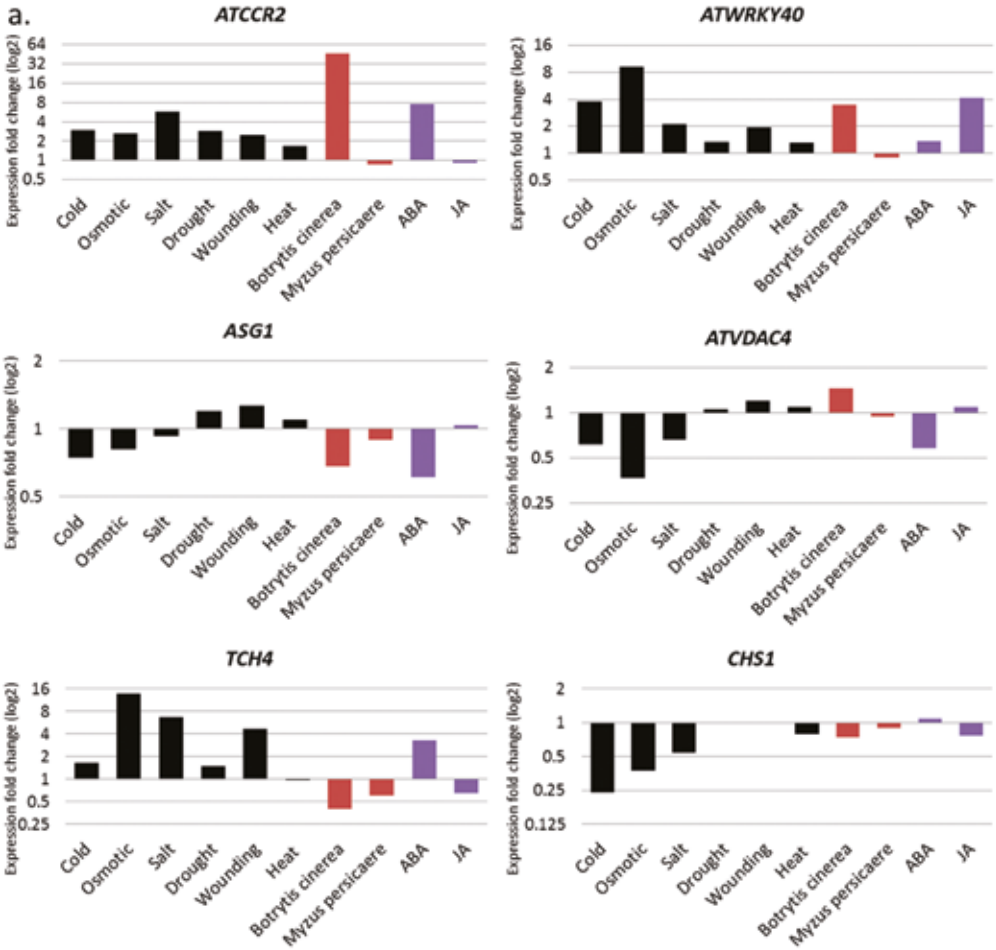


Figure S5. Genetic associations common for plant response to the main clusters of the genetic correlation network: parasitic plant and aphid on the one hand versus fungus, caterpillar, thrips and drought on the other hand. Genetic associations were estimated with a contrast analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 6 SNPs are associated with enhanced resistance to abiotic stresses and reduced resistance to biotic stresses; the bottom 7 SNPs show the inverse. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.


a.
ATCCR2
ATWRKY40
ASG1
ATVDAC4
TCH4
CHS1
Expression fold change (log2)
Cold
Osmotic
Salt
Drought
Wounding
Heat
Botrytis cinerea
Myzus persicaere
ABA
JA

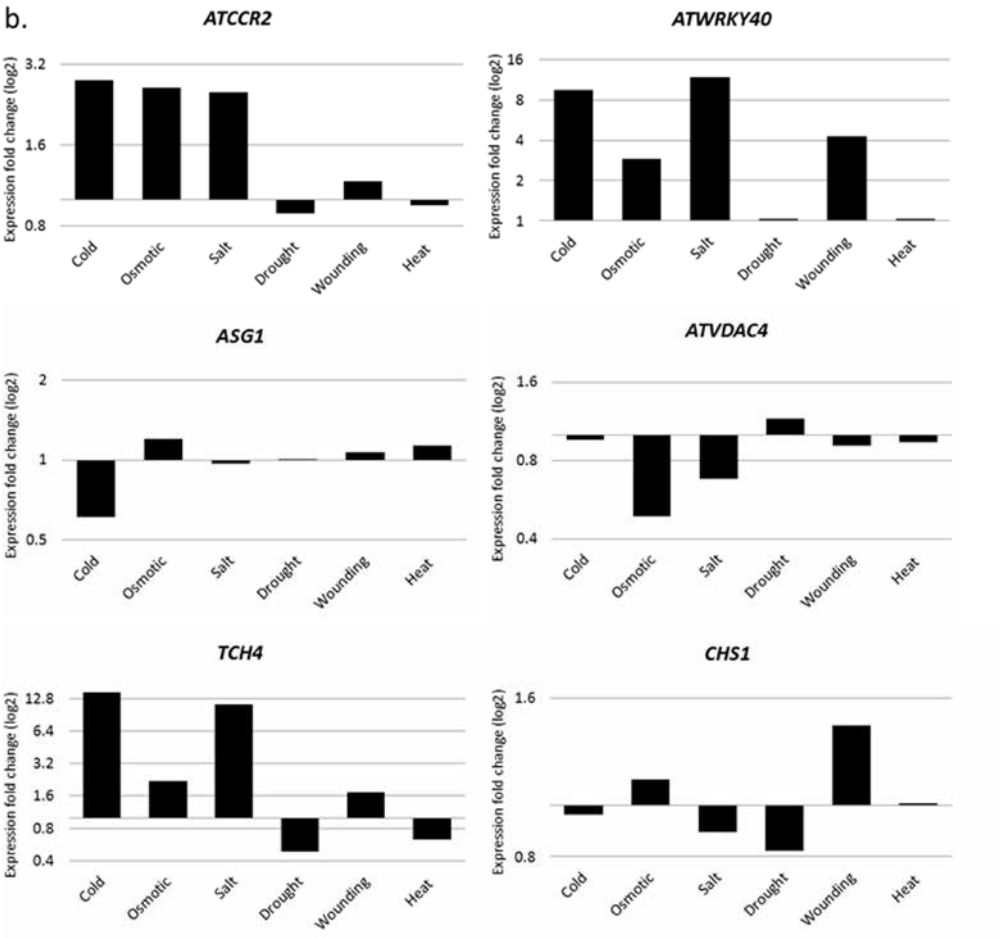


Figure S6. **Expression data of 6 candidate genes (resulting from MTMM analysis, see Table 2b) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues.** Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

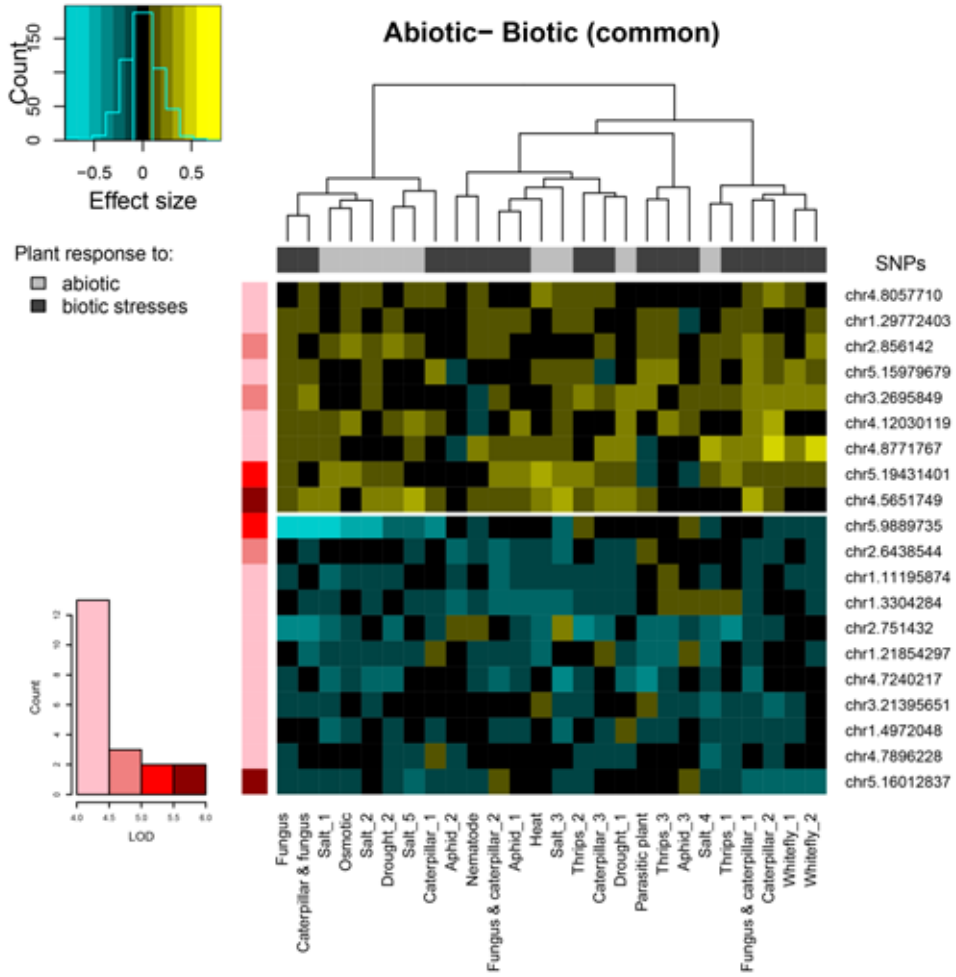


Figure S7. Genetic associations common for plant responses to abiotic and biotic stresses. Genetic associations were estimated with a contrast analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 9 SNPs are associated with enhanced resistance to abiotic and biotic stresses; the bottom 11 SNPs are associated with reduced resistance to abiotic and biotic stresses. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

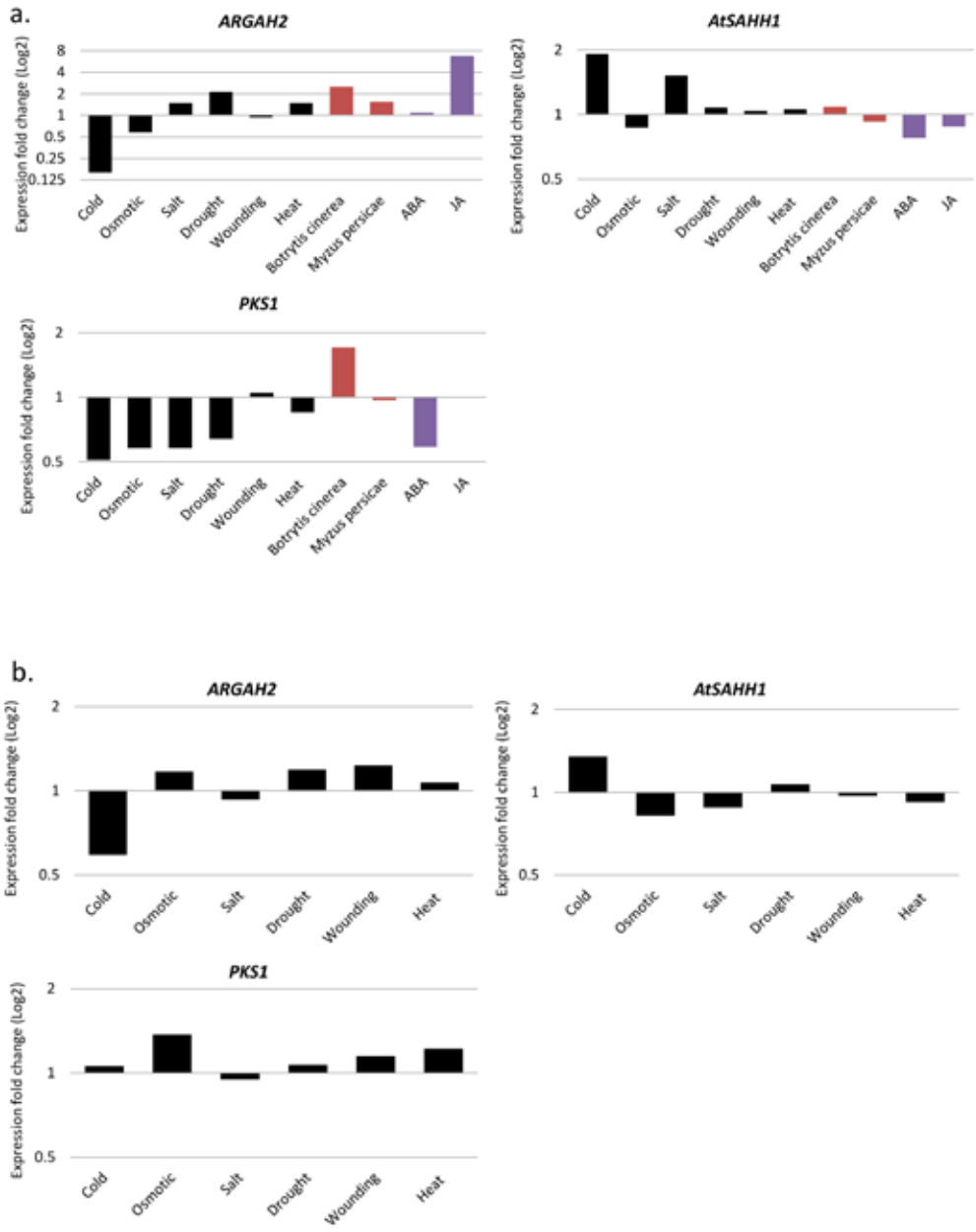


Figure S8. Expression data of 3 candidate genes (resulting from MTMM, see Supplementary Table S5) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

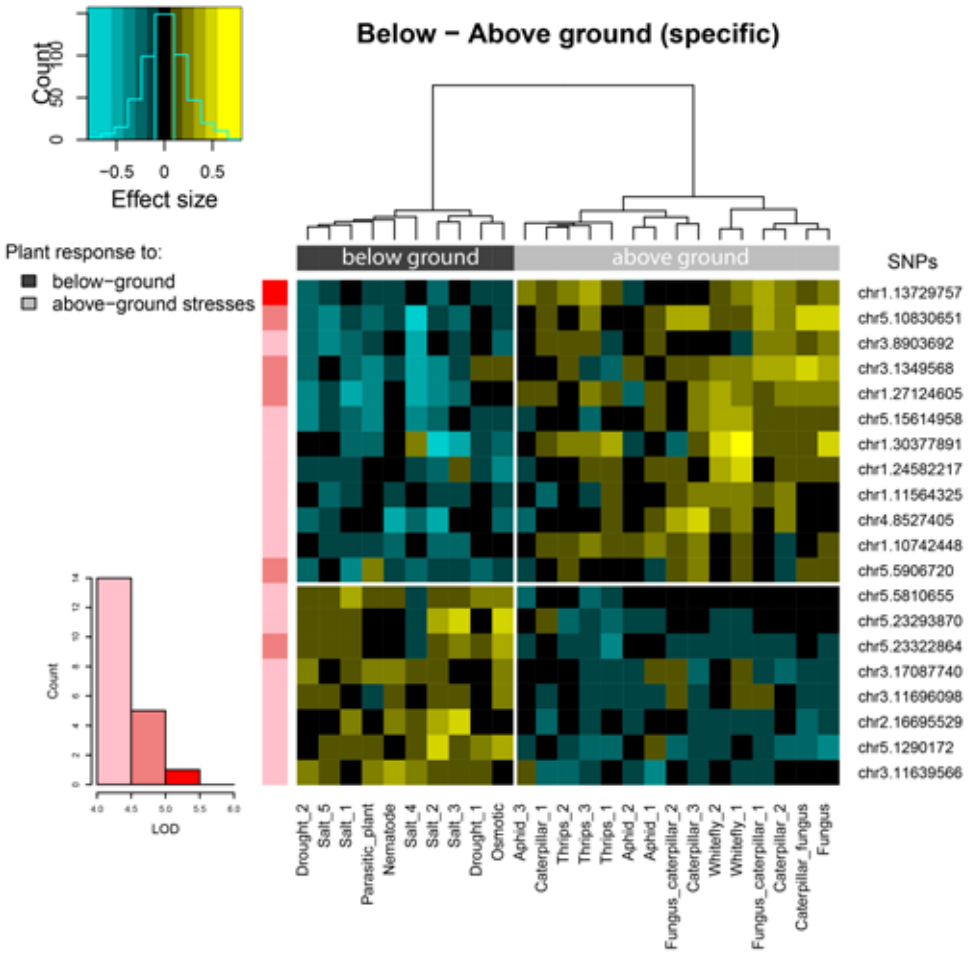


Figure S9. **Genetic associations specific for plant responses to either below- or aboveground stress.** Genetic associations were estimated with a contrast analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the belowground-aboveground contrast are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 12 SNPs are associated with enhanced resistance to aboveground stresses and reduced resistance to belowground stresses; the bottom 8 SNPs show the inverse. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

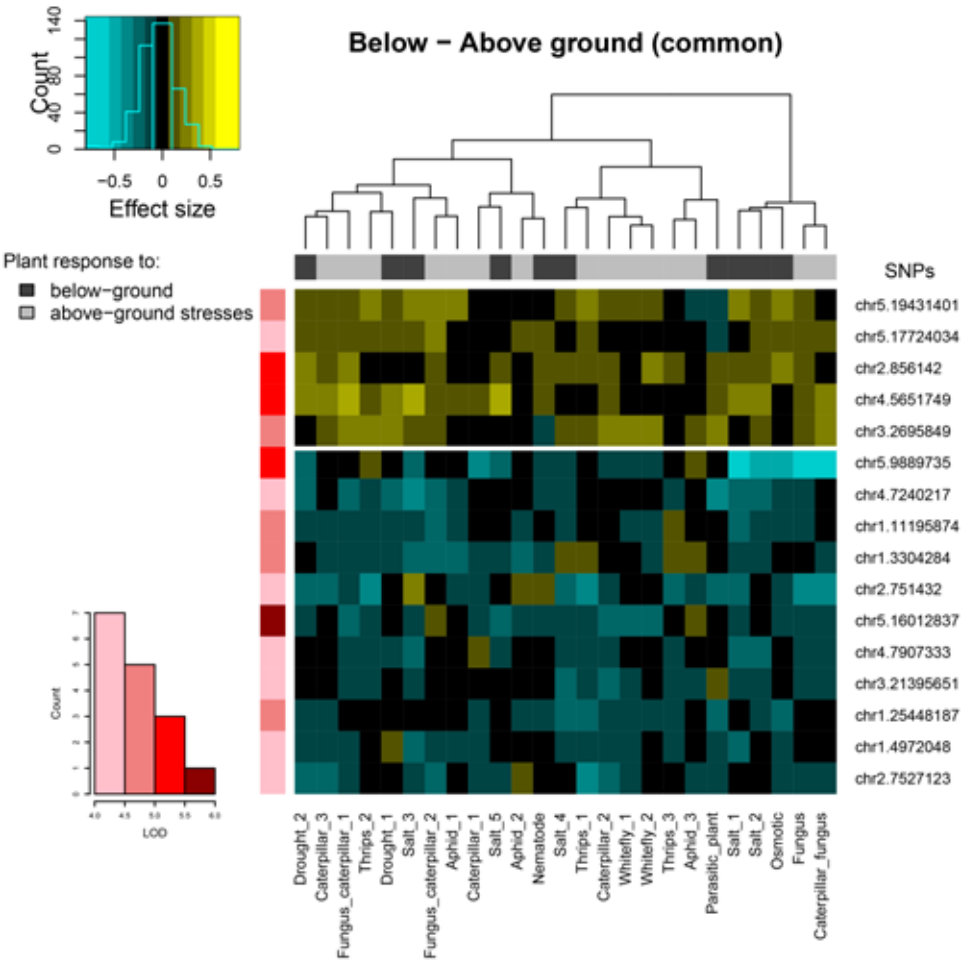


Figure S10. Genetic associations common for plant responses to below- and aboveground stresses. Genetic associations were estimated with a contrast analysis using MTMM. SNPs with a significance above LOD score 4 ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effect size estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 5 SNPs are associated with enhanced resistance to above- and belowground stresses; the bottom 11 SNPs are associated with reduced resistance to above- and belowground stresses. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

Chapter 7

General discussion

Nelson H. Davila Olivas

Introduction

Ecogenomics is defined as a multidisciplinary approach to addressing ecological and evolutionary questions, by making use of the powerful high-throughput tools developed by molecular biologists (Dicke *et al.*, 2004; Kant & Baldwin, 2007; Anderson & Mitchell-Olds, 2011). Techniques used in ecogenomics include (a) QTL mapping and Genome Wide Association mapping, (b) transcriptome, metabolome and proteome profiling, and (c) population genomics and the use of transgenic methodologies. These techniques can be applied independently, or jointly to investigate a particular phenomenon (Anderson & Mitchell-Olds, 2011), such as transcriptome changes elicited by generalist and specialist insects, insects of different feeding guilds, and combined stresses (Voelckel & Baldwin, 2004; Bidart-Bouzat & Kliebenstein, 2011; Appel *et al.*, 2014). Ecogenomics has also been applied to dissect the genetic architecture of defence and developmental traits (Prasad *et al.*, 2012; Zust *et al.*, 2012; Dittmar *et al.*, 2014).

Plants encounter in their habitat a diversity of perturbations that interfere with plant development and have an adverse effect on plant fitness (Buchanan *et al.*, 2000; Stam *et al.*, 2014). These perturbations can be caused by other organisms such as insects, bacteria, viruses, nematodes, fungi, and neighbouring plants, so-called biotic stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Stam *et al.*, 2014). On the other hand, external conditions such as drought, flooding, extreme temperatures, nutrient deficiency or surplus, so-called abiotic stresses, can also lead to perturbations (Fahad *et al.*, 2015; Mickelbart *et al.*, 2015). Biotic and abiotic stresses trigger changes in the plant at different levels of biological organization such as gene expression, metabolite production, protein biosynthesis, growth rate, reproduction and yield (Fu *et al.*, 2009; Keurentjes *et al.*, 2011; Stam *et al.*, 2014). Some of these changes enable plants to acclimate to abiotic stresses or actively defend themselves against biotic stresses (Howe & Jander, 2008; Dangl *et al.*, 2013; Mickelbart *et al.*, 2015).

A growing body of literature has reported that when several stresses occur simultaneously, they elicit a response that is different from the individual stresses (Rizhsky *et al.*, 2004; Mittler & Blumwald, 2010; Vile *et al.*, 2012; Prasch & Sonnewald, 2013; Rasmussen *et al.*, 2013; Kissoudis *et al.*, 2014; Rivero *et al.*, 2014; Sewelam *et al.*, 2014; Suzuki *et al.*, 2014). These observations are relevant because in natural conditions and agro-ecosystems, combined stresses are the rule rather than the exception (Atkinson & Urwin, 2012; Stam *et al.*, 2014; Prasch & Sonnewald, 2015). Technological advances in sequencing, genotyping and high throughput phenotyping allow to move the field of plant stress biology to the next level, that is to understand how plants respond to combined stresses (Keurentjes *et al.*, 2011; Atkinson & Urwin, 2012; Stam *et al.*, 2014; Prasch & Sonnewald, 2015).

In this general discussion I will integrate the main findings of this thesis. The findings will be contrasted to research conducted by others in the plant stress biology field. The feasibility of obtaining plants that are resistant to multiple stresses is discussed from the point of view of genetic trade-offs and experimental limitations. Ecogenomics strategies chosen in this thesis for gene discovery will be discussed, particular attention is given to the use of insects in quantitative genetic studies; alternatives are discussed and proposed. Finally, I will discuss technological advances that will enable to apply ecogenomics to study stress responses in non-model organisms.

Trade-offs between resistance to insect herbivores belonging to different guilds

Plants share *ca.* 350 million years of evolutionary history with insects (Edwards *et al.*, 1995; Gatehouse, 2002). As a result of selection pressure imposed by insects, plants have evolved defensive mechanisms to fend off insects (Gatehouse, 2002; Schoonhoven *et al.*, 2005; Mithofer & Boland, 2012). Insects of different guilds, e.g. leaf chewers and phloem feeders, are known to trigger divergent defence mechanisms (De Vos *et al.*, 2005; Appel *et al.*, 2014). Plant hormones have emerged as major players in controlling the specificity of plant defence responses (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Erb *et al.*, 2012; Pieterse *et al.*, 2012). For instance, defence responses against leaf chewers are mediated by the jasmonic acid (JA) signalling pathway, whereas responses to phloem feeders are mediated by the salicylic acid signalling pathway (SA) (De Vos *et al.*, 2005; Verhage *et al.*, 2010; Pieterse *et al.*, 2012).

In Chapter 2, a trade-off was observed between the resistance to the leaf chewer *Pieris rapae* and the phloem feeder *Myzus persicae* across a large number of *Arabidopsis thaliana* accessions. Furthermore, in Chapter 6 this trade-off was found to be based on a common genetic architecture represented by a strong negative genetic correlation between resistance to caterpillars and aphids. This strong negative correlation suggested that common genes affect resistance to both insects in opposite directions (i.e. an allele that codes for higher resistance to *P. rapae* is associated with higher susceptibility to *M. persicae*). Interestingly, plant defences to *P. rapae* and *M. persicae* are mediated by JA and SA signalling pathways respectively, which are known to antagonize each other (Sendon *et al.*, 2011; Vos *et al.*, 2013a; Caarls *et al.*, 2015). This cross talk between SA and JA has been investigated meticulously and several of the molecular components for this cross talk have been identified (Vos *et al.*, 2013a; Caarls *et al.*, 2015). This cross talk may provide a mechanistic explanation for the trade-off observed between *P. rapae* and *M. persicae*. In fact,

natural variation for SA-JA cross talk has been observed in *A. thaliana* suggesting a role in the regulation of plant defences under natural conditions (Koornneef *et al.*, 2008). In Chapter 6, multi-trait genome wide association analysis targeted to the identification of genes harbouring alleles with contrasting effect on phloem feeders and leaf chewers identified several genes involved in JA-mediated responses (*LOX5*, *MYB11, MES16*) and SA-mediated responses (*MYB107)*. However, none of the cross talk players described in the literature (e.g. *NPR1, MPK4, WRKY50, WRKY51, WRKY70*) was found among the candidate genes. This suggests that there is little genetic variation in these main players of SA-JA cross talk. Given the importance of this cross talk in keeping the balance between plant defences and growth, I imagine a scenario in which alteration of this balance may be deleterious for the plant. Thus, mutations in these main players may be strongly selected against. In fact, low genetic variation in genes involved in the signalling pathways that control plant defences have been reported in *A. thaliana* including *NPR1* (Bakker *et al.*, 2008). These genes are thought to be under purifying selection given the scarcity of non-synonymous mutations (Bakker *et al.*, 2008).

I also observed trade-offs among resistance to other stresses, such as drought and *P. rapae* (Chapters 2 and 6), that are genetically determined as indicated by their negative genetic correlations. Genetic trade-offs have implications for natural and artificial selection of any pair of traits in the multivariate space (Juenger, 2013). For example, the trade-off observed between responses of *A. thaliana* to feeding by *P. rapae* and *M. persicae* (Chapters 2 and 6) indicates that it is not possible to select for lines that are resistant to both insect herbivores.

Plant responses to combined stresses cannot be predicted from the responses to the single stress situations

In complex environments, such as natural and agricultural ecosystems, plants experience stresses that commonly occur simultaneously (Atkinson & Urwin, 2012; Stam *et al.*, 2014; Suzuki *et al.*, 2014). Several efforts have been made to mimic such combinations of stresses under laboratory conditions. Interestingly, these studies have concluded that phenotypic and gene expression changes can often not be predicted based on the responses to the individual stresses (Atkinson & Urwin, 2012; Kissoudis *et al.*, 2014; Stam *et al.*, 2014; Suzuki *et al.*, 2014).

In Chapter 3 and 4, I observed differences in the phenotypic and transcriptomic responses to combined and single stresses. For instance, we observed that *P. rapae* pre-treatment resulted in a delay of disease development elicited by *B. cinerea*. This delay in disease development was also observed at the transcriptome level. On the

other hand, no differences were observed in biomass reduction when plants were exposed to *P. rapae* alone or after prior inoculation by *B. cinerea*. The transcriptome responses to *P. rapae* and the double stress *B. cinerea* plus *P. rapae* converged over time. These results suggest that the responses to combined stresses depend on the types of stress involved. On the other hand, the phenotypic and transcriptome effects of combined stresses are expected to be a result of the interaction among the mechanisms that regulate the response to each individual stress, such as phytohormones, and therefore the results are normally discussed accordingly. However, interactions between the phytohormones do not always explain the observations. For example, herbivory by *P. rapae* activates JA-signalling, whereas *Turnip crinkle virus* (TCV) activates SA signalling (De Vos *et al*., 2006). Because JA and SA signalling pathways are known to antagonize each other, I would expect that herbivory by *P. rapae* facilitates infection by TCV. In contrast, it resulted in enhanced resistance to TCV because of ethylene-mediated priming of the SA-signalling pathway (De Vos *et al*., 2006). In another example, drought is known to activate ABA-signalling, whereas biotrophic pathogens activate SA-signalling. SA and ABA are known to antagonize each other (Vlot *et al*., 2009; Pieterse *et al*., 2012). Under this assumption, I would expect that plants stressed by drought would be more susceptible to biotrophic pathogens. However, both antagonistic and synergistic interactions have been reported (Mohr & Cahill, 2003; Achuo *et al*., 2006; Goel *et al*., 2008).

Rasmussen *et al.* (2013) estimated that 61% of the transcriptome changes in *A. thaliana* plants exposed to combined abiotic and biotic stresses could not be predicted from the responses to single stresses. Furthermore, in the same study it was observed that potentially antagonistic responses were prioritized in only 5 – 10% of the cases. These results and the results presented in Chapters 3 and 4 of this thesis suggest that there are unique interactions that occur under combined stresses and that the approach of using the phytohormone interactions for explaining the outcomes is rather simplistic. In fact, responses to stress are not only known to display cross talk at the phytohormone signalling pathways but also exhibit interactions at the physiological and phenotypic level (Kissoudis *et al*., 2014). For example, plants under drought stress close their stomata, which may limit the entrance of plant pathogens. In fact, pathogen effectors that interact with ABA-signalling components and result in abnormal stomatal opening have been identified (Goel *et al*., 2008; Hurley *et al*., 2014).

The aim of studying combinations of stresses is to understand how plants respond to stresses in more natural situations and, thus, to develop crops that have a broad spectrum resistance. However, given the number of possible combinations of stresses, it is unrealistic to think we can experiment with all of them. Are there other possibilities to develop broad spectrum resistance? Several strategies have been proposed such

as the use of (1) transcription factors that commonly regulate responses to several stresses, (2) pyramiding of genes that do not antagonize each other, and (3) priming through use of chemicals such as BABA (Kissoudis *et al.*, 2014). Univariate and multi-trait Genome Wide Association analysis identified several regions of the genome associated with resistance to the combined stresses addressed in this thesis (Chapter 5 and 6). Furthermore, genes differentially expressed upon combination of stresses were described in Chapters 3 and 4. An example of a gene providing susceptibility to more than one stress, *RMG1*, is presented in Chapter 6. Phenotypic assays with selected candidate genes are in progress. Upon further characterization these genes can be used to develop plants that are more resistant to combined stresses using transgenic strategies or through identification of orthologues in crops.

Alternatives to using insects for quantitative genetic studies of insect resistance.

Quantitative genetics aims to understand the genetic architecture of complex traits that are governed by the influence of many genes (Griffiths *et al.*, 2015). Insect damage results in severe crop yield losses and, thus, breeders and scientists are constantly searching for new sources of resistance (Schoonhoven *et al.*, 2005; Smith & Clement, 2012). It is necessary to make a distinction between insects of different feeding guilds. Whereas resistance to sap feeders such as the Hessian fly, plant hoppers and aphids seems to be monogenic (Rossi *et al.*, 1998; Boissot *et al.*, 2010; Smith & Clement, 2012; Stuart *et al.*, 2012), similar to resistance to certain pathogens, resistance to leaf-chewing insects is usually a polygenic trait (Smith & Clement, 2012; Keith & Mitchell-Olds, 2013; Kliebenstein, 2014).

Several studies have aimed at unravelling the genetic basis of insect resistance to leaf chewers in different plant species including *A. thaliana.* These studies have reported QTLs associated with insect resistance, but few of them have identified the causal loci (Jander *et al.*, 2001; Pfalz *et al.*, 2007; Ordas *et al.*, 2009; Schranz *et al.*, 2009; Prasad *et al.*, 2012). Several studies have reported low heritability for resistance to leaf chewers (Kliebenstein *et al.*, 2002; Pfalz *et al.*, 2007; Kliebenstein, 2014). This lack of success in the identification of the causal genes may reside in the complex architecture of resistance to leaf chewers (Keith & Mitchell-Olds, 2013; Kliebenstein, 2014). Resistance to leaf chewers is a complex trait that is governed by many loci each of them contributing just a small proportion of the phenotypic variance. This kind of genetic architecture is similar to resistance to abiotic stresses (Roy *et al.*, 2011; Mickelbart *et al.*, 2015). In Chapters 2 and 6, I also found low to moderate heritability for insect resistance to two specialist leaf chewers (*Pieris rapae* and *Plutella xylostella*). For simple traits (i.e. one or a few genes causing a large effect),

low heritability may not be a problem. However, for complex traits (i.e. hundreds of genes of small effect) traditional mapping techniques (QTL mapping or GWA) lack the power for QTL detection (Kliebenstein *et al.*, 2002; Pfalz *et al.*, 2007; Korte & Farlow, 2013; Kliebenstein, 2014).

Working with sexually reproducing insects, especially leaf chewers, in quantitative genetic studies presents several disadvantages because they also display genetic variation and also interact with the environment. This results in an interaction-phenotype that is dependent on two organisms (plant and insect) that both display genetic variation and genotype by environment interactions which, in turn, is reflected in the low heritability and repeatability of the experiments (Kliebenstein, 2014). On the other hand, for those herbivorous insect species that reproduce asexually, such as aphids, it is possible to work with clones and insect genetic variation may not be a problem. Interestingly, low heritability was recorded for resistance to aphids in Chapters 2 and 6.

From here onwards I will focus on the issue of genetic variation within an insect species when investigating genetic variation in plant resistance to stress. A solution to this problem is to increase the sample size in the experiments which often is not possible due limited space, resources and laborious phenotyping procedures. Large-scale high-throughput phenotyping may be a good solution but these platforms are still in the development phase (Grosskinsky *et al.*, 2015; Kloth *et al.*, 2015). Another solution that has been proposed to solve this problem is to measure the content of defensive compounds because they rely solely on plant genetic variation and are characterized by high heritability contrary to the interactions that these compounds mediate with insect herbivores (Kliebenstein *et al.*, 2001; Kliebenstein *et al.*, 2002; Chan *et al.*, 2011; Kliebenstein, 2014). This kind of studies has successfully identified most of the key enzymes involved in glucosinolate metabolism in the Brassicaceae family (Halkier & Gershenzon, 2006; Kliebenstein, 2014). Once a gene has been successfully identified and cloned, one can perform bioassays with insects and evaluate how genetic variation in that particular gene affects the level of resistance. A pitfall of this method is that the search is limited to specific classes of compounds. An alternative is to perform untargeted metabolomic analysis (Keurentjes, 2009). Untargeted metabolomic analysis for the Hapmap population, the population used in this thesis, is in progress (R. Kooke and J.J.B. Keurentjes, personal communication). Preliminary data from GWA analysis on a few glucosinolates identified several of the usual suspects with QTLs harbouring significant $-\log_{10}(P)$-values 10-fold higher than the ones observed for most of the traits evaluated in Chapters 5 and 6 of this thesis. Targeted and untargeted metabolomic studies under untreated conditions, may render information about constitutive plant defence mechanisms. But what about induced defences?

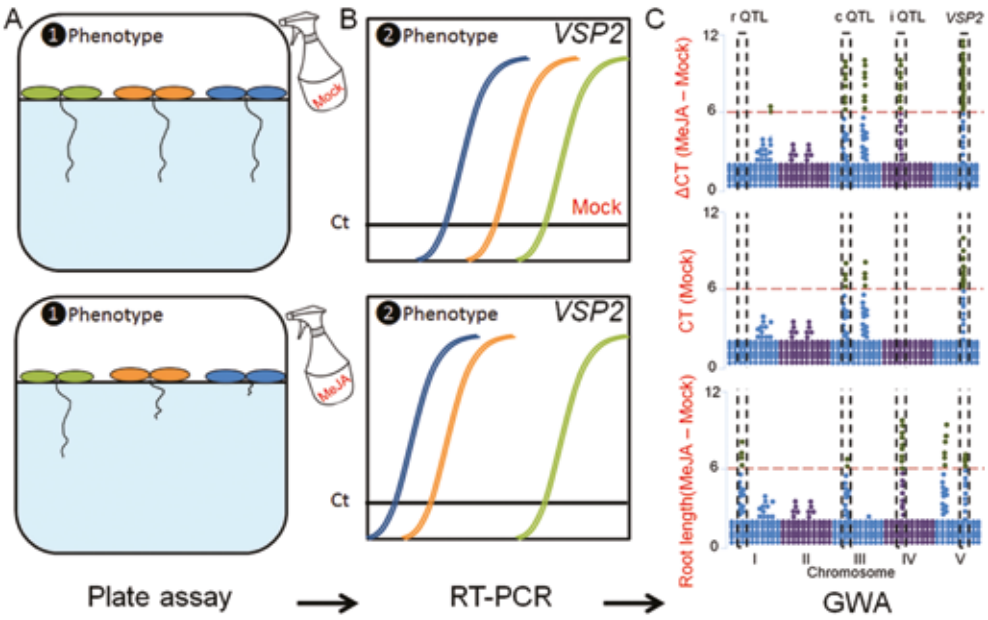


Figure 1. Genetic dissection of constitutive and induced plant defences against insect herbivores. (A) Plant assay where different accessions (indicated by different colours) are grown. A group of accessions is grown under control conditions (Mock) and another group is exposed to methyl jasmonate (MeJA). A well described phenotype for the response to MeJA in *A. thaliana* is the inhibition of root growth (Staswick *et al*., 1992). Therefore the rooth length under Mock and MeJA can be quantified as phenotype. The change in root length will represent the response to MeJA. (B) Tissue from the plants is collected and used for RT-PCR using markers genes of JA-mediated responses such as *VSP2* and *MYC2* (Verhage *et al*., 2011; Vos *et al*., 2013b). Accessions that display little responsiveness to MeJA (green) will neither show root growth inhibition nor induction of gene expression. The opposite will be expected for responsive accessions (blue and orange). (C) Genome-wide association (GWA) can be performed on the change in (1) root length and (2) marker gene induction in response to MeJA. These two phenotypes will shed light on inducible defences (i QTLs). Note that variation in marker gene expression under mock conditions will reflect to some extent the constitutive level of defences in the population (c QTLs). Further, note that for the change in root length phenotype one may expect to identify QTLs associated with variation in root length itself and they may have nothing to do with defences (r QTLs). Once candidate genes are confirmed through allelic complementation or mutant analysis, their role in defence against insects can be tested.

Here, I propose an experiment that may inform about both basal and induced levels of plant defences mediated by the JA signalling pathway. This kind of experiment may be extended to plant defences mediated by other phytohormones. In this experiment, I propose to measure a proxy for a phenotype associated with resistance (Figure 1A). For example, effective defence responses against leaf chewers are mediated by JA (De Vos *et al*., 2005; Pieterse *et al*., 2009; Stam *et al*., 2014). It has been estimated that *P. rapae* elicits defence responses that are mediated for 67-84 % by the JA signalling pathway (Reymond *et al*., 2004). Thus, a phenotype induced by JA treatment could be used as a proxy for defence responses.

A well-known phenotype triggered by JA treatment in *A. thaliana* is root growth inhibition (Staswick *et al.*, 1992; Berger *et al.*, 1996). In fact, natural variation in root growth inhibition in response to MeJA has been reported for a few accessions (Matthes *et al.*, 2008). Furthermore, several marker genes, such as *VSP2* and *MYC2,* have been validated and are used as proxy for JA-mediated responses (Verhage *et al.*, 2011; Vos *et al.*, 2013b). Measuring expression levels for these marker genes can also be used as proxy for a phenotype of JA-mediated responses (Figure 1B). Measuring variation in the expression of marker genes under control conditions may identify QTLs associated with constitutive levels of defences.

Using gene expression to characterize a phenotype, one would expect QTLs harbouring these genes as a result from a GWA analysis as demonstrated by Atwell *et al.* (2010). The other QTLs are expected to contain genes that belong to the gene network involved in the response to MeJA (Figure 1C). Once candidate genes are confirmed by mutant analysis or allelic complementation, they can be evaluated through insect bioassays. A shortcoming of the proposed method is that, although there is an overlap between the responses elicited by MeJA applications and insect feeding, important differences have been observed (Bruinsma *et al.*, 2009; Zhang *et al.*, 2010). For example, MeJA elicited a volatile profile in *B. oleracea* that was different from the blend emitted by plants infested either with *P. rapae* or *P. xyllostella* (Bruinsma *et al.*, 2009).

Use of *Arabidopsis thaliana* as a model for fundamental and applied research

Model organisms have been useful in science to gain knowledge on certain phenomena and to articulate hypotheses that can be tested in other organisms. As such, *A. thaliana* has played a fundamental role in advancing our knowledge on plant biology (Koornneef & Meinke, 2010; Krämer, 2015). Several phenomena are now thoroughly understood by the use of genetic screenings in *A. thaliana,* such as flowering time, hormonal signalling pathways and defences to abiotic and biotic stresses (Shindo *et al.*, 2005; Ma *et al.*, 2009; Park *et al.*, 2009). In several cases, knowledge on *A. thaliana* has been successfully translated into crops. For example, several transgenic lines of different crops (e.g. rice, potato, tomato, canola) harbouring genes from *A. thaliana* have been generated that are more resistant to certain abiotic stresses (Yang *et al.*, 2010; Lata & Prasad, 2011). Alternatively, once a gene has been identified in *A. thaliana* as being involved in a certain process, orthologous genes in crops can be identified and characterized. For example, ABA receptor orthologues in tomato have been identified and characterized. Interestingly, tomato receptors were able to inhibit

the activity of protein phosphatase type 2Cs PP2Cs from both tomato and *A. thaliana*, emphasizing the conservation of protein function across species (Gonzalez-Guzman *et al.*, 2014). Another example is *BOS1,* a transcription factor that in both *A. thaliana* and tomato provides tolerance to salt stress as well as resistance to infection by *B. cinerea* (Mengiste *et al.*, 2003; AbuQamar *et al.*, 2009). These studies demonstrate the potential of the knowledge generated on *A. thaliana* for translation into crops.

In this thesis *A. thaliana* resistance against a broad range of abiotic and biotic stresses was dissected at the genetic level (Chapters 5 and 6). Furthermore, responses at the transcriptional level were also investigated (Chapters 3 and 4). These studies generated a rich knowledge of putative candidate genes involved in resistance against abiotic and biotic stresses. Functional characterization of candidate genes is ongoing. An example is presented in Chapter 6 where loss of function mutants for *RMG1* were proven to provide resistance to *P. rapae* and osmotic stress. Upon further validation, candidate genes can be investigated in crops through transgenic strategies or search for orthologues as described above.

Despite the importance of the information generated in model organisms, the diversity of the organisms that they represent is too vast and care must be taken when extrapolating information. There are about 300,000 species of flowering plants and it is unrealistic to expect that the full complexity of ecological interactions and secondary compounds in nature can be represented by one organism (Kreft & Jetz, 2007; Mithofer & Boland, 2012). For example, *A. thaliana* does not interact with nitrogen-fixing bacteria, an important process occurring in legumes, with fundamental and applied importance (Stacey *et al.*, 2006). Furthermore, ecological interactions between insects and plants may be mediated by compounds that are not present in *A. thaliana*. For example, nicotine in *Nicotiana attenuata* plays a double role as pollination stimulant and defence against herbivory (Wu *et al.*, 2007).

In the future, I expect that *A. thaliana* will continue to play an important role as a research organism. Especially in the last years thousands of accessions have been genotyped and re-sequenced opening the possibilities for studies on ecology and evolution of traits along the native range of the species (Weigel, 2012; Krämer, 2015). For example, identification of genetic variants influencing local adaptation and fitness under natural conditions have been reported in *A. thaliana* (Hancock *et al.*, 2011; Dittmar *et al.*, 2014). An example of such a study is also presented in Chapter 2 of this thesis. But what is beyond *A. thaliana*? And which technological advances can help to develop an ecogenomics approach in non-model species?

Arabidopsis thaliana was proposed as a model system due to the availability of its full genome sequence, amenability for genetic screenings and extensive range of mutants (Page & Grossniklaus, 2002; Koornneef & Meinke, 2010). In the last few years the

reduction in costs of Next Generation Sequencing has opened up the possibility for accessing the genomes and transcriptomes of non-model organisms (Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). For example, RNA-seq has been applied to understand flooding survival of closely related *Rumex* species that deploy different strategies (van Veen *et al.*, 2013). Another important technological advancement that can facilitate the study of non-model species is the application of CRISPR-Cas for manipulation of genomes (Belhaj *et al.*, 2015; Guo *et al.*, 2015). This system allows accurate editing of genomes and has been used to investigate gene function in several organisms such as the mosquito *Aedes aegypti*, mice and several plant species (Belhaj *et al.*, 2015; Guo *et al.*, 2015; Hall *et al.*, 2015; Hashimoto & Takemoto, 2015). The investigation of transcriptome changes in non-model species using RNA-seq and further candidate gene validation using accurate genome editing with the CRISPR-Cas system will, without doubt, accelerate gene discovery in non-model species.

This thesis is a contribution to the understanding of how plants respond to different abiotic and biotic stresses either as single stress or when applied in combination. Particular emphasis is given to plant responses to insects and how they are altered by a combination of stresses. A wealth of candidate genes was generated by applying an ecogenomics approach, mainly through transcriptome analysis, large-scale phenotyping and Genome Wide Association analysis. Functional characterization of these genes is in progress. These genes are likely to constitute a rich source of resistance to abiotic, biotic and combined stresses that in the future can be applied for crop improvement.

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Summary

In natural and agricultural ecosystems, plants are exposed to a wide diversity of abiotic and biotic stresses such as drought, salinity, pathogens and insect herbivores. Under natural conditions, these stresses do not occur in isolation but commonly occur simultaneously. However, plants have developed sophisticated mechanisms to survive and reproduce under suboptimal conditions. Genetic screenings and molecular genetic assays have shed light on the molecular players that provide resistance to single biotic and abiotic stresses. Induced defenses are attacker specific and phytohormones play an essential role in tailoring these defense responses. Because phytohormones display antagonistic and synergistic interactions, the question emerges how plants elicit an effective defense response when exposed to conflicting signals under multiple attack. Recent studies have shed light on this issue by studying the effects of combinations of stresses at the phenotypic, transcriptomic and genetic level. These studies have concluded that the responses to combined stresses can often not be predicted based on information about responses to the single stress situations or the phytohormones involved. Thus, combined stresses are starting to be regarded as a different state of stress in the plant. Studying the effects of combinations of stresses is relevant since they are more representative of the type of stresses experienced by plants in natural conditions.

In a coordinated effort, responses of *Arabidopsis thaliana* to a range of abiotic and biotic stresses and stress combinations have been explored at the genetic, phenotypic, and transcriptional level. For this purpose we used an ecogenomic approach in which we integrated the assessment of phenotypic variation and Genome-Wide Association (GWA) analysis for a large number of *A. thaliana* accessions with an in-depth transcriptional analysis. The focus of this thesis is especially on (but not limited to) three stresses, i.e. drought, herbivory by *Pieris rapae* caterpillars, and infection by the necrotrophic fungal pathogen *Botrytis cinerea*. These stresses were chosen because the responses of *A. thaliana* to these three stresses are highly divergent but at the same time regulated by the plant hormones JA and/or ABA. Consequently, analysis of responses to combinatorial stresses is likely to yield information on signaling nodes that are involved in tailoring the plant's adaptive response to combinations of these stresses. Responses of *A. thaliana* to other biotic and abiotic stresses are included in an integrative study (Chapter 6).

We first investigated (Chapter 2) the extent of natural variation in the response to one abiotic stress (drought), four biotic stresses (*Pieris rapae* caterpillars*, Plutella xylostella* caterpillars*, Frankliniella occidentalis* thrips*, Myzus persicae* aphids) and two combined stresses (drought plus *P. rapae,* and *B. cinerea* plus *P. rapae*). Using 308 *A. thaliana* accessions originating from Europe, the native range of the species,

we focused on the eco-evolutionary context of stress responses. We analyzed how the response to stress is influenced by geographical origin, genetic relatedness and life-cycle strategy, *i.e.* summer versus winter annual. We identified heritable genetic variation for responses to the different stresses. We found that winter annuals are more resistant to drought, aphids and thrips and summer annuals are more resistant to *P. rapae* and *P. xylostella* caterpillars and to the combined stresses of drought followed by *P. rapae* and infection by the fungus *B. cinerea* followed by herbivory by *P. rapae*. Furthermore, we found differential responses to drought along a longitudinal gradient.

We further investigated, using *A. thaliana* accession Col-0, how phenotypic and whole-genome transcriptional responses to one stress are altered by a preceding or co-occurring stress (Chapters 3 and 4). The whole-transcriptomic profile of *A. thaliana* triggered by single and combined abiotic (drought) and biotic (herbivory by caterpillars of *P. rapae*, infection by *B. cinerea*) stresses was analyzed by RNA sequencing (RNA-seq). Comparative analysis of plant gene expression triggered by single and double stresses revealed a complex transcriptional reprogramming. Mathematical modelling of transcriptomic data, in combination with Gene Ontology analysis highlighted biological processes specifically affected by single and double stresses (Chapters 3). For example, ethylene (ET) biosynthetic genes were induced at 12 h by *B. cinerea* alone or drought followed by *B. cinerea* inoculation. This induction was delayed when plants were pretreated with *P. rapae* by inducing ET biosynthetic genes only 18 hours post inoculation. Other processes affected by combined stresses include wound response, systemic acquired resistance (SAR), water deprivation and ABA response, and camalexin biosynthesis.

In Chapter 4, we focused on the stress imposed by *P. rapae* herbivory alone or in combination with prior exposure to drought or infection with *B. cinerea*. We found that pre-exposure to drought stress or *B. cinerea* infection resulted in a significantly different timing of the caterpillar-induced transcriptional changes. Additionally, the combination of drought and *P. rapae* induced an extensive downregulation of *A. thaliana* genes involved in defence against pathogens. Despite the larger reduction in plant biomass observed for plants exposed to drought plus *P. rapae* feeding compared to *P. rapae* feeding alone, this did not affect weight gain of this specialist caterpillar.

In Chapter 5, we used univariate GWA to (1) understand the genetic architecture of resistance to the different stresses and (2) identify regions of the genome and possible candidate genes associated with variation in resistance to those stresses. In Chapter 5 a subset of the stresses addressed in Chapter 1 (*i.e.* drought, herbivory by *P. rapae* and *P. xylostella*, and the combined stresses drought plus *P. rapae* and *B. cinerea* plus *P. rapae*) were investigated. Results from GWA were integrated with expression

data generated in Chapters 3 and 4 or available from the literature. We identified differences in genetic architecture and QTLs underlying variation in resistance to (1) *P. rapae* and*P. xylostella* and (2) resistance to *P. rapae* and combined stresses drought plus *P. rapae* and *B. cinerea* plus *P. rapae*. Furthermore, several of the QTLs identified contained genes that were differentially expressed in response to the relevant stress. For example, for *P. xylostella* one of the QTLs contained only two genes encoding cysteine proteases (*CP1* and *CP2*). The expression data indicated that these genes were induced by *P. rapae* and *P. xylostella* herbivory.

In Chapter 6, the genetic architecture underlying plant resistance to 11 single stresses and some of their combinations was investigated. First, the genetic commonality underlying responses to different stresses was investigated by means of genetic correlations,, revealing that stresses that share phytohormonal signaling pathways also share part of their genetic architecture. For instance, a strong negative genetic correlation was observed between SA and JA inducers. Furthermore, multi-trait GWA identified candidate genes influencing the response to more than one stress. For example, a functional *RMG1* gene seems to be associated with susceptibility to herbivory by *P. rapae* and osmotic stress since loss of function mutants in *RMG1* displayed higher resistance to both stresses. Finally, multi-trait GWA was used to identify QTLs with contrasting and with similar effects on the response to (a) biotic or abiotic stresses and (b) belowground or aboveground stresses.

Finally, In Chapter 7, I discuss the feasibility of obtaining plants that are resistant to multiple stresses from the point of view of genetic trade-offs and experimental limitations. The ecogenomic approach for gene discovery taken in this thesis is discussed, and recommendations are especially given on the use of herbivorous insects in quantitative genetic studies of stress resistance. Furthermore, alternatives to the use of insects in quantitative genetic studies of stress resistance are discussed and proposed. Finally, I discuss the feasibility of using an ecogenomic approach to study stress responses in other plant species than the model plant of molecular genetics, *A. thaliana*.

A wealth of candidate genes was generated by taking an ecogenomic approach, in particular transcriptome analysis and GWA analysis. Functional characterization of these genes is a next challenge, especially in the context of multiple stress situations. These genes constitute a rich source of potential factors important for resistance to abiotic, biotic and combined stresses that in the future may be applied for crop improvement.

Acknowledgements

Four years have passed and it is time to thank all the people that were part of my academic formation and social life and have made this chapter of my life a great adventure full of learning and personal growth.

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My project was embedded in a bigger project, called “Learning from Nature”. Within this project, I had the opportunity to collaborate with a great group of ambitious PhD students and post-docs with whom I shared ideas, frustrations and good moments. My gratitude to the “core team”; **Pingping Huang**, **Silvia Coolen**, **Manus Thoen**, **Karen Kloth**, **Dr. Willem Kruijer** and **Dr. Joost van Heerwaarden** with whom I collaborated these four years and that are co-authors in several of the chapters of this thesis. Many thanks to the professors involved in the project, special thanks to **Dr. Corné Pieterse**, **Dr. Saskia van Wees, Dr. Mark Aarts**, **Dr. Joost Keurentjes**, **Dr. Fred van Eeuwijk** with whom I directly interacted and discussed ideas that are set out all over this thesis. Within this project, several companies were involved and contributed with ideas and resources. I am extremely thankful to **Dr. Alexander Wittenberg**, **Dr. Marcel Prins** and **Dr. Raymond Hulzink** (Keygene N.V., Wageningen) who did RNA-seq for my project and which data are the basis of two chapters of this thesis. Many thanks to **Dr. Paul Passarinho** (Genetwister Technologies B.V., Wageningen) and **Dr. Sjoukje Heimovaara** (Royal Van Zanten B.V., Rijsenhout) who were also part of this project. Many thanks to the technicians who were instrumental during the data collection phase **Léon Westerd**, **Gerrie Wiegers**, **Frank Becker** and **Hans van Pelt**.

During these four years I also got the chance to interact with PhDs, postdocs and professors of other departments and in one or another way contributed to my formation with ideas and discussing literature. Many thanks to **Dr. Padraic Flood**, **Charles Moreira**, **Dr. Johanna Bac-Molenaar**, **Dr. Rik Kooke** and **Dr. Edouard Severing** of the Laboratory of Genetics; **Christos Kissoudis**, **Dr. Alejandro Lucatti**, **Dr. Colette Broekgaarden** of Plant Research International; **Dr. Jan van Kan** of the Laboratory of Phytopathology, **Dr. Gerrit Gort** of Biometris and **Dr. Marcel van Verk** and **Dr. Richard Hickman** of the Plant-Microbe Interactions group of Utrecht University.

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Some people say that a PhD project is a lonely process but that was not the case for me. I was surrounded by a great group of people that I call family. Many thanks **Joao & Mira**, **Alejandro & Hanna**, **Foteini & Argyris**, **Luigi & Alice**, **Andres & Sanne, Andresinho & Evy, Sebastian & Ana, Andre Silva and Charles & girls.** Despite the fact that I was far away from home you made this place my home. To **Kim M.C.A Vermeer**, my girlfriend, my partner, my confident and my friend. Completing this thesis could not have been possible without your support. Many thanks my love for your support during these years, for being strong when I was weak, for believing in me when I did not, for telling me what I need to hear in difficult periods "*You can do it, you are a finisher, not a deserter*".

Finally, without solid foundations, you cannot build a great building, and the values and principles that my parents taught me as a child led me all the way to this moment. Therefore, I would like to devote the last words of thanks to the people who built the foundations and made me the man I am; my parents and grandparents. Gracias a mis padres y abuelitos, quienes me forjaron con principios y valores que llevo conmigo donde quiera que voy. Porque me enseñaron que no hay metas imposibles, que todo se alcanza con trabajo duro y empeño. Muchas gracias también a la familia Castilblanco-Flores, a quienes también considero familia, y que con su trabajo duro y disposición para ayudar a otras personas se han ganado mi respeto y admiración. Sin el apoyo que me dieron en los primeros pasos de mi carreara todo esto no hubiera sido posible.

Curriculum Vitae

Nelson H. Davila Olivas was born on August 6, 1985 in Estelí, Nicaragua. Estelí is the third largest city of Nicaragua and it is located in the North highlands of the country. Growing up in a country, the economy of which relies largely on agriculture, and being raised in a family of accountants and farmers, Nelson grew up with a passion for agriculture. Driven by motivation, discipline and perseverance he obtained a scholarship from the People's Republic of China to take his bachelor degree at Zamorano University in Honduras, one of the most prestigious colleges of agriculture in Latin America. He obtained his bachelor degree in 2007 where he graduated with the best academic performance in Agricultural Science and Production.

During his bachelor, the mixture of theory and practical work provided him with knowledge in themes related to farming and agricultural production, agribusiness, agro-industry and agro-biotechnology. Especially during this time, he developed a fascination for biotechnology and recognized that agriculture worldwide was moving towards the use of biotechnological innovations. This fascination for biotechnology led him to start a journey in search of knowledge, experience and understanding of biotechnological applications. This path led him to different corners of the globe. First he did an internship at University of Florida where he learned about the use of chemical mutagenesis, tissue culture and genetic transformation in Bahia grass. Subsequently, he moved to Ohio State University, where he learned the use of molecular markers for population genetic studies of the Soybean - Soybean aphid interaction.

After gaining some experience with biotechnological tools and concepts his curiosity was not fully satisfied and he decided that the next step was to pursue an MSc degree. After exploring several programs at Universities in the USA, he decided that his next step was across the Atlantic. In 2009, he was recipient of the Bonn Scholarship Plus for talented students from developing countries to follow the MSc curriculum in Plant Sciences at the University of Bonn in Germany. During his MSc studies he learned about the molecular basis of desiccation tolerance in the resurrection plant *Craterostigma* and salt tolerance in the model plant *Arabidopsis*. Furthermore, during his MSc he did an internship at Wageningen University in The Netherlands, where he learned about the molecular and physiological basis of drought tolerance in potato. Beside the knowledge and experience obtained during this period, he found his new passion "*doing science*".

After obtaining his MSc degree in 2011 he was accepted as a PhD student in a 4-year multidisciplinary project in the Laboratory of Entomology at Wageningen University. Nelson's PhD project was embedded within the Perspectief program 'Learning from Nature' funded by the Dutch Technology Foundation (STW). This program was a multidisciplinary team effort carried out at different universities and innovative plant breeding and biotechnology companies such as Keygene, Genetwister, Royal Van Zanten, Syngenta, Rijk Zwaan among others, with the common goal of understanding plant responses to abiotic and biotic stresses. Results of this 4-year journey are presented in this thesis.

Publications

Published or Accepted

Kannan B, Davila Olivas NH, Lomba P, Altpeter F. 2015. In vitro chemical mutagenesis improves the turf quality of bahiagrass. *Plant Cell, Tissue and Organ Culture (PCTOC)* **120**(2): 551-561.

Michel AP, Rouf Mian MA, Davila Olivas NH, Cañas LA. 2010. Detached leaf and whole plant assays for soybean aphid resistance: differential responses among resistance sources and biotypes. *Journal of Economic Entomology* **103**(3): 949-957.

Davila Olivas NH, Coolen S, Huang P, Severing E, van Verk MC, Hickman R, Wittenberg AHJ, De Vos M, Prins M, van Loon JJA, Aarts MGM, van Wees SCM, Pieterse CMJ, Dicke M. 2015b. Effect of prior drought and pathogen stress on *Arabidopsis* transcriptome changes to caterpillar herbivory. *in press. New Phytologist*.

Submitted

Bac-Molenaar JA, Caldas Paulo MJ, Kooke R, Kruijer WT, Davila Olivas NH, Oztolan-Erol N, Moreira CN, El-Soda M, Aarts MGM, Vreugdenhil D, Keurentjes JJB, van Eeuwijk FA, Malosetti M. 2015. Detecting constitutive QTLs for plant biomass by Multi-Environment Genome Wide Association mapping. *Submitted*.

Coolen S*, Proietti S*, Hickman R*, Davila Olivas NH*, Huang P*, van Verk MC, van Pelt JA, Wittenberg AHJ, De Vos M, Prins M, Aarts MGM, van Loon JJA, Dicke M, Pieterse CMJ, van Wees SCM. 2015. Whole transcriptome analysis of *Arabidopsis thaliana* under sequential biotic and abiotic stresses. *Submitted*.

Davila Olivas NH, Frago E, van Loon JJA, Thoen MPM, Kloth KJ, Becker F, van Heerwaarden J, Gort G, Keurentjes JJB, Dicke M. 2015a. Natural variation in life-history strategy of *Arabidopsis thaliana* determines stress responses to drought and insects of different feeding guilds. *Submitted*.

Thoen MPM*, Davila Olivas NH*, Kloth KJ*, Coolen S*, Huang P*, Aarts MGM, Bac-Molenaar JA, Bakker J, Bouwmeester HJ, Broekgaarden C, Bucher J, Busscher-Lange J, Cheng X, Fradin EF, Jongsma MA, Julkowska MM, Keurentjes JJB, Ligterink W, Pieterse CMJ, Ruyter-Spira C, Smant G, Testerink C, Usadel B, van Loon JJA, van Pelt JA, van Schaik CC, van Wees SCM, Visser RGF, Voorrips R, Vosman B, Vreugdenhil D, Warmerdam S, Wiegers GL, van Heerwaarden J, Kruijer W, van Eeuwijk FA, Dicke M. 2015. Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping. *Submitted*.

In prep

Davila Olivas NH, Kruijer WT, Wijnen CL, van Loon JJA, Dicke M. 2016. Genome Wide Association analysis reveals distinct genetic architectures for single and combined stress responses in *Arabidopsis thaliana*. *in prep*.

* Shared first authorship

Educational Statement of the Graduate School Experimental Plant Sciences

Issued to: Nelson Horacio Davila Olivas

Date: 26 February 2016

Group: Laboratory of Entomology

University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Insect resistance of plants in the context of multiple stresses	2011
► **Writing or rewriting a project proposal**	
Hotel grant Proposal "Metabolic responses to combinations of multiple abiotic and biotic stresses"	Sep 2013
► **Writing a review or book chapter**	
► **MSc courses**	
GEN-30306 Genetic Analysis, Tools and Concepts	2011-2012
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*9.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, University of Amsterdam	Nov 30, 2012
PE& RC student day (Exploring life in the extremes and the extreme in life)	Nov 01, 2012
EPS PhD Student Days 'Get2Together', Soest (NL)	Jan 29-30, 2015
► **EPS theme symposia**	
EPS theme 4 Symposium 'Genome biology', Wageningen University	Dec 09, 2011
EPS theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten day, Wageningen University	Feb 10, 2012
EPS theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten day, Utrecht University	Jan 24, 2013
EPS theme 3 (Metabolism and Adaptation), University of Amsterdam	Mar 24, 2013
EPS theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten day, University of Amsterdam	Feb 25, 2014
► **NWO Lunteren days and other National Platforms**	
Annual Meeting of the Netherlands Entomological Society	Dec 16, 2011
Annual Ecology Meeting. Lunteren (NL)	Feb 07-08, 2012
NWO-ALW Meeting Experimental Plant Sciences, Lunteren (NL)	Apr 02-03, 2012
NWO-ALW Meeting Experimental Plant Sciences, Lunteren (NL)	Apr 22-23, 2013
NWO-ALW Meeting Experimental Plant Sciences, Lunteren (NL)	Apr 14-15, 2014
NWO-ALW Meeting Experimental Plant Sciences, Lunteren (NL)	Apr 13-14, 2015
► **Seminars (series), workshops and symposia**	
Workshop	
6th Workshop Plant-insect interactions, University of Amsterdam	Nov 23, 2011
7th Workshop Plant-insect interactions, Leiden University	Dec 28, 2012
8th Workshop Plant-insect interactions, Wageningen University	Sep 24, 2013
9th Workshop Plant-insect interactions, Utrecht University	Nov 03, 2014

Symposia	
Intraspecific pathogen variation	Dec 01, 2013
All-inclusive Breeding: Integrating high-throughput science	Oct 16, 2014
Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding	Dec 11, 2014
Mini-symposium on the occasion of the Farewell of Maarten Koornneef	Apr 11, 2013
EPS Flying Seminars	
Seminar by Dr. Javier Palatnik, 'Biogenesis and function of plant microRNAs'	Aug 25, 2011
Seminar by Dr. Rober Furhang, 'Plant Phenomics, photosynthesis and the global food security challenge'	Sep 02, 2011
Seminar by Dr. Jill Farrant, University of Capetown, South-Africa 'Use of resurrection plants as models to understand how plants tolerate extreme water loss: A systems biology approach with applications for making drought tolerant crops	Jun 26, 2012
Seminar by Professor Sir David C. Baulcombe, Cambridge University "Plant versus virus: defense, counter defense and counter counter defense"	Oct 10, 2012
Seminar by Prof. Yukihiro Sugimoto, Kobe University, Japan "Strigolactones, new plant hormones. Importance of their stereochemistry for bioactivity as germination stimulant"	Oct 16, 2012
Seminar by Dr. Ruth Finkelstein, University of California "'ABA signaling networks in Arabidopsis"	Nov 13, 2012
Seminar by Dr. Tom Mitchell-Olds, Duke University, USA "Strong selection on the genes controling complex traits in complex environments"	Dec 10, 2012
Seminar by Dr. Deflet Weigel, Max Planck Institute for Developmental Biology, Tuebingen,Germany. ARABIDOPSIS THALIANA AND ITS RELATIVES AS MODEL SYSTEMS FOR THE STUDY OF EVOLUTIONARY QUESTIONS	Feb 27, 2013
Seminar by Dr. Kazuki Saito, RIKEN Plant Science Centre, and Chiba University, Japan. 'Metabolomics-based functional genomics - from Arabidopsis to crops and medicinal plants'	Apr 08, 2013
Seminar by Dr. David Weller, Washington State University, USA "Soilborne Pathogens and their Natural Biocontrol Agents in Cereal-Based Production Systems of the U.S. Pacific Northwest	Sep 25, 2013
Seminar by Dr. Noah Whiteman, University of Arizona, USA "Evolution of plant-herbivore interactions: insights from genomics"	Jul 17, 2014
Seminar by Dr. Joy Bergelson, University of Chicago, USA "Maintaining an ancient balanced polymorphism for resistance amidst diffuse interactions"	Sep 26, 2014
Entomology Colloquium	
Seminar by Dr. Hans Smith, Wageningen University, ": Learning in parasitic wasps: genes, brains and behaviour"	Oct 18, 2011
Seminar by Dr.H.C Sharma, ICRISAT, "Host plant resistance to insects in grain legumes: Potential and limitations"	Nov 16, 2011
Lab of Genetic Seminars	
Seminar by. Dr. Joost Keurentjes, Wageningen University"In Search of Missing Heritability: Establishing Haplotype Mapping in Arabidopsis"	Oct 18, 2011
Seminar by Pingping Huang, "Natural variation in *Arabidopsis thaliana* as a tool for identify new tolerance pathway for combinatorial stress responses"	Nov 04, 2011
Seminar by Nihal, "Genetic Analysis of Nitrogen Use Efficiency in *Arabidopsis thaliana* using Association Mapping"	Nov 04, 2011
Seminar by Natalia Rosero "Potato genetical genomics"	Jan 31, 2012
Seminar by Padraic Flood "Quantitative genetic analysis of photosyntesis in *A. thaliana*"	Jan 31, 2012
Seminar by Xiaoqian " How manipulating meiosis can help plant breeding "	Mar 13, 2012
Seminar by Ana Carolina " Mapping Genes Involved in Zn Homeostasis in *Arabidopsis thaliana* "	Mar 13, 2012
Seminar by Charles Moreira" Association Mapping for Phosphorus Stress in *Arabidopsis thaliana* "	Mar 27, 2012

Seminar by Dr. Hans de Jonge " FISH technology in the genomics era: examples from tomato and potato	Mar 27, 2012
Semiar by Diana " Overexpression of MYB29 transcription factor increases the beneficial effects for agriculture and human health of Brassica oleracea plants "	May 01, 2012
Seminar by Jelle " Molecular mechanisms of nutrient-dependent ageing"	May 01, 2012
Seminar by Martijn Heddes " Centromere mediated genome elimination where and when?"	May 15, 2013
Seminar by Joost v/d Heuvel " Yo-yoing towards the mechanistic understanding of resource allocation "	May 15, 2013
Seminar by Roy Bigger, Biorad "An introduction to qPCR "	Jun 24, 2013
Seminar by Joost van Heerwaarden"Phenotype, shmenotype. Mapping adaptive genes in *Arabidopsis thaliana* using predicted trait values	Jun 24, 2013
Seminar by Alfred " Comparison of Zn regulatory mechanism in *Arabidopsis thaliana* and Noccaea caerulescens "	May 17, 2014
Seminar by Rik " Trans-generational stress continued: RNAseq and biotic interactions "	May 17, 2014
Padraic Flood " High-Throughput Phenotyping of Photosynthesis and Growth for Plant Phenomics: good enough for nature methods? "	Jul 15, 2014
► **Seminar plus**	.
► **International symposia and congresses**	
14th International Symposium on Insect-Plant Relationships, Wageningen (NL)	Aug 13-17, 2011
ICAR International Arabidopsis Research Conference. Vienna (Austria)	Jul 04-07, 2012
5th European PhD retreat. Ghent (Belgium)	Jul 23-26, 2013
XVI International Congress on Molecular Plant-Microbe Interactions, Rhodes (Greece)	Jul 06-10, 2014
► **Presentations**	
Insect resistance of plants in the context of multiple stresses_ STW meeting - Talk	Jan 26, 2012
ICAR International Arabidopsis Research Conference - Poster	Jul 03-07, 2012
Exploiting natural variation in *A. thaliana* as source of resistance to biotic and abiotic stresses_ STW meeting - Poster	Sep 27-28, 2012
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting - Poster	Apr 23, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Sep 27-28, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Jan 29, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting - Talk	May 17, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Jun 18, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Jul 23-26, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Oct 30, 2013
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Mar 28, 2014
XVI International Congress on Molecular Plant-Microbe Interactions - Talk	Jul 06-10, 2013
The ABCs of R: A learnding by doing approach - Talk	Sep 17, 2014
Exploiting natural variation in *A. thaliana* as source of resistance_STW meeting -Talk	Sep 25-26, 2014
The ABCs of R: A learnding by doing approach - Talk	Oct 02, 2014
► **IAB interview**	
► **Excursions**	
CBSG matchmaking event, visit to Monsanto and Rijk Zwaan	Oct 18, 2012
Entomology PhD excursion 2013	Oct 28-Nov 01, 2013
EPS company visit (In2care and Genetwister)	Sep 19, 2014
Subtotal Scientific Exposure	*33.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
6th Utrecht PhD School on Enviromental Signaling	Aug 22-24, 2011
Basic statistics for PhD students	Dec 13, 14, 15, 20 & 21, 2011
Genome Wide Association course (Learning from nature programme)	Feb 23, 2012
Introduction to R for statistical analysis	Jun 11-12, 2012
PhD Summer School 'Natural Variation of Plants'	Aug 21-24, 2012
The power of RNA-seq	Dec 16-18, 2012
Linear Models	Jun 05-07, 2013
Generalized Linear Models	Jun 13-14, 2013
► **Linear Mixed Models**	Jun 20-21, 2013
Introduction to Genomic Data Analysis using HapMap and 1000 Genomes Projects - 2nd Edition, Barcelona, Spain	Jan 20-24, 2014
► **Journal club**	
IPI Meetings in Entomology + Genetic literature discussions	2011 -2015
► **Individual research training**	
Subtotal In-Depth Studies	*12.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Information Literacy	Dec 06.12-2011
Techniques for writing and presenting a scientific paper	Dec 04-07, 2012
► **Organisation of PhD students day, course or conference**	
EPS PhD party 2011 and EPS PhD party 2012	2011 and 2012
Organisation of Insect Plant Interactions bi-weekly Meetings	2012-2013
EPS Flying seminars (Deflet Weigel, David Weller, Noah Whiteman)	2013 and 2014
EPS company visit (In2Care, Genetwister)	Sep 19, 2014
► **Membership of Board, Committee or PhD council**	
Member of EPS PhD council	2011-2014
Subtotal Personal Development	*5.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**61.2**

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 ECTS credits

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

Learning from nature to protect crops

Plants are under the constant threat of biotic and abiotic stresses. Yet, devastating pests and diseases only rarely occur in nature and plants have managed to sustain for millions of years in this hostile environment. This is due to and has resulted in a tremendous degree of natural variation in mechanisms that plants exploit to defend themselves against pathogens and insects and to deal with abiotic stresses. In agriculture, however, we have exploited only very little of this diversity of defenses and as a consequence environment-malignant pesticides remain a dominant method to control pests and diseases. The current threat of climatic changes and limiting resources for agriculture (water, fertilizer) require improved resistance to abiotic stresses.

Ambition and goal

With this multidisciplinary and innovative STW programme we want to mine the natural reservoir of plant defense mechanisms. This will be done by using state-of-the-art high-throughput technologies to explore the natural potential and exploit mechanisms, genes and markers to develop novel resistance mechanisms against biotic and abiotic stresses for plant breeding.

In nature plants have co-evolved with a large variety of attackers. Therefore, wild species, such as *Arabidopsis thaliana*, harbour a fantastic reservoir of natural adaptive mechanisms to respond to (a)biotic stresses that to date have not been systematically explored. In the past decade, *Arabidopsis* has been adopted world-wide as the ideal model for plant science and an impressive molecular genetic toolbox has since been developed (e.g. the full genome sequence, the availability of well-characterized *Arabidopsis* populations, full-genome microarrays and metabolomics protocols). Hence, exploring natural variation in the defense responses of *Arabidopsis* to a large variety of (a)biotic stresses will yield important new insights into how plants selectively adapt to stresses, and provide novel concepts for sustainable agriculture and resistance breeding.

Objectives

1. To explore natural varation in resistance to abiotic and biotic stresses in *Arabidopsis* populations through an integrated multidisciplinary approach.
2. To identify mechanisms underlying natural resistance to abiotic and biotic stresses in *Arabidopsis*
3. To develop methods to analyze complex datasets on different types of resistance
4. To exploit information gained on natural variation in *Arabidopsis* to identify molecular markers that can assist in breeding for resistance to abiotic and biotic stresses in crop plants.

Focus and results at the end of the programme

To this end *Arabidopsis* ecotype and RIL populations can be exploited to analyze the degree of resistance to a diversity of microbial pathogens, herbivorous insects and abiotic stresses and their interaction. Using large-scale bioinformatics this information can be integrated with transcriptomics and metabolomics, to select genotypes and lines that can be used for in-depth analysis of the resistance mechanisms. The information gained from this comprehensive approach will lead to the identification of genes and molecular markers for different resistance mechanisms. These mechanisms will be characterized at the molecular, biochemical and physiological level and can subsequently be used to screen large numbers of lines of various crop species for orthologous genes involved in similar resistance mechanisms.

Innovation

Never before has the natural variation in plant defenses against different biotic and abiotic stresses and their interaction been investigated in such a comprehensive, multidisciplinary programme. To date, solutions to individual (a)biotic stresses have been sought. However, this has not resulted in a systems approach that results in durable solutions for a range of stresses.

Appendix

Due to the large amount of information presented, including the supplementary data of several of the chapters of this thesis in the printed version was not feasible. Thus, an electronic version is provided through the following link:

https://www.dropbox.com/sh/26rid0hw4pzplrw/AACQpa3h-crc_GvEztWGTOKTa?dl=0

Through this link you will be able to access the supplementary data for chapters 2, 4, 5 and 6 and supplementary figures and tables for chapter 3.

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