Exploring the genetics underlying the responses to consecutive combinations of biotic stresses and drought in *Arabidopsis thaliana*

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Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 7 October 2016

at 11 a.m. in the Aula.

per La meglio gioventù

Content

List of Abbreviations:

FW   Rosette fresh weight

DW   Rosette dry weight

WC   Water content in rosette

PLA   Projected rosette area

ACC   Accessions

GWA   Genome-wide association

QTL   Quantitative trait loci

GxE   Genotype by environment interaction

CHAPTER 1

General Introduction

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Plants growing in natural environments are exposed to a broad range of biotic and abiotic factors that are known to cause stress symptoms in many species. These stresses can be: pathogen attack, insect herbivory, drought, extreme temperatures, UV radiation, salinity, etc. (Pareek et al., 2010; Robert-Seilaniantz et al., 2010). Biotic and abiotic stress-inducing determinants often adversely impact plant growth and development, frequently leading to severe annual yield losses in agricultural production (Pierik et al., 2013; Pieterse et al., 2012; Stam et al., 2014).

Stress responses in plants

In response to a myriad of biotic and abiotic stress triggers, plants mobilize signaling molecules, thereby boosting the accumulation of so-called second messengers such as reactive oxygen species (ROS), calcium ions (Ca^{2+}), phospholipids, mitogen-activated protein kinases (MAPKs), G-proteins, and phytohormones (Smékalová et al., 2014; Takahashi et al., 2011; Tuteja and Sarvajeet, 2012). Various stress signaling pathways are interconnected in an overarching network, influenced by key regulators, such as transcription factors and MAPKs, responding to, and affecting the production of stress-related plant hormones and ROS (Caarls et al., 2015; Fujita et al., 2006; Pieterse et al., 2009b; Rejeb et al., 2014; Robert-Seilaniantz et al., 2011). Under stress conditions, ROS are generated rapidly and act as a common message to activate MAPKs (Jalmi and Sinha, 2015). MAPK- and Ca^{2+}-induced activation of the calcium-dependent protein kinases (CDPKs) are two major signaling cascades triggered in response to both biotic and abiotic stresses (Colcombet and Hirt, 2008). The last step of the delineated defense mechanism is the subsequent induction of transcription of stress-responsive genes. While the resultant production of an array of plant hormones has been associated with a variety of stress triggers (of both biotic and abiotic nature), high accumulation of abscisic acid (ABA) was recorded under specific abiotic conditions, such as drought, osmotic imbalance, salinity, and low temperature (Tuteja, 2007). Further, this hormone was shown to negatively interact with biotic stress response cascades. For instance, the drought-associated accumulation of ABA coincided with decreased levels of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Adie et al., 2007; Koornneef and Pieterse, 2008). Moreover, ABA can modulate

the crosstalk between the JA-regulated signaling pathway and those under control of salicylic acid (SA), gibberellins (GAs), and auxins (AUXs) (Abe et al., 2003; Dombrecht et al., 2007; Kazan and Manners, 2013; Yamaguchi-Shinozaki and Shinozaki, 2005).

Genes involved in response to biotic and abiotic stress

Reactive oxygen species (ROS) as stress-response gene activators

In the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*), a few hundred genes involved in the ROS scavenging network were identified (Mittler et al., 2004). Among them, *SUPEROXIDE DISMUTASE 2* (*FSD2*), *FSD3*, *ASCORBATE PEROXIDASE 1* (*APX1*), *APX2*, and *MONODEHYDROASCORBATE REDUCTASE 4* (*MDAR4*) were shown to be transcriptionally induced upon heat, cold, drought, and salinity stresses (Mittler et al., 2004). Furthermore, zinc finger and WRKY transcription factor genes (ZF- and WRKY-TFs) are induced by ROS. For example, the zinc finger protein ZAT12 is required for the expression of *APX1* and responds transcriptionally to multiple triggers, including intense light, freezing, osmotic and oxidative stress, wounding, and pathogen infection (Fujita et al., 2009; Rizhsky et al., 2004), while another zinc finger protein, ZAT6, positively regulates responses to drought, salinity, as well as bacterial infection (Shi et al., 2014). Concurrently, *WRKY25* is transcriptionally induced under multiple conditions, such as wounding, heat, osmotic and oxidative stress, and appears to be regulated by ZAT12 (Rizhsky et al., 2004), whereas WRKY70 interacts with ZAT7 in regulating plant disease response and the SA- and JA-mediated signaling pathways (Li et al., 2004) .

Mitogen-activated protein kinases (MAPKs)

MAPKs often engage in cross-talk with ROS signaling pathways. For instance, the OXIDATIVE SIGNAL-INDUCIBLE 1 (OXI1) kinase plays a role in ROS sensing and is required for activation of MPK3 and MPK6 (Forzani et al., 2011; Smékalová et al., 2014). These two are involved in disease resistance, drought and salt tolerance, and abscisic acid (ABA) signaling (Frei dit Frey et al., 2014; Tsugama et al., 2012; Yu et al., 2010). Moreover, MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1 (MEKK1), a common MAP kinase kinase kinase (MPKKK), is activated by ROS under various biotic and abiotic conditions.

In biotic stress situations, MEKK1 mobilizes MKK4/5 and stimulates expression of *MPK3/6* for innate immunity response, while under abiotic stress, the kinase activates MKK1/MKK2 for the expression of *MPK4* and *MPK6*, thereby affording cold and salt acclimation (Jalmi and Sinha, 2015).

Hormone signaling-regulated transcription factors

Transcription factors, such as ERF1, BOS1, RD26, MYB2, MYC2, RD22, ANAC019 and ANAC055 are associated with both ABA and JA-ET signaling (Koornneef and Pieterse, 2008; Shinozaki and Yamaguchi-Shinozaki, 2007). They are expressed under multiple biotic and abiotic stresses, including drought, salinity, osmotic imbalance, as well as infection by *Fusarium oxysporum* and *Pieris rapae* herbivory (Abe et al., 2003; Anderson et al., 2004; Fujita et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Vos et al., 2013). Further, multiple transcription factors regulate the expression of defense- and wounding-responsive genes. For instance, ANAC019 and ANAC055 are expressed under drought stress, they interact with MYB2 and MYC2 to up-regulate the expression of wounding-responsive genes *VSP1* and *LOX2* (Bu et al., 2008; Kazan and Manners, 2013).

Cross-talk between drought, pathogen, and insect herbivore response mechanisms

Drought is one of the most ubiquitous negative abiotic environmental factors influencing plants. Under drought stress, the accumulation of ABA triggers stomatal closure to reduce water loss via transpiration (Rai and Takabe, 2006; Xiong and Ishitani, 2006). As a consequence, water uptake and assimilation are diminished, resulting in lower nutrient take up, which leads to lower biomass (Verslues et al., 2006). Adaptive responses to drought involve metabolic, osmotic, and structural adjustment, as well as production of proteins with DNA damage-control and DNA repair functions (Ingram and Bartels, 1996). ABA-dependent and ABA-independent drought-response signaling pathways were described by Shinozaki and Yamaguchi-Shinozaki (2007). In addition, JA and ET have been implicated as important regulators of drought response (Bray, 1997; Shinozaki et al., 2003; Riera et al., 2005; Huang et al., 2008).

Plant pathogenic fungi can generally be classified as necrotrophs or biotrophs depending on whether they kill their host cells or not. *Botrytis cinerea* (hereafter referred to as *Botrytis*) is a necrotrophic fungus and is considered to be the second most important plant pathogen (Dean et al., 2012), infecting over 200 cultivated plant species and causing significant economic damage to crops worldwide. It kills plant tissue prior to feeding deploying diverse mechanisms that cause decay, e.g., enzymatic degradation of the cell wall, generation of toxic reactive oxygen compounds, and secretion of host non-selective toxins (Diaz et al., 2002; El Oirdi et al., 2011; Geraats et al., 2002; Rowe et al., 2010; Thomma et al., 1998; Thomma et al., 1999). While plant defense against biotrophic pathogens commonly involves SA, fighting necrotrophic invaders often requires the action of JA and ET (Pieterse et al., 2009). ABA and SA have been shown to exert negative effects on resistance to necrotrophic fungi (El Oirdi et al., 2011; Liu et al., 2015; Vos 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 chains (Agrawal, 2011; Cyr and Pace, 1993; Johnson, 2011). JA is an important primary signal in herbivore-induced local and systemic defense mechanisms in various plant–herbivore interactions, while ABA plays a modulating role in the JA-responsiveness (Bodenhausen and Reymond, 2007; Howe and Jander, 2008; Soler et al., 2013; Vos et al., 2013). *Pieris rapae* (hereafter *Pieris*), the Small Cabbage White butterfly, is among the most destructive pests of cruciferous plants as it has adapted to the glucosinolate-derived glycoside toxins produced by crucifers as chemical deterrents (Hopkins et al., 2009). SA is reported to inhibit the JA-dependent defense pathway induced by *Pieris* feeding (Koornneef et al., 2008).

One of the signaling pathways that modulate the cross-talk between *Botrytis*, *Pieris*, and drought stresses is the MYC2-related signaling pathway. MYC2 is a positive regulator of drought stress response induced by ABA as well as a negative regulator of the *Botrytis* defense gene *PDF1.2* through interaction with ERF1 within the JA-signaling pathway. What is more, MYC2 down-regulates the expression of other pathogen defense genes, such as *PR3* and *PR4*, while

boosting that of the *Pieris* defense-responsive gene *VSP2* (Anderson et al., 2004; Lorenzo et al., 2004;Dombrecht et al., 2007; Vos et al., 2013).

Effects of consecutive stresses on plants

Abiotic stresses can significantly affect plant responses to biotic strains and vice versa, depending on the timing, nature, and severity of the afflicting events (Atkinson and Urwin, 2012; Appel et al., 2014; Rejeb et al., 2014). Both positive and negative interactions among various stress factor combinations have been reported (Suzuki et al., 2014). For instance, a drought-stress-associated increase in cellular ABA concentrations causes plant stomata to close, which has a negative impact on photosynthesis. This often leads to elevated canopy temperatures and reduced relative humidity of the afflicted plant, which may render its microenvironment suboptimal for herbivores, adversely influencing their fitness and reproduction (Haile, 2000). At the gene level, drought-induced accumulation of ABA leads to up-regulation of specific transcription factors, such as CBF, NAC, and MYB. The TF activation, in turn, triggers elevated expression of pathogenesis-related (PR) genes, thus rendering the plant resistant to subsequent biotic stress. For example, the drought tolerance gene *Di19*-encoded ZF-TF modulates the expression of *PR1*, *PR2*, and *PR5* in *A. thaliana* (Liu et al., 2013).

Furthermore, increased ABA concentrations have a positive impact on callose deposition, which may boost plant resistance to fungal and bacterial pathogens (Mauch-Mani and Mauch, 2005). Studies with tomato plants showed that a drought-associated surge in endogenous ABA levels caused a 50% reduction in *Botrytis* infection (Achuo, 2006), while moderate drought stress in *Nicotiana benthamiana* led to decreased growth of its bacterial invader, *Pseudomonas syringae* pv. *tabaci* (Ramegowda et al., 2013).

On the other hand, exposure to herbivorous insects and pathogens can have both positive and negative impacts on plant response to subsequent drought stress (Ramegowda and Senthil-Kumar, 2015). For example, Brome Mosaic Virus (BMV), Cucumber Mosaic Virus (CMV), and Tobacco Mosaic Virus (TMV) infections enhance drought tolerance of *N. benthamiana* through reduction of

transpiration rate and a boost in osmoprotectant levels (Xu et al., 2008). The reverse is true of the infection of *A. thaliana* by the Turnip Mosaic Virus (TuMV), which causes lesions, mosaics, and mottling that reduce photosynthetic capacity, thereby rendering the plant more susceptible to subsequent drought stress (Prasch and Sonnewald, 2015).

Tailored responses of plants to stress combinations

Generally, plant responses to individual stress factors are different from those involved in countering a simultaneous or sequentially combination of detrimental triggers. Most studies have focused on a single time point in the stress response, representing only a snapshot of the transcriptional changes that are induced by a single or combinatorial stress (Stein and Waters, 2011; Shaik and Ramakrishna, 2013). Coding sequences involved in response to multiple factors can be identified through comparison of differentially expressed genes (DEGs) under various individual and combined stress treatments (Prasch and Sonnewald, 2013; Rasmussen et al., 2013). Transcriptome analysis on simultaneous effects of drought, heat, and virus infections in *Arabidopsis* resulted in the identification of 11 genes involved in all relevant single, double, and triple stress treatments (Prasch and Sonnewald, 2013). It was impossible though to determine how single stress responses led to responses to stress factor combinations. For instance, expression of the resistance gene *RPS6* was only responsive in case of simultaneous heat, drought, and virus treatments, but not under any of the double or individual stresses (Rasmussen et al., 2013a). As the responses of plants to blends of triggering factors cannot be predicted based on mechanisms countering their individual components, there is likely to be a unique combinatorial stress response, as has been suggested by Ramegowda and Senthil-Kumar (2015).

RNA-sequencing and genome-wide association mapping

Microarray and RNA-sequencing (RNA-seq) approaches are very useful in providing large, yet comprehensive, sets of gene expression data. However, for financial reasons, this strategy can be applied to examine but a limited number of genotypes at once. Natural variation, partly reflecting the adaptation to different environments, is one of the most important resources for genetic studies in *Arabidopsis* (Koornneef et al., 2004; Alonso-Blanco et al., 2005; Weigel, 2012). Due to the aforementioned constraints, most investigative efforts addressing plant responses to multiple individual stresses have focused on only a few genotypes (Stein and Waters, 2011; Rasmussen et al., 2013; Shaik and Ramakrishna, 2013). To fully understand the genetic architecture of complex traits, such as plant adaptation to a diversity of stress factors, a much wider survey of the natural genetic variation within a species is mandatory (if variation is present). Genome-wide association (GWA) analysis, relying on a large number of well-genotyped plant accessions, provides an important tool for natural genetic variation research approaches. Although the interest in natural variation and GWA mapping is rapidly increasing (Wijnen and Keurentjes, 2014; Ogura and Busch, 2015), no comprehensive evaluation of natural genetic variation in plants tolerant to simultaneous and sequential biotic and abiotic stress factor combinations was available at the time the hereby presented PhD research project had begun.

Scope of the thesis

In the research endeavors described in this thesis, *Arabidopsis* was used as a model organism to study plant responses to different sequential combinations of biotic factors (infection with *Botrytis* or herbivory by *Pieris*) and drought. The main objective was to identify genes that contribute to tolerance to the aforementioned sequential stress combinations.

The investigations described herein are part of a collaborative project, involving three PhD students, embedded within the national 'Learning from Nature' research program. My contribution involved application of large-scale experimental approaches to examine the genetics and physiology of variation in plant tolerance to sequential combinations of the indicated biotic/abiotic factors, mainly focusing on the response to drought or osmotic stress, in combination with biotic factors. With three single stress-inducing determinants, i.e., drought or osmotic stress, *Pieris* herbivory and *Botrytis* infection, six sequential combinations of treatments could be applied. RNA-seq was used to analyze the dynamics of transcriptome changes over time that occurred in *Arabidopsis* accession Col-0 in response to the single and combined treatments, whereas the GWA approach was employed to identify genes characterized by natural genetic variation contributing to different phenotypic responses to the investigated trigger combinations.

The main results of the 'Learning from Nature' program, integrating the GWA analyses of the aforementioned large set of *Arabidopsis* accessions (the HapMap population) challenged with 11 single environmental factors and several of their consecutive combinations, are reported in a comprehensive, multi-author paper presented as **Chapter 2**.

Responses to the consecutive biotic and drought/osmotic stresses were mapped using two types of the GWA methods. Genotype-by-environment (G×E) effects and phenotypic plasticity of several singe nucleotide polymorphisms (SNPs) relevant to stress responses were highlighted and the biological functions of some candidate genes were validated by gene expression and mutant analyses in **Chapter 3**.

Responses to consecutive drought and *Botrytis* infection were delineated using univariate GWA-mapping. Investigation results pertaining to phenotypic plasticity of significant SNPs as well as biological function of selected candidate genes are presented in **Chapter 4**.

Major outcomes of the whole-transcriptome profile analysis of *Arabidopsis* Col-0 exposed to three single stress triggers (*Pieris*, *Botrytis*, drought) and six consecutive combinations thereof are reported in a multi-author paper presented as **Chapter 5**.

The most relevant or significant findings of the whole-transcriptome profiling of *Arabidopsis* Col-0 exposed to biotic and subsequent drought stresses and the alternative splicing (AS) events identified under different conditions were investigated in detail and are described in **Chapter 6**.

Phenotypic responses of 16 *Arabidopsis* accessions challenged with different single or combinatorial stresses and the changes in expression levels of nine drought-responsive genes under alternative stress conditions are expounded upon in **Chapter 7**.

All findings presented in this thesis, in concert with their implications for future research, are comprehensively discussed in **Chapter 8**.

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CHAPTER 2

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, but stress responses are usually investigated for single stresses only. 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. After removing the effects of general robustness, for the 30 most significant SNPs average QTL-effect-sizes were larger 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.

- Our approach allowed for an unprecedented comprehensive genetic analysis of how plants deal with a wide spectrum of stress conditions.

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. Under natural conditions, these stresses do not occur in isolation but are commonly present simultaneously (Rizhsky *et al.*, 2004; Bergelson & Roux, 2010; 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). 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; Bergelson & Roux, 2010; Pieterse *et al.*, 2012; Stam *et al.*, 2014; Brachi *et al.*, 2015; Julkowska & Testerink, 2015; Kerwin *et al.*, 2015). Yet, investigating this in a targeted experimental way 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 the 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 (De Vos *et al.*, 2006; 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).

In plant breeding, resistance and tolerance to multiple stresses is a common selection target (Braun *et al.*, 1996). A well-known strategy to achieve resistance and tolerance is by evaluation of candidate varieties in multi-environment trials, i.e., field trials at multiple locations during several years (van Eeuwijk *et al.*, 2010; Malosetti *et al.*, 2013). In such trials, multiple stresses can occur, but their occurrence and the intensity with which they occur is not guaranteed and, therefore, plant breeders developed the concept of managed stress trials in which specific and well-defined stress conditions are imposed for a single or a small number of stresses (Cooper & Hammer, 1996; Cooper *et al.*, 2014). Recently, the urge to manage environmental factors even more precisely has led to the development of phenotyping platforms, where, again, mainly single stresses are investigated (Fiorani & Schurr, 2013; Granier & Vile, 2014; Kloth *et al.*, 2015).

Most studies, outside plant breeding, 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 full 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 carefully standardized 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). The standardization of these 15 stress conditions is an important element of the study because it allows for phenotyping of well-defined stress responses. We developed a tailored multi-trait GWA analysis that allowed the identification of candidate genes associated with plant responses to multiple stresses that were validated by gene expression and mutant analyses.

Materials and Methods

Arabidopsis thaliana population

In this study we included 350 Arabidopsis thaliana (L.) Heynh. accessions from the Hapmap population (http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls). 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.

Definition of the target traits

For every experiment, the target traits were derived from the individual plant data using the following strategy. First, when residuals deviated from normality, a logarithmic, arcsine or square root transformation was applied to the original observations. Second, genotypic (accession) means for each treatment were calculated using a mixed model to account for design effects. Different mixed models were used in the experiments, reflecting the different designs. In all cases, accession effects were modelled as fixed, and the accession means were the best linear unbiased estimator (BLUE) of these effects. Third, for traits measured in treatment and control conditions, differences or residuals (when regressing treatment on control values) were defined, in order to obtain a measure of stress tolerance that was corrected for the expression of the same trait under control conditions. Finally, within each experiment, the traits were replaced by the first principal component if the latter explained more than half of the variation in all traits in this experiment; in all other cases the original traits were retained. An overview of final traits and their corresponding sections in the Supplementary Methods can be found in Table 1. In case of replacement by the first principal component, original traits and the variance explained by the first principal component are listed (Supplementary Methods Tables M1-M5). In total, phenotypic data for 73 individual traits were obtained by 10 different research groups. All calculations were performed in R, unless stated otherwise. Mixed model analysis was performed with the R-package ASReml (Butler *et al.*, 2009). In all equations the term *E* denotes residual error.

All other terms represent fixed effects unless stated otherwise. A colon (:) is used to define interactions between terms.

Statistics

Genetic correlation networks and heritability

Pairwise marker-based genetic correlations between traits, genomic correlations, 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.

Narrow sense heritability (Table S1) was estimated using the mixed model $Y_i = \mu + A_i + E_i$ where Y_i represents the phenotypic means of accessions ($i=1,...,350$), and A_i and E_i random genetic and residual effects. The vector of additive genetic effects follows a multivariate normal distribution with covariance $\sigma_A^2\ K$, K being a marker-based relatedness matrix. The residual errors are independent, with variance σ_E^2. We obtained REML-estimates of σ_A^2 and σ_E^2, and estimated heritability as $h^2 = \sigma_A^2\ /\ (\sigma_A^2\ +\ \sigma_E^2)$. This is an estimate of narrow-sense heritability, since the model for the genetic effects only captures additive effects, and σ_E^2 is the sum of environmental and non-additive genetic effects (see e.g. Kruijer *et al.* (2015)).

Multi-trait mixed models

Following Zhou and 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, $vec(E)$ follows a zero mean normal distribution with covariance $V_e \otimes I_n$, where V_e accounts for the non-genetic correlations between traits.

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 $V_e = diag(\sigma_{e,1}^2, ..., \sigma_{e,p}^2)$ was chosen for the environmental covariances. For V_g a second order factor analytic structure was chosen $V_g = \sigma_g^2(\lambda\lambda^t + diag(\tau_1^2, ..., \tau_p^2))$, where σ_g^2 represents a scale parameter, the magnitude of genetic effects, the *p x 2* matrix λ contains the trait specific scores belonging to the factor analytic part of the model that provides a rank two variance-covariance structure between traits, and $diag(\tau_1^2, ..., \tau_p^2)$ provides trait specific residual genetic variances (Piepho, 1997; Meijer, 2009). The model was fitted with the R-package ASRreml (Butler *et al.*, 2009).

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, as explained below.

Compressed kinship was calculated as the average kinship within genetic groups. Genotypes were assigned to k genetic groups by performing Ward clustering based on the squared Euclidean distance along the first $k - 1$ principal components calculated from a matrix of standardized SNP scores, followed by cutting the resulting dendrogram into k distinct clusters (van Heerwaarden *et al.*, 2012; Odong *et al.*, 2013; van Heerwaarden *et al.*, 2013).

The use of a compressed kinship matrix requires a choice of the level of compression, as determined by the number of genetic groups over which the individual kinship is averaged. This choice needs to balance the gain in computational efficiency with model fit (Zhang *et al.*, 2010) and the ability of the compressed matrix to capture the correlation between genetic dissimilarity and phenotypic differences, which is ultimately the reason for including a kinship matrix in the association model. There are currently no standard methods to determine the optimum level of compression, at least not when used in a multi-trait setting. We determined the appropriate level of compression for each association model based on the model likelihood, convergence and correspondence between kinship and phenotypic and geographical similarity. The latter was quantified as the Frobenius norm of the difference between the complement of the compressed kinship matrix, expanded to a block matrix of full rank, and the Euclidean distance matrix of phenotypic traits or geographic coordinates. We considered a range of 4 to 100 groups. Correspondence with phenotypic and geographical dissimilarity increased steeply from 4 to around 35 groups, after which correspondence with geographic distance increased more slowly and the correspondence with phenotypic distance showing a local decrease until 58 groups. Model likelihood was relatively stable above 4 groups but convergence was erratic depending on the modeled contrasts. For each model the number of groups was therefore chosen to be the minimum number of groups needed to achieve a level of correspondence approximating that found at 35 groups, under condition of model convergence.

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 effects $\beta_1, \dots, \beta_p$ (contained in B). To identify general QTLs with trait-specific effects, for individual markers, the null hypothesis $\beta_1 = \beta_2 = \cdots \beta_p = 0$ 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 $\beta_1 = \beta_2 = \cdots \beta_p = \beta \neq 0$ was tested. To identify potentially 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: $\beta_1 = \beta_2 = \cdots \beta_{p1} = \alpha_{abiotic}$; $\beta_{p1+1} = \beta_{p1+2} = \cdots \beta_p = \alpha_{biotic}$ and $H_0: \alpha_{abiotic} = \alpha_{biotic}$ versus $H_a: \alpha_{abiotic} \neq \alpha_{biotic}$. For the Wald test for the hypothesis $\beta_1 = \dots = \beta_p$ we first fit the MTMM $Y = XB + G + E$ with XB only containing trait specific means $\mu_1, \dots, \mu_p$, and next test hypotheses on the marker effects. The contrast is defined through a partitioning of the traits in two groups (e.g. resistance against biotic or abiotic stress). Using the R-package asreml (Butler *et al.*, 2009) we perform Wald tests for the following hypotheses:

1. $H_0 : \beta = 0$, in the constrained model $\beta_1 = \dots = \beta_p = \beta$.
2. $H_0 : a_1 = a_2$, in the constrained model where a_1 is the effect on all traits in the first group, and a_2 for traits in the second group.

Simulations to compare power for full MTMM, contrast MTMM and univariate analysis

We further compared the different Wald tests using simulations, described in more detail in the Supplementary Methods (SM.12). Specifically, we compared the performance of the general MTMM (i.e. testing the hypothesis $\beta_1 = \beta_2 = \cdots \beta_p = 0$) with the MTMM used for the contrasts (i.e. $H_0: \alpha_{group\,1} = \alpha_{group\,2}$, where, within two predefined groups of traits, all SNP-effects equal $\alpha_{group\,1}$ respectively $\alpha_{group\,2}$). We simulated phenotypic data for given genotypic data, either assuming SNP-effects positive (but not equal) within one group of traits and negative for the other (Scenario A), or the sign of each SNP effect being chosen randomly (Scenario B). The simulation results as presented in Fig. S11

(see later) clearly indicate that the Wald test for the contrast has superior power under scenario A, while the general MTMM performs best under scenario B. In both cases, univariate analysis of the trait with the highest heritability is outperformed by at least one of the MTMM analyses. As a consequence, univariate GWAS and GWAS with the general and contrast MTMM give different rankings of SNPs.

Selecting candidate genes

A significance threshold of $P < 0.0001$ was chosen after implementation of genomic control (see below). For MTMM this resulted in 43 SNPs meeting this criterion. Such a threshold of 0.0001 is not uncommon in studies involving single trait GWAS (e.g., El Soda et al. 2015; van Rooijen et al. 2015; Kooke et al. 2016). Given the total number of analysed SNPs (i.e. 199589 SNPs having a minor allele frequency above 0.05) and under the null hypothesis of no QTLs and independence of the markers, we arrive at a naive estimate for the expected number of false positives of around 20, which is considerably smaller than the 43 SNPs with $P < 0.0001$ recorded in the full MTMM, suggesting that about half of the significant SNPs must be true positives. Furthermore, following the procedure described by Benjamini and Hochberg (1995), we estimated the false discovery rate to be 0.45, a number very comparable to our naive estimate above. SNPs within a 20kb region were considered to be part of one LD block. This resulted in 30 genomic regions. For presentation purposes, each LD block was represented in Figures and heatmaps by the SNP with the on average strongest (absolute) effect across all traits. For the GWA contrast analyses, the same procedure was followed to define LD blocks and representative SNPs.

Correcting for genomic inflation

The Wald test is known to suffer from some inflation (Zhou & Stephens, 2014), which we correct for by using genomic control (GC) (Devlin & Roeder, 1999; Devlin *et al.*, 2001), which divides the observed test statistics $T_1, ..., T_p$ by the genomic inflation factor. For both the unconstrained MTMM and the MTMM for contrasts described above, we observed inflation for small as well as large *P*-values (i.e. also more *P*-values close to one than expected). Consequently, the usual genomic control procedures based on the observed versus expected median of test statistics gave too optimistic inflation factors. We therefore applied an alternative genomic control procedure, in which we regress the observed $-\log_{10}(P)$ values on the expected ones, and correct the observed $-\log_{10}(P)$ values for the slope. The genomic inflation factor was 1.24 for the full MTMM, with similar values for the other MTMM analyses (between 1.07 and 1.38). For the full MTMM without correction for population structure (i.e. taking the kinship to be the identity matrix), the inflation factor was 2.36.

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. Correcting for the respective control means that in the residual signal for a trait, effects of earliness, flowering time, general robustness, vigour, etc., have been removed already. Therefore, the traits as analysed represent a kind of stress *per se* response from which all kinds of disturbances have already been eliminated. 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 carefully standardized stress treatments, the trait definitions and phenotyping, see Supplementary Methods.

Table 1. Phenotypes assessed. The dataset contains three plant stress categories applied to *Arabidopsis thaliana*; 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. Yellow indicates abiotic stress, green indicates biotic stress and blue indicates combinations of biotic and abiotic stress.

	Stress	Trait name	Section of Supplementary Methods	Trait phenotype	Treatment
Abiotic stresses	Salt	Salt_1	SM.1	Main root length, number of lateral roots and straightness	75 mM NaCl
		Salt_2	SM.1	Main root length	125 mM NaCl
		Salt_3	SM.1	Number of lateral roots	125 mM NaCl
		Salt_4	SM.1	Main root angle	125 mM NaCl
		Salt_5	SM.2	Biomass	25 mM NaCl
	Drought	Drought_1	SM.2	Biomass	Drought
		Drought_2	SM.3	Biomass	Drought
	Osmotic	Osmotic	SM.2	Biomass	PEG8000
	Heat	Heat	SM.2	Number of siliques	35 °C

Biotic stresses	Parasitic plant	Parasitic plant	SM.4	Attachments	*Phelipanche ramosa*
	Nematode	Nematode	SM.5	Offspring, eggmass	*Meloidogyne incognita*
	Whitefly	Whitefly_1	SM.6	Survival, whiteflies	*Aleyrodes proletella*
		Whitefly_2	SM.6	Reproduction, eggs	*A. proletella*
	Aphid	Aphid_1	SM.7	Behavior T1, probing	*Myzus persicae*
		Aphid_2	SM.7	Behavior T2, probing	*M. persicae*
		Aphid_3	SM.7	Offspring, aphids	*M. persicae*
	Thrips	Thrips_1	SM.8	Feeding damage	*Frankliniella occidentalis*
		Thrips_2	SM.8	Behavior T1	*F. occidentalis*
		Thrips_3	SM.8	Behavior T2	*F. occidentalis*
	Caterpillar	Caterpillar_1	SM.9	Leaf area consumed	*Pieris rapae*
		Caterpillar_2	SM.3	Biomass	*P. rapae*
		Caterpillar_3	SM.3	Number of damaged leaves and feeding sites	*P. rapae*

Abiotic and biotic stress	Fungus	Fungus	SM.10	Number of spreading lesions	*Botrytis cinerea*
	Double stress	Fungus and caterpillar_1	SM.3	Biomass	*B. cinerea* and *P. rapae*
		Fungus and caterpillar_2	SM.3	Number of damaged leaves and feeding sites	*B. cinerea* and *P. rapae*
		Caterpillar and fungus	SM.10	Number of spreading lesions	*P. rapae* and *B. cinerea*
	Double stress	Drought and fungus	SM.10	Number of spreading lesions	Drought and *B. cinerea*
		Drought and caterpillar	SM.3	Number of damaged leaves and feeding sites	Drought and *P. rapae*
		Caterpillar and osmotic_1	SM.9	Projected leaf area	*P. rapae* and PEG8000
		Caterpillar and osmotic_2	SM.9	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 (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; $h^2=0.6$) (Table S1). The traits related to combined stresses have predominantly low

heritabilities; however, it should be emphasized that the combined stresses especially relate to combinations involving fungal and caterpillar stress.

### 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). 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 basis underlying the responses to sets of single and combined stresses (Figure S2).
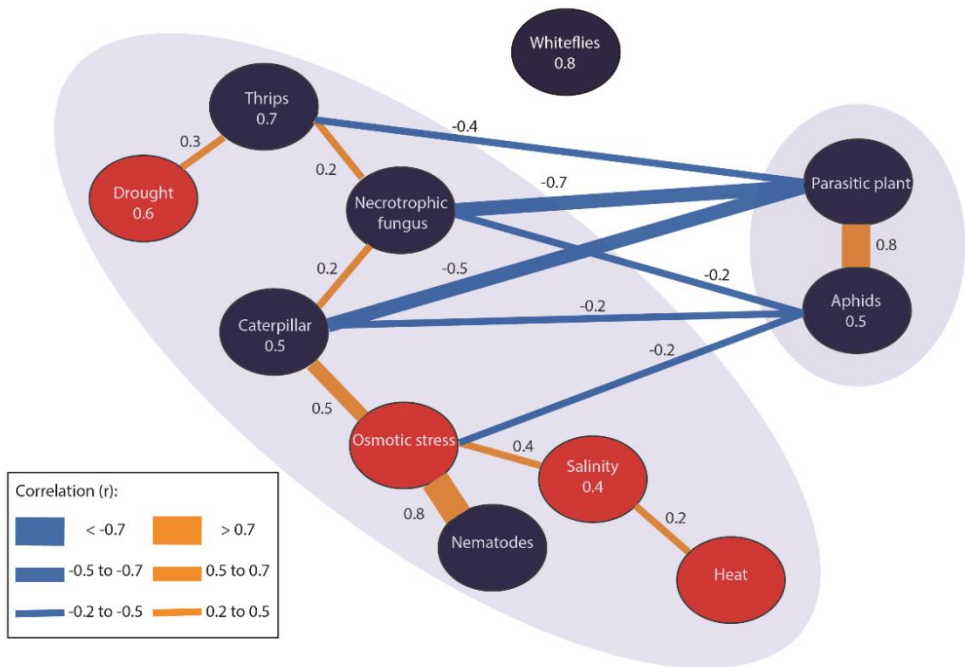
Figure 1. Mean genetic correlations between responses of *Arabidopsis thaliana* to abiotic (red) and biotic (dark blue) plant stresses. Thickness of lines represents the strength of mean genome-wide correlations, annotated with r values (orange=positive, blue=negative correlation). The more shared genetic 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.

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 and 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 significant SNP with the strongest effect was chosen to represent the locus (Figure 2; Table S2). Clustering of stresses by estimated SNP-effect profiles (Figure 2) indicates 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 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 (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* (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.
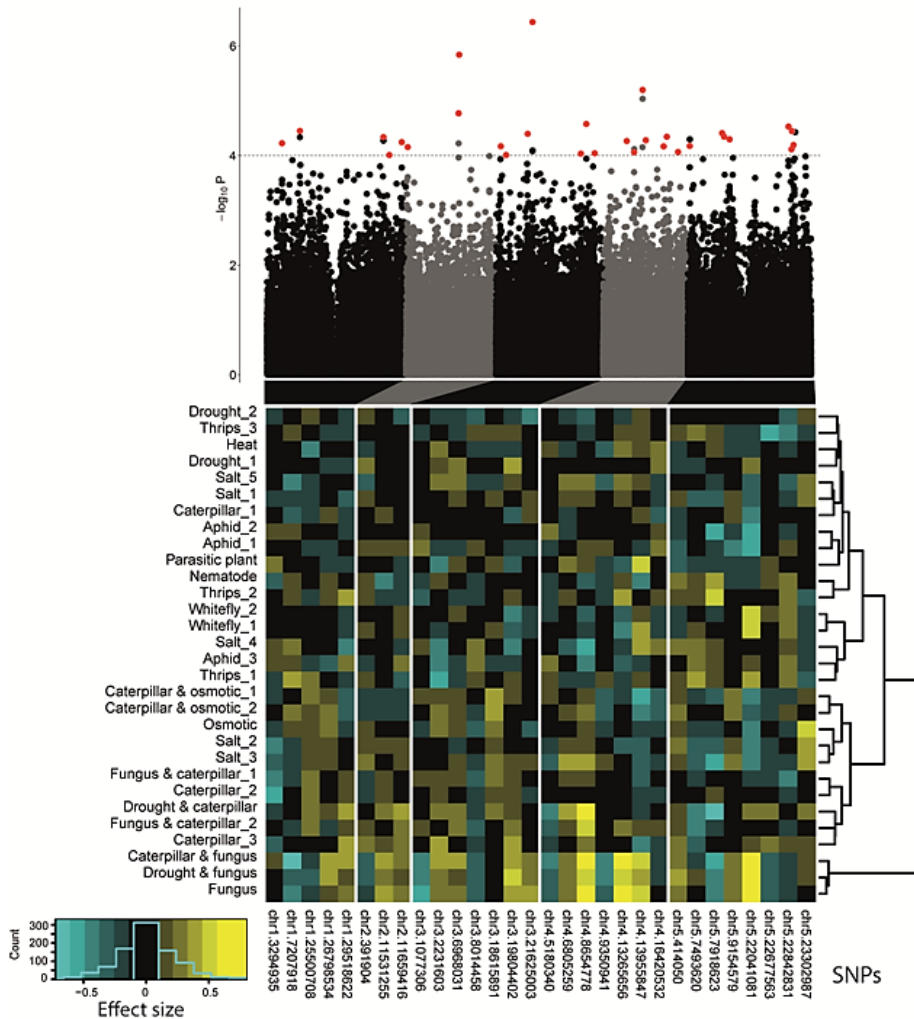
Figure 2. Multi-trait mixed-model (MTMM) GWA mapping with 30 different stress responses of *Arabidopsis thaliana*. The top panel shows the 214k SNPs with their corresponding -log$_{10}$(P) values for the five chromosomes. The lower panel depicts the trait-specific effect sizes of the rare alleles for significant SNPs (*P*<0.0001) as estimated by the full MTMM. When several SNPs were located within the 20 kb linkage disequilibrium half-windows around the most significant SNP in a region, the effects for the SNP with the on average strongest absolute effects are shown (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, using Ward's minimum variance method. The key shows the frequency distribution for the effect sizes of the SNPs.

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Table 2. Candidate Arabidopsis thaliana 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**	Responsiveness	References
chr2.11659416	AT2G27250	CLV3	One of the three CLAVATA genes controlling the size of the shoot apical meristem (SAM) in Arabidopsis	unknown	(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.	Heat, salt & heat, heat & Silwet	(Martiniere et al., 2012; Peret et al., 2012; Rasmussen et al., 2013; 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.	flagellin	(Yu et al., 2013)
chr5.7493620	AT5G22570	WRKY38	Member of WRKY Transcription Factor; Group III	SA, Pseudomonas	(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.	Cold, flagellin	(Jurkowski et al., 2004; Keisa et al., 2011; Chin et al., 2013; Rasmussen et al., 2013)
chr5.23302987	AT5G57560	TCH4	Encodes a cell wall modifying enzyme, rapidly upregulated in response to environmental stimuli	Heat, heat & silwet, heat & salt, heat & high light, high light, high light & cold, high light & salt	(Braam & Davis, 1990; Xu et al., 1996; Purugganan et al., 1997; Iliev et al., 2002; Rasmussen et al., 2013)

* 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*	Responsiveness	Reference
chr1.30381439	AT1G80820	CCR2	*CINNAMOYL COA REDUCTASE*. Encodes a cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis.	Cold & flagellin & silwet	(Luderitz & Grisebach, 1981; Lauvergeat et al., 2001; Zhou et al., 2010; Rasmussen et al., 2013)
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*.	Cold & flagellin & silwet	(Chen et al., 2010a; Pandey et al., 2010; Liu et al., 2012; Rasmussen et al., 2013)
chr1.6038270	AT1G17610	CHS1	*CHILLING SENSITIVE 1*, mutant accumulates steryl-esters at low temperature.	Cold & high light	(Rasmussen et al., 2013; 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.	ABA, salt	(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.	Cold	(Sung et al., 2007; Bond et al., 2009; Finnegan et al., 2011)
chr5.23293119	AT5G57560	TCH4	Encodes a cell wall-modifying enzyme	Heat, heat & silwet, heat (Braam & Davis, 1990; Xu et al., & salt, heat & high light, 1996; Purugganan et al., 1997; high light, high light & Iliev et al., 2002; Rasmussen et cold, high light & salt al., 2013)	
chr5.23293870	AT5G57490	VDAC4	Encodes a voltage-dependent anion channel (VDAC: AT3G01280/VDAC1)	*Pseudomonas*	(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)	unknown	(Chen et al., 2010b)

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

Phytohormonal signaling underlying contrasts in stress responses

The MTMM framework allowed imposing constraints on the values of the estimated QTL effects, thereby providing a powerful testing framework for QTLs that have a common effect for the stresses belonging to one particular group of stresses as contrasted to the effect for another group of stresses (see Materials and Methods section 'Multi-trait GWAS'). 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 (Figure S4). Seven of these SNPs, were in LD with one or more genes known to be involved in JA-, SA- or resistance-related signal transduction (Table S4). Among these genes are *LOX5*, whose product is involved in facilitating aphid feeding (Nalam *et al.*, 2012a; Nalam *et al.*, 2012b), *MYB107* encoding a transcription factor responsive to SA (Stracke *et al.*, 2001; Chen *et al.*, 2006), the JA-inducible genes *TPS02* and *TPS03* encoding terpene synthases (Huang *et al.*, 2010) and *MES16*, encoding a methyl jasmonate esterase (Christ *et al.*, 2012). Using TAIR10 annotations, we found that in total there are 371 genes that have an annotation related to JA and SA signaling (JA-SA genes). Our GWA analysis identified significant SNPs inside or in a 20 kb neighbourhood of five of those. In the remainder of the genome, i.e. non JA-SA, we identified 162 genes close to or with significant SNPs. So, in candidate regions for JA-SA, we had a ratio of 5/371 = 1.35% significant genes, while in non-candidate regions, we found 162/27863 = 0.58%. This is an enrichment of 2.33 times, significant at α=0.05 (Fisher exact probability test, mid-P value < 0.046; Rivals *et al.* (2007)). Following Atwell *et al.* (2010), an upper bound for the false discovery rate is then 1 / 2.33 = 0.43.

In addition to screening for SNPs with contrasting effects, we screened for SNPs with a similar effect across the above-mentioned trait clusters (Figure S5)

and found candidate genes involved in oxidative stress and plant responses to salinity and pathogens (Table S5).

QTLs underlying contrasts in responses to biotic and abiotic stresses

We expected a negative correlation between the responses to abiotic and biotic stresses due to antagonistic interactions between ABA and the SA and JA/ET pathways (Anderson *et al.*, 2004; Fujita *et al.*, 2006; De Torres Zabala *et al.*, 2009; Kissoudis *et al.*, 2015). Testing for this contrast within the GWA analysis using our MTMM approach significantly identified 43 SNPs with a QTL effect that changed sign between biotic and abiotic conditions. For presentation purposes, traits were grouped by a cluster analysis across SNPs, while SNPs were grouped by clustering 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). 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). Transcription data (Figure S6) support the notion that these genes play a contrasting role in responses to abiotic and biotic stresses and reveal an antagonistic responsiveness between ABA and JA treatment (*TCH4*) or a specific responsiveness to either ABA (*AtCCR2*, *ASG1*, *ATVDAC4*) or JA (*ATWRKY40*). This is in line with the hypothesis that there are antagonistic effects between abiotic stress responses, predominantly involving the ABA pathway, and wound and biotic stress responses involving the JA-ET or SA pathways (Kissoudis *et al.*, 2015). Previous studies have, however, also revealed an overlap in abiotic and biotic plant responses, such as similar transcriptomic perturbations after salinity and pathogens stress (Ma *et al.*, 2006). A screen for QTLs with similar effects on resistance to biotic and abiotic stress (Figure S7) identified three genes annotated to be responsive to stress stimuli (Table S6). Transcriptional data show that these genes respond differentially to different (a)biotic stresses and phytohormones (Figure S8). *ARGAH2*, encoding an arginase enzyme with a role

in the metabolism of polyamines and nitric oxide, is involved in both SA- and JA-mediated resistance to both biotrophic and necrotrophic pathogens, and is also responsive to abiotic stimuli such as temperature, salt and light intensity (Figure S8) (Jubault *et al.*, 2008; Gravot *et al.*, 2012; Rasmussen *et al.*, 2013). *PKS1* is known to be involved in adaptation in plant growth in response to light (Fankhauser *et al.*, 1999; Molas & Kiss, 2008), but also seems to be responsive to *Botrytis* (Figure S8). These genes are promising candidates for consistent effects across biotic and abiotic stresses.

e
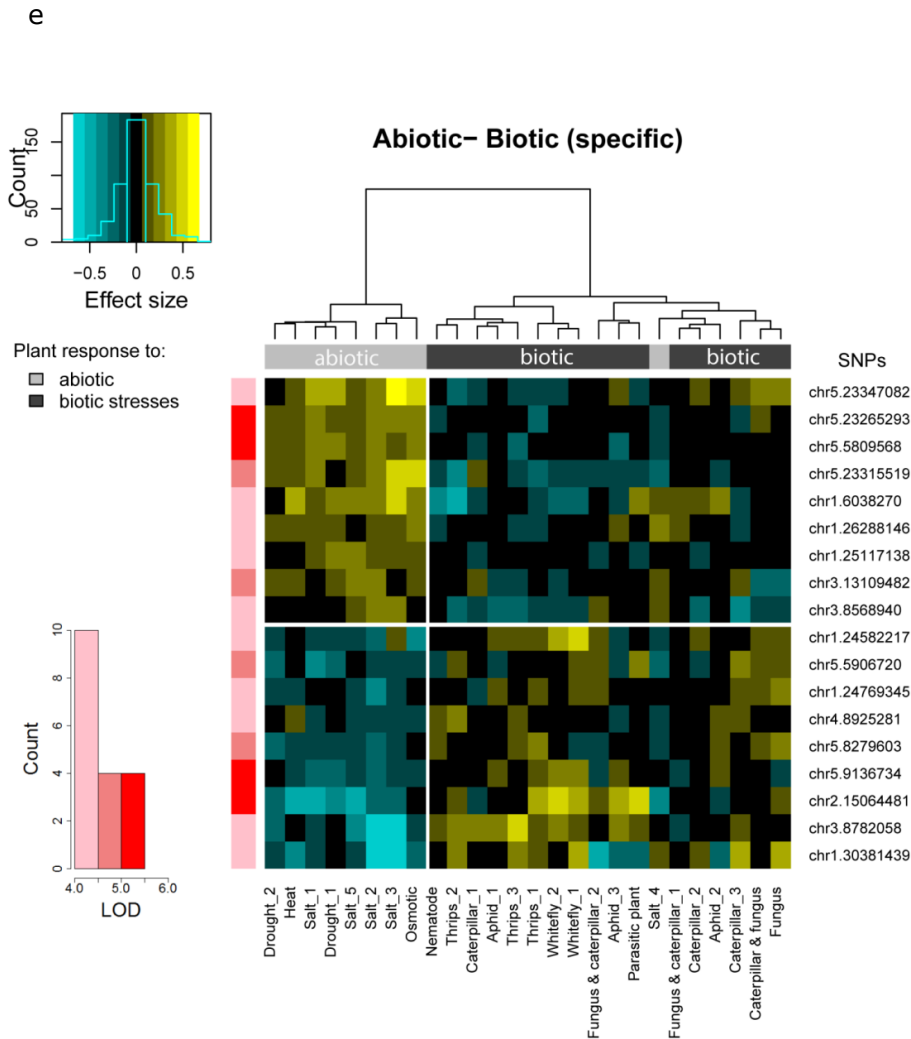
Figure 3. Genetic associations specific for contrasting responses of *Arabidopsis thaliana* to abiotic and biotic stresses. Genetic associations (in red) were estimated with a contrast-specific GWA analysis using MTMM. For exploratory purposes, significant SNPs ($P \leq 10^{-4}$) for the biotic-abiotic contrast were clustered on their trait-specific effect sizes as estimated in the full MTMM, that is, without imposing a contrast restriction on the SNP effects. If there was another SNP in LD that had a higher effect size, this SNP was used a representative for the LD block. Negative effects (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effects (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 were clustered on the basis of SNP effects using Ward's minimum variance method. The key shows the frequency distribution of SNPs across effect sizes.

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 (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 aboveground tissues, yet a downregulation in the root tissues (Winter *et al.*, 2007). Marker chr5.16012837 showed the strongest signal for similar effects on responses to below- and aboveground stresses (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 SM.11), being more resistant to caterpillar feeding and osmotic stress (Figure 4). *RMG1* (AT4G11170) encodes an NB-LRR disease resistance protein, which acts as a pattern-recognition receptor (PRR) that recognizes evolutionarily conserved pathogen-derived signatures, and transcription is 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 stress. Gene expression data show that *RMG1* is upregulated by several abiotic and biotic stresses (Figure 4). In addition, gene ontology enrichment analysis of the co-expression network of *RMG1* shows an overrepresentation of genes involved in immune responses and maintenance of ion homeostasis. The latter is based upon co-expression with five genes

encoding glutamate receptors (*GLR1.2*, *GLR1.3*, *GLR2.5*, *GLR2.8*, and *GLR2.9*), putatively involved in ion-influx-mediated long-distance signaling of wound, pathogen and salt stress (Ma *et al.*, 2006; Mousavi *et al.*, 2013; Choi *et al.*, 2014; Kissoudis *et al.*, 2015). 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.
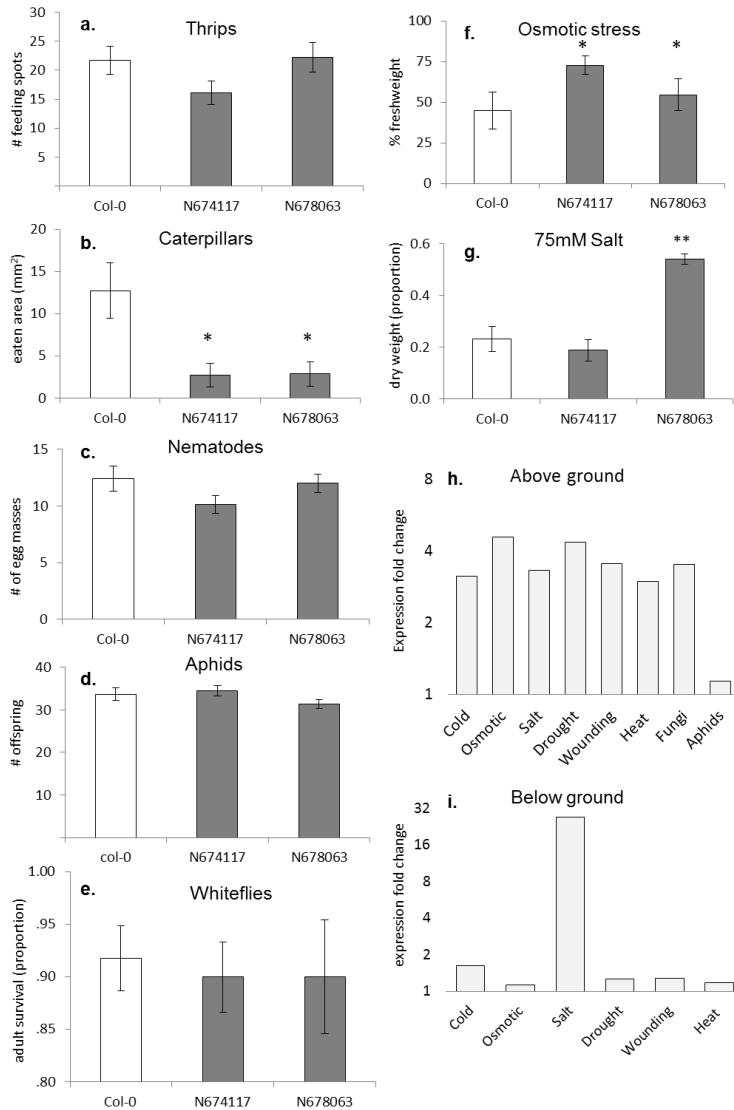
Figure 4. Phenotypes of *RMG1* T-DNA mutant screenings for *Arabidopsis thaliana*.
Phenotypes are given for two T-DNA lines in the *RMG1* gene and for Col-0 as control. a.
Number of thrips feeding spots on a detached leaf, 6 days post infestation (N=24); b. Leaf
area consumed by *P. rapae* caterpillars (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 < 0.10, *: 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).

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

We developed a novel mixed-model approach to multi-trait GWA mapping with a special feature for testing contrasts between groups of stresses to identify the genetic architecture underlying a total of 30 stress response traits in Arabidopsis. The strength of our statistical approach was that our multi-trait mixed model accounted simultaneously for dependencies between genotypes and between traits, providing a natural and appropriate correction for multiple testing, while maximizing power for the detection of QTLs for the stress contrast under study. As we addressed a large number of stresses, our phenotyping experiments were distributed across a series of laboratories and were not performed simultaneously. To mitigate as much as possible the occurrence of QTLs induced purely by experiment-specific differences in plant management and environmental control, our phenotypic responses were defined in terms of control-corrected responses. This type of correction will emphasize QTLs for resistance and tolerance per se and will decrease detection power for QTLs related to development and viability.

The extensive phenotyping executed in this study was done under carefully controlled conditions in climate chambers. Ideally, phenotyping should be done in nature because that is where genetic variation is exposed to natural selection (Bergelson & Roux, 2010; Brachi *et al.*, 2010; Brachi *et al.*, 2013). Here, we have phenotyped the plant population to 15 different stresses under laboratory conditions and our data show an interesting pattern based on genetic correlations that matches with phytohormonal signalling underlying stress responses (Figure 1). This indicates that the genetic architecture recorded here is biologically relevant. Drought and salt stress responses share signal-transduction mechanisms (Zhu, 2002) which is represented by the genetic correlations recorded (Figure 1). Insect damage is commonly associated with drought or osmotic stress and this is also clear from overlap in underlying phytohormonal signalling (Pieterse *et al.*, 2012). Figure 1 shows that drought stress and osmotic stress correlate with insect stresses. Extending studies of genetic variation and the genetic architecture underlying responses to multiple stresses to natural conditions will be an important next step (Bergelson & Roux, 2010).

Through the approach developed here, candidate genes for 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 ($P < 0.007$, Table S2). This suggests 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. However, the combined stresses included in this study especially involve fungal and caterpillar stresses. Future studies including other combined stresses are needed to further investigate the suggested pattern.

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 (Korte *et al.*, 2012; Zhou & Stephens, 2014; section 'Multi-trait GWAS' in Materials and Methods). For a comparison of the MTMM analysis with single-trait analyses, see 'Simulations' in Materials and Methods, SM. 12 and Figures S11 and S12. 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.

Acknowledgements

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Supporting Information

Additional supporting information may be download via link

https://www.dropbox.com/sh/hb95t5dwvxbsc1u/AAB2Z7Q1DPSwCCU-oaTBBi2Pa?dl=0

Supplementary tables

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

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

Table S3: 125 candidate genes derived from the Multitrait Mixed Model analysis.

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.

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.

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.

Supplementary figures

Figure S1: Narrow sense heritability for *Arabidopsis thaliana* resistance to abiotic and biotic stresses.

Figure S2: Genetic and phenotypic correlation matrix.

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.

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.

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.

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.

Figure S7: Genetic associations common for plant responses to abiotic and biotic stresses.

Figure S8: Expression data of 3 candidate genes (resulting from MTMM, see Supplementary Table S6) in plants exposed to biotic or abiotic stress factors, relative to control conditions.

Figure S9: Genetic associations specific for plant responses to either below- or aboveground stress.

Figure S10: Genetic associations common for plant responses to below- and aboveground stresses.

Figure S11: Power of MTMM in simulations.

Figure S12: Comparison of SNPs identified by MTMM and Univariate GWAS.

Supplementary methods

SM.1 Salt

SM.2 Abiotic

SM.3 Caterpillar – combinatory stress

SM.4 Parasitic plants

SM.5 Nematodes

SM.6 Whiteflies

SM.7 Aphids

SM.8 Thrips

SM.9 Drought – combinatory stress

SM.10 Fungus – combinatory stress

SM.11 Screening of T-DNA lines

CHAPTER 3

Genome wide association analysis reveals candidate genes controlling a negative effect of biotic stresses on consecutive osmotic stress response

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Abstract

While the response of *Arabidopsis thaliana* to either drought, herbivory or fungal infection has been well-examined, the consequences of exposure to a series of such (a)biotic stresses are not well studied. This work reports on the genetic mechanisms underlying the *Arabidopsis thaliana* (Arabidopsis) response to a priming pre-treatment with a biotic agent, followed by drought, a 'combinatorial stress' treatment. Two genome-wide association (GWA) mapping approaches were employed on a set of 350 Arabidopsis accessions. Plants were exposed separately to either fungal infection using *Botrytis cinerea* or herbivory using *Pieris rapae* caterpillars, and then subjected to a PEG treatment to simulate drought. Alternatively, plants were subjected to PEG without any pre-treatment. Multi-environment (ME) GWA-mapping identified a number of SNPs associated with candidate genes for which allelic variation affects the plant response to combinatorial stresses in a gene-by-environment interaction (GxE) mode. Univariate GWA-mapping identified additional SNPs that affect phenotypic plasticity in response to the combinatorial stress treatment when compared to the single PEG treatment. SNPs associated with a gene of unknown function and *DROUGHT INDUCED 19 (DI19)* were identified by both approaches, supporting their potential involvement in the combinatorial stress response. Several SNPs were found to be in linkage disequilibrium with known stress-responsive genes such as *PEROXIDASE 34* (*PRX34), BASIC LEUCINE ZIPPER 25* (*bZIP25*), *RESISTANCE METHYLATED GENE 1* (*RMG1*), and *WHITE RUST RESISTANCE 4 (WRR4)*. New roles were found for some of these known stress-responsive genes, e.g. for *PRX34* in their response to the combined *Pieris* and PEG treatment, and *RMG1* to *Botrytis* and PEG. In general, antagonistic interactions were found between biotic and drought stress responses, and found a negative effect of biotic stresses on consecutive osmotic stress response. The results of this study illustrate the complexity of combinatorial stress responses, but also offer new leads to improve tolerance of crop species to multiple stresses.

Keywords

Arabidopsis thaliana, genome-wide association mapping, genotype-by-environment interaction, QTL x E, (a)biotic stress response, multiple stress.

Introduction

In the field, plants are simultaneously or sequentially exposed to various biotic and abiotic stress inducing factors which limit plant performance and cause a substantial annual reduction in agricultural production (Hirt and Shinozaki, 2004). Plant responses to biotic and abiotic stresses are mainly regulated by abscisic acid (ABA), in response to abiotic stresses such as drought and salt (Shinozaki and Yamaguchi-Shinozaki, 2007); jasmonic acid (JA) and ethylene (ET), in response to necrotrophic fungi and chewing insects; and salicylic acid (SA), which activates the plant defence response upon infection with biotrophic fungi (Pieterse et al., 2009; Dodds and Rathjen, 2010). Alongside these major compounds, plant hormones such as brassinosteroids (BRs), auxins (AUXs), and cytokinins (CKs) are also involved in regulating the response of plants to biotic and abiotic stresses (Hirt, 2009; Pareek et al., 2010; Pieterse et al., 2012). Negative interactions have been observed between JA and SA, ABA and SA, ABA and JA, and ABA and ET; while positive interactions have been observed between ABA and AUX (Yoshioka and Shinozaki, 2009).

The interaction between biotic and abiotic stress responses is often mediated by different plant hormones and the outcome can be synergistic, antagonistic, or neutral. The impact of abiotic stresses on plant responses biotic strains can significantly, and vice versa (Atkinson and Urwin, 2012; Appel et al., 2014; Rejeb et al., 2014). For instance, drought increased ABA concentrations have a positive impact on callose deposition, which may boost plant resistance to fungal and bacterial pathogens (Mauch-Mani and Mauch, 2005; Achuo et al., 2006; Ramegowda et al., 2013). Turnip Mosaic Virus (TuMV), which causes lesions, mosaics, and mottling that reduce photosynthetic capacity, thereby rendering the plant more susceptible to subsequent drought stress (Prasch and Sonnewald, 2015).

Generally, plant responses to individual stress factors are different from those involved in countering detrimental triggers that are either simultaneous or sequentially combined. The consecutive exposure to biotic and abiotic stresses triggers specific multiple-stress-responsive gene expression, indicating the existence of a unique combinatorial stress signalling network (Atkinson and Urwin, 2012; Sewelam et al., 2014). For instance, Rasmussen et al (2013)

showed that 61% transcription changes in response to the combinatorial stress cannot be predicted from the single stress responses. Prasch and Sonnewald (2013) showed that the expression of the resistance gene *RPS6* was only regulated in case of simultaneous heat, drought, and virus treatments, but not under any of the double or individual stresses. Transcriptome analysis on simultaneous effects of drought, heat, and virus infections in *Arabidopsis* resulted in identification of 11 genes involved in all relevant single, double, and triple stress treatments (Prasch and Sonnewald, 2013), with those responding to stress factor combinations impossible to determine in presence of single triggers (Rasmussen et al., 2013).

Most studies on the response of plants to multiple stresses have only focused on a limited number of genotypes (Narsai et al., 2013; Shaik and Ramakrishna, 2013; Sewelam et al., 2014; Ramegowda and Senthil-Kumar, 2015), which is fine to understand the general physiology of the response, but insufficient to understand the genetic architecture of such response. For Arabidopsis it is attractive to investigate the natural genetic variation of a trait using genome-wide association (GWA) mapping, which gives high genetic resolution for traits which have high heritability and are controlled by a modest number of genes (Baxter et al., 2010; Li et al., 2010; Chao et al., 2012; Bac-Molenaar et al., 2015; Ogura and Busch, 2015).

In the research reported here, the genetic architecture of the Arabidopsis response to a biotic stress, followed by drought, was examined. Arabidopsis plants were either exposed to *Pieris rapae* larvae, to induce response to herbivory, or to the necrotrophic fungus *Botrytis cinerea*, to induce a fungal infection response, and then subjected to a PEG treatment which induces an osmotic stress, simulating drought. These two treatments will be further referred to as '*Pieris* and PEG' and '*Botrytis* and PEG'. Control plants were subjected to the single PEG treatment without any pre-treatment. The purpose of our study was to 1) determine the heritability of the single and combined treatments for different growth parameters; 2) identify SNPs associated with these phenotypes through different GWA approaches; 3) identify candidate genes linked to these SNPs, for which allelic variation contributes to the trait variation; and 4)

determine if there is any genotype by environment interaction affecting the phenotypes, that could be linked to allelic variation.

Materials and Methods

Plant material and growth conditions.

For the GWA-mapping experiments, the Arabidopsis HapMap population (Li et al., 2010) was used. Seeds of most T-DNA insertion mutants (Supplementary Table 1) were ordered from the European Arabidopsis Stock Centre (http://Arabidopsis.info/), except for seeds of the *drought-induced19* (*di19*) mutant (Liu et al., 2013), which were obtained from the authors.

Prior to experiments, seeds were stratified at $4^{o}C$ in the dark for 5 days. Thereafter, one seed per accession was sown on one Grodan® Rockwool cube of 40 X 40 X 40 mm. Plants were watered 3 times / week (on Monday, Wednesday, and Friday, respecitvely) with a nutrient solution developed for Arabidopsis with pH = 7 and EC = 1.4 mS/cm. The nutrient solution consisted of 1.7 mM NH_4^+, 4.5 mM K^+, 0.4 mM Na^+, 2.3 mM Ca^{2+}, 1.5 mM Mg^{2+}, 4.4 mM NO_3^-, 0.2 mM Cl^-, 3.5 mM SO_4^{2-}, 0.6 mM HCO_3^-, 1.12 mM PO_4^{3-}, 0.23 mM SiO_3^{2-}, 21 µM Fe^{2+} chelated using 3% diethylene triaminopentaacetic acid, 3.4 µM Mn^{2+}, 4.7 µM Zn^{2+}, 14 µM BO_3^{3-}, 6.9 µM Cu^{2+}, and <0.1 µM MoO_4^{4-}. Plants were grown in a climate-controlled growth chamber set to short day condition, i.e. 10 h day/14 h night, at $21^{o}C$ day/$19^{o}C$ night temperatures and 70% relative humidity. Irradiation was set at 200 µmol $m^{-2}s^{-1}$.

## Preparation of Pieris rapae caterpillar and Botrytis cinerea spores

Pieris rapae caterpillars were reared on cabbage plants (*Brassica oleracea* convar. capitata var. alba) under greenhouse conditions (24°C, with natural daylight). Butterflies were fed on the nectar of flowering plants such as *Lantana camara*. When flowers were scarce, additional food solution consisting of 20% honey and 10% sucrose was introduced to butterflies. Inbreeding of the existing population was minimized by adding wild butterflies and caterpillars from the Dutch Flevopolder. After starving for 1h, first-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described by Van Wees et al., (2013).

Botrytis cinerea strain B05.10 (Staats and Van Kan, 2012) was grown for 2 weeks at room temperature on half-strength potato dextrose agar (PDA; Difco Laboratories) plates containing 100 µg/ml penicillin and 200 µg/ml streptomycin. Spores were collected, filtered through glass wool and then re-suspended in half-strength potato dextrose broth (PDB; Difco Laboratories) to a final density of 1×10^5 spores/ml. Three hours after incubation, spores were used for inoculation by applying two 5-µl droplets on two Arabidopsis leaves as described by Van Wees et al., (2013).

Single and combinatorial stresses

The HapMap population : For single osmotic treatment, 19-day-old plants were irrigated with Hyponex nutrient solution that contained 7.7% w/v Polyethylene Glycol (PEG) 8000 for 6 days ("PEG"). For combinatorial stress, 17-day-old plants were exposed to one *Pieris* 1st instar larva or inoculated with two 5-µl droplets of *Botrytis* spore suspension for 1 day. The next day, the caterpillars were removed and the plant continued to grow in the control conditions for 1 day, followed by irrigation with Hyponex solution that contained 7.7% w/v Polyethylene Glycol (PEG) 8000 for 6 days ("*Pieris* and PEG" and "*Botrytis* and PEG"). Plants that did not receive any stress treatment were used as a control (Figure 1).
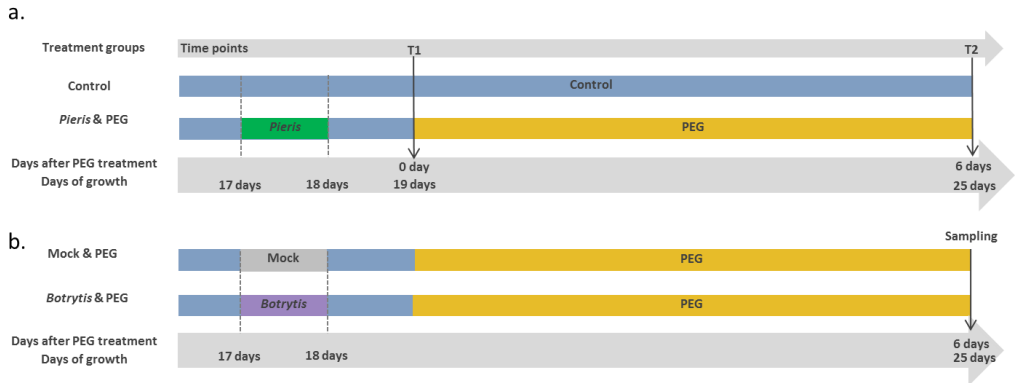
Figure 1. Experimental design for treatment of Arabidopsis plants subjected to Mock and PEG, *Botrytis cinerea* infection and PEG, or *Pieris rapae* herbivory and PEG, and control conditions. Conditions are indicated in different colours: control (dark blue); PEG (yellow); *Botrytis* treatment (purple); Mock treatment (grey); and *Pieris* treatment (green). Vertical arrows indicate sampling time points (T1, T2, Sampling). Vertical dashed line indicates before and after biotic treatment.

Testing T-DNA mutants: In addition to the four treatments described in the previous paragraph, For single *Pieris* treatment, 17-day-old plants were exposed to either one *Pieris* 1st instar larva or two 5-µl droplets of *Botrytis* spore suspension for 1 day. Thereafter, the caterpillars were removed and the plant was allowed to grow in control conditions for 6 days ("*Pieris*" and "*Botrytis*") (Figure 2).

Testing extreme accessions from the HapMap population: This experiment was carried out with the same design as described in the previous paragraph.
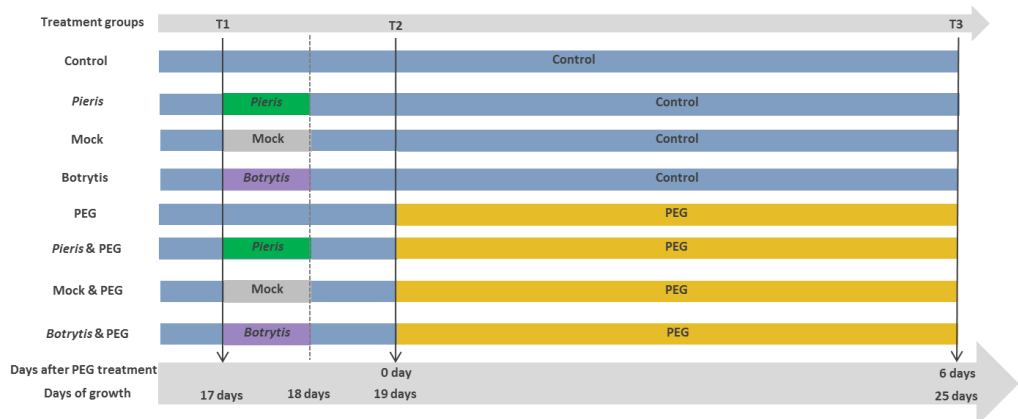
Figure 2. Experimental design for treatment of Arabidopsis plants subjected to PEG, *Botrytis cinerea* infection*, Pieris rapae* herbivory*, Mock, PEG, Mock and PEG, Botrytis* infection and PEG, or *Pieris rapae* herbivory and PEG, and control conditions. Conditions are indicated in different colours: control (dark blue); PEG (yellow); *Botrytis* treatment (purple); Mock treatment (grey); and *Pieris* treatment (green). Vertical arrows indicate sampling time points (T1 – T3). Vertical dashed line indicates after biotic treatment

Plant phenotyping

For all plants used for GWA mapping, projected Leaf Area (PLA) were measured. For the combined *Pieris* and PEG treatment, plants were measured directly after the *Pieris* pre-treatment (T1), and 6 days after the PEG treatment (T2) (Figure 1.a). For the other treatments the rosettes of too many plants were overlapping and the data was not used for mapping. Instead, for the *Botrytis* and Mock & PEG treatments, rosette fresh weight (FW) and dry weight (DW) were measured after the PEG treatment. (Figure 1.b). Rosette water content (WC) was calculated using the formula: $\frac{fresh\,wight - dry\,weight}{fresh\,weight}$.

Genome-Wide Association mapping

For PLA, residuals were calculated from the regression analysis between the PLA after *Pieris* treatment (T1) and PLA after PEG treatment (T2), whereas, residuals of DW and WC were calculated from the regression between the *Botrytis* & PEG and mock & PEG. These residual values were used for mapping. For Multi-Environment (ME) GWA mapping (Korte et al., 2012) a minor allele frequency (MAF) of 0.05 was used and a –log(p-value) of 4 as arbitrary threshold value for significance as described by El-Soda et al., 2015). Univariate GWA-mapping was performed using the scan_GLS software as described by

Kang et al. (2010) and Kruijer et al (2015). In brief, this approach involves performing generalized least squares (GLS) calculations conditional on the variance components, which were estimated in the model without markers and can efficiently handle genetically identical individuals. Thereafter, SNPs with MAF<0.05 were excluded. The proportion of explained phenotypic variance was the criterion to identify significant SNPs as described by (Sun et al., 2010). The information of SNPs in the linkage disequibrium (LD) with the significant SNPs were identified by the online LD tool (http://dev3.ab.wur.nl/AthaLD), as well as the information of coding sequence substitution and amino acid substitution.

Selection of candidate genes

Gene ontology annotation of selected candidate genes for the quantitative trait loci (QTL) identified after the GWA analysis was found in The Arabidopsis Information Resource (TAIR; www.Arabidopsis.org/tools/bulk/go/index.jsp). Genes characterized by one or more of the following criteria were considered as candidates: 1) Genes known to be responsive to biotic and abiotic stimulus and expressed under abiotic stress and hormonal (JA and ABA) treatments; 2) Genes with reported physiological functions in response to stress conditions and expressed under abiotic stresses and hormonal (JA and ABA) treatments (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). 3) Genes with reported physiological functions in response to stress conditions and responsive to biotic and abiotic stimuli.

T-DNA mutants of the candidate genes were ordered from the European Arabidopsis Stock Centre if available as homozygotes.

Comparing drought and PEG8000-induced osmotic stress

Four-week-old Col-0 plants were subjected to a PEG treatment as described above and compared to soil-grown plants subjected to drought by withholding water for 6d. The expression of five drought-responsive genes; *RD26* (*AT4G27410*)*, MYC2* (*AT1G32640*)*, RD29b* (*AT5G52300*)*, RD29a* (*AT5G52310*)*, and *P5CS1* (*AT2G39800*) (Shinozaki and Yamaguchi-Shinozaki, 2007) was determined by real-time quantitative reverse transcription PCR (qRT-PCR) under both treatments.

RNA was extracted from rosettes following the protocol described by Onate-Sanchez and Vicente-Carbajosa (2008). cDNA was synthesized from 800 ng of total RNA using an iScript cDNA synthesis kit (Bio-Rad). cDNA was diluted ten times before qRT-PCR. The gene with ID At3g15930 was selected as reference for qRT-PCR as the most stably expressed across different drought, osmotic, and *Botrytis* treatments based on the transcriptome database Genevestigator (refgenes.org/rg). The qRT-PCR was performed using iQ SYBR$^®$ Green Supermix (cat. no. 170-8885) on a Bio-Rad CFX96 real-time PCR system set at 95°C for 4 minutes, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. Relative gene expression was calculated using the 2(-ΔΔCT) method (Livak and Schmittgen, 2001). The standard error were calculated from at least 3 plants per accession. Primers of the five drought-responsive genes mentioned in the previous paragraph are provided in Supplementary Table 2. Primers of all investigated candidate genes are provided in Supplementary Table 3.

Statistical analysis

The broad sense heritability was calculated using the equation $H^2 = \frac{Var(G)}{Var(G)+Var(E)}$, in which G = Genotype, E = Environment and Var = Variance. Pearson's correlation coefficient 'r', one-way and two-way ANOVAs were performed using GenStat for Windows 16th Edition (VSN International Ltd., Hemel Hempstead, UK). Student's t-test was used to compare the growth of the same accession between treatments. Three-way ANOVA was performed as described by (Sokal RR, 1981). Statistical tests of FW, DW and WC were performed on 4 plants per accession, whereas gene expression was performed on 3 plants per accession.

Results

Drought stress can be mimicked by exposure to polyethylene glycol

To minimize non-genetic, environmental, variation affecting the phenotypes of the Arabidopsis Hapmap population used for GWA analysis, plants were grown on rockwool, watered with nutrient solution, rather than on a peat-based substrate often used in growing Arabidopsis. This however limits the application of drought stress by withholding water, as rockwool is much more resilient to drying than peat-based substrates. To induce a stress mimicking drought, a polyethylene glycol (PEG) 8000 treatment was applied, which gave similar plant phenotypes after six days as plants grown on a peat-sand mixture left without water for six days. To show that also plant physiology was comparable, the expression of five genes involved in the ABA-dependent or the ABA-independent pathway of drought response (Shinozaki and Yamaguchi-Shinozaki, 2007) was determined in plants subjected to either treatment (Figure 3). Except for *RD29b*, the change of expression of these genes compared to control conditions, was indistinguishable. *RD29b* expression was induced in both treatments, only slightly, but significantly, more in the PEG treatment. Nevertheless, we conclude that the two treatments are sufficiently comparable to consider the PEG treatment as physiologically equivalent to drought by withholding watering, which allowed us to use rock-wool-based growth set-up that supports more uniform growth of Arabidopsis under control and stress treatments than a peat-based growth set-up.
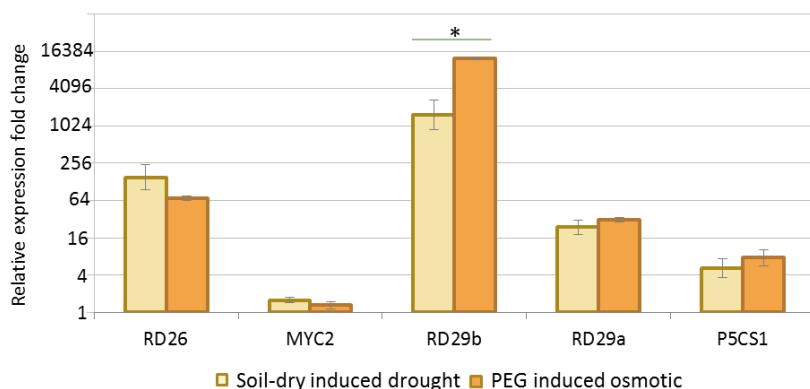
Figure 3. Relative expression of five drought-responsive genes in rosettes of plants either grown on a peat-based mixture, subjected to drought by withholding water (light orange) or grown on rock wool, watered with a nutrient solution containing PEG8000 (PEG), to induce an osmotic stress response (dark orange). Relative gene expression was determined by comparing expression in treated plants with expression in control plants (either growing in a well-watered peat mixture or on rock wool watered with a nutrient solution without PEG). A Student's t-test was used to compare expression of each gene in the different conditions. * indicates a significant difference ($p<0.05$). Standard errors were calculated based on at least four plants per treatment.

Phenotyping the Hapmap population under the Botrytis and the combinatorial stress

To obtain relevant trait values for genetic characterization of quantitative trait loci (QTLs) involved in the response to a combined stress treatment, plants of the Hapmap population were grown on rock wool and either pre-treated with *Pieris rapae* larvae (*Pieris* treatment), to induce herbivory, or with *Botrytis cinerea* (*Botrytis* treatment), to induce fungal infection, before exposure to a PEG treatment to induce osmotic stress (Figure 1-2). In this experiment, rosette fresh weight (FW) and projected rosette leaf area (PLA) were determined in the *Pieris* treatment and FW, PLA, rosette dry weight (DW) and the (FW-DW)/FW ratio, representing water content (WC) for the *Botrytis* treatment. The rosette FW measurements of plants exposed to different treatments were all significantly correlated, suggesting no major environmental disturbances or experimental errors (Supplementary Table 4). Broad sense heritabilities (H^2) were calculated for the measured traits in control and treated plants, which ranged from 0.37 to 0.63 (Supplementary Table 5), suggesting moderate to high heritability and good prospects of identification of QTLs upon mapping. Two-way ANOVA was performed to test the interaction between treatments and

accessions for PLA, FW, DW, and WC. Significant main effects of treatments and accessions were observed, but no but no significant interactions between accessions and treatments were found (Supplementary Table 6).

## *Univariate GWA-mapping of residuals identified SNPs associated with phenotypic plasticity*

Rather than mapping each trait for the respective treatment, we decided to map the residual values for each trait and accession combination obtained after regression of the trait value of stress-treated plants on the trait value of control-grown plants, or of combinatorial stress-treated plants on single stress-treated plants. The residuals represent the variations of the accessions (genotype) in response to stresses (environment) (Filiault and Maloof, 2012). To observe the effect of *Pieris* priming on plant growth in response to subsequent PEG, residuals was calculated from a regression of PLA of the *Pieris* and PEG stress treatment on PLA of the *Pieris* treatment alone. When these PLA residual values were used for GWA analysis, 60 associated SNPs were found, directly linked to 47 genes (Supplementary Figure 2.a, Supplementary Table 7), but with more genes in linkage disequilibrium (LD) with these SNPs. Eleven SNPs were mapped to a genomic region that spans only 19 kb on chromosome (Chr) 2, in which six genes are located (Figure 4.a), corresponding to TAIR gene IDs At2g36540-At2g36590. The combined boxplot of the haplotypes combining nine of these 11 SNPs showed a that accessions containing the non-Col-0 allele had comparably lower rosette PLA under the *Pieris* and PEG treatment vs. the single *Pieris* treatment than accessions containing the Col-0 allele (Figure 5.a and Supplementary Figure 2). SNPs m73992 -97 were located at gene with TAIR IDs At2g36560, significantly reduced PLA was observed in when comparing accessions carrying the non-Col allele to the accessions carrying Col-0 allele (Figure 5.b).

When the residuals resulting from the regression of the DW upon *Botrytis* and PEG treatment on the DW upon PEG treatment alone were used for GWA mapping, 26 associated SNPs were found, residing in 17 genes (Supplementary Figure 2.b, Supplementary Table 8). Nine associated SNPs mapped to a locus spanning 9.7kb of Chr 1, corresponding to four genes with TAIR IDs At1g56280-

At1g56310 (Figure 4.b). The combined boxplot of the haplotypes combining these nine SNPs (m33786 – m33809) showed a that accessions containing the non-Col-0 allele had comparably higher rosette DW under the *Botrytis* and PEG treatment vs. the single PEG treatment than accessions containing the Col-0 allele (Figure 5.c). Six (m33789 – m33798) of these 9 SNPs, residing in At1g56290, the non-Col-0 allele of the six SNPs exhibited significant higher DW under the *Botrytis* and PEG treatment vs. the single PEG treatment than accessions containing the Col-0 allele (Figure 5.d). The SNP marker m33786, residing in the *DROUGHT INDUCED 19 (DI19)* gene (At1g56280), significantly distinguished accessions carrying the non-Col-0 allele from those carrying the Col-0 allele (Figure 5.e), with the former allele contributing to a relatively higher DW after the combined treatment.

When mapping the residuals for WC, comparing the combined *Botrytis* and PEG treatment with the single PEG treatment, 23 associated SNPs were found, corresponding to 17 genes (Supplementary Figure 2.c, Supplementary Table 6). A set of SNPs identifies one region of 14.5 kb on Chr. 5, comprising genes with TAIR IDs At5g06480-At5g06530 (Figure 4.c, Supplementary Table 9). One of these genes is *ATP-BINDING CASSETTE TRANSPORTER G22* gene (At5g06530), mutation of which is known to cause increased water respiration and drought susceptibility (Kuromori et al., 2011). Accessions carrying the non-Col-0 haplotype for SNP markers m164494 and particularly m164506, residing in this region, had a lower relative WC upon *Botrytis* and PEG stress vs. PEG stress alone, when compared to accessions carrying the Col-0 allele (Figure 5.f & g)

Figure 4. Genomic region on (a) Genomic region on Chromosome 2 from (15324282bp to 1346000bp) contains six genes At2g36540-At2g365090 which were associated to 11 significant SNPs for *Pieris* and PEG responses (Supplementary Table 7). (b) Chromosome 1 from (21072000bp to 21088000bp) contains six genes At1g56280-At1g56330 which were associated to nine significant SNPs for *Botrytis* and PEG responses(Supplementary Table 8); (b) Chromosome 5 from 1970000bp to 2005000bo) contains five genes At5g06490 – At5g06530 which were associated to nine significant SNPs for *Botrytis* and PEG responses (Supplementary Table 9). Red colour indicates SNPs in high LD (LD > 0.8) with SNP m73995 (a), m33786 (b), or m164484 (c), which exhibited the highest –log(p) score among the SNPs. Yellow colour indicates SNPs in LD (LD > 0.3) with the SNP m33786, m73995 or m164484. Yellow boxes indicates gene exons. Horizontal dashed line indicates bonferroni threshold. Figure 4 is the GWA-mapping results which was obtained using an accelerated mixed model (AMM) method from online tool GWAPP (https://gwas.gmi.oeaw.ac.at). Same association region was found by both AMM and GLS (this project) methods.
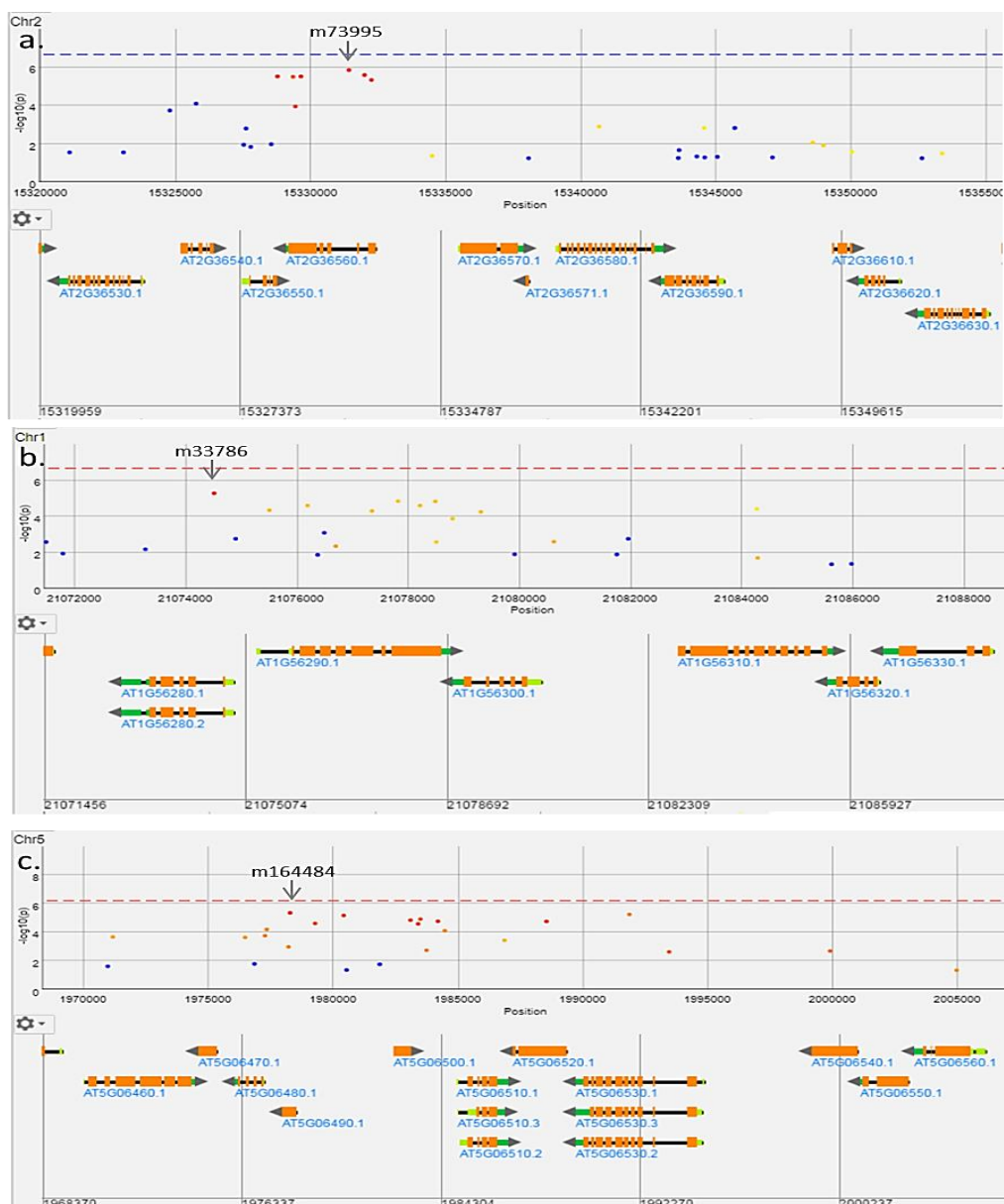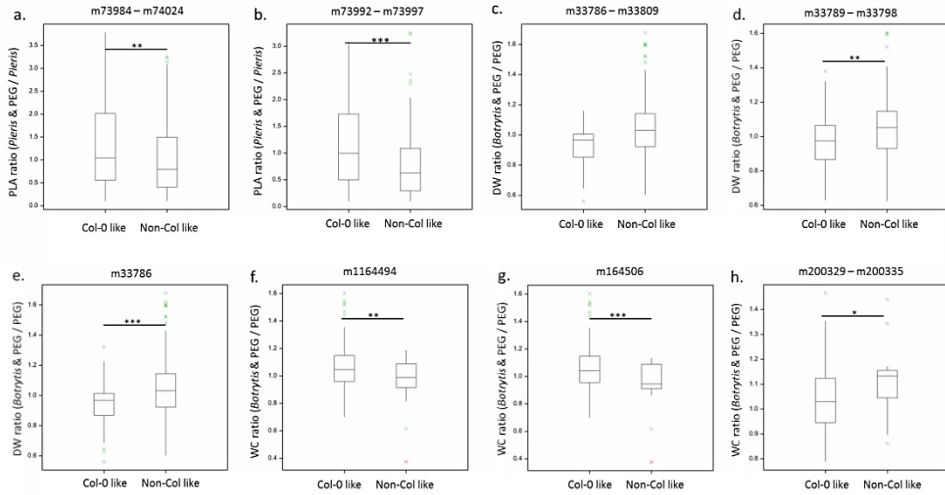
Figure 5. Boxplots shows the effect of two types alleles (Col-0 like and Non-Col like) of the combination of (a) 11 significant SNPs (m73984 – m74024) and (b) six (SNPs m73992 - m73997, residing in At2g36560, on project rosette area (PLA) under the *Pieris* and PEG treatment vs. the single *Pieris* treatment; the combination of (c) nine significant SNPs (m33786 – m33809), (d) six significant SNPs (m33789 – m33798, residing in At1g56290), and (e) one significant SNP m33786 (residing in *DI19*) on rosette dry weight (DW) under the *Botrytis* and PEG treatment vs. the single PEG treatment; Significant SNPs (e) m1164494, (f) m164506, and m200329 – m200335 on rosette water content (WC) under the *Botrytis* and PEG treatment vs. the single PEG treatment. Student's t-test was sued to compare the difference of the two groups. *indicates the difference between the Col-0 like allele and the Non-Col allele is *<0.05, **<0.01, ***<0.001.

Multi-environment GWA-mapping identified SNPs interacting with the stress treatments

Next to the univariate GWA mapping approach, we also employed Multi-environment (ME) GWA-mapping using a multi trait mixed model (MTMM) approach (El-Soda et al., 2015). This approach has the advantage over the first one that it can test multiple environment responses and thus is better in identifying SNPs representing QTL-by-environment interaction (QxE). This method was used to map PLA at the two time-points at which it was determined during the *Pieris* and PEG treatment, with T1 representing the *Pieris* pre-treatment and T2 representing the *Pieris* and PEG treatment (Figure 1). This revealed 39 associated SNPs (Supplementary Figure 3.a and supplementary table 9). Seven of these SNPs mapped to a region of Chr 2 containing the genes with TAIR IDs At2g36550 and At2g36560. This region was also found in the univariate GWA analysis (Supplementary Figure 2.a). Accession carrying the non-Col allele of gene with TAIR ID At2g36560 exhibited reduced PLA under the *Pieris* and PEG treatment vs. the single *Pieris* treatment when compared to accessions containing the Col-0 allele (Figure 5.b)

When mapping the QxE effect on DW, comparing the combined *Botrytis* and PEG treatment with the single PEG treatment, 10 associated SNPs, residing in nine genes were found (Supplementary Figure 3.b and supplementary table 10). One of these was the SNP identifying the *DI19* gene also identified upon univariate GWA analysis (Figure 4.b). The SNP m33786 was associated with DW and was mapped to an intron of gene *Di19.* However, this SNP was in the intron but the Non-col like allele of SNP m33786 is more tolerant to the consecutive combination of *Botrytis* and PEG stress in comparison to the Col-0 like allele (Figure 5.e). When mapping the effect on WC for the same treatments, 43 associated SNPs were found, residing in 30 genes (Supplementary Figure 3.c and supplementary table 10). Four of these SNPs identify a region on Chr 3 containing associated with seven genes with TAIR IDs At3g22670-At3g22730 (Supplementary Table 10). The combined boxplot of the haplotypes combining these four SNPs showed a that accessions containing the non-Col-0 allele did not exhibit differences in WC under the *Botrytis* and PEG treatment vs. the single *Botrytis* treatment than accessions containing the Col-0 allele (Supplementary

Figure 4.a). In addition, six associated SNPs mapped to a region on Chr. 5 containing seven genes with TAIR IDs At5g48120-At5g48180. Boxplot of the haplotypes combining these six SNPs showed a that accessions containing the non-Col-0 allele did not exhibit differences in WC under the *Botrytis* and PEG treatment vs. the single PEG treatment than accessions containing the Col-0 allele (Supplementary Figure 4.b). Two SNPs m200317 and m200318 were residing in *MET18* gene (At5g48120). No significant differences in WC between accessions carrying the non-Col-0 alleles of the two SNPs under the *Botrytis* and PEG treatment vs. the single PEG treatment when compared to accessions containing the Col-0 allele (Supplementary Figure 4.c). SNPs m200329, m200331 and m200335, residing in the genes with TARI IDs At5g48130 and At5g48140. Boxplot of the haplotypes combing the three SNPs showed that accessions containing Non-Col-0 allele exhibited higher WC under the *Botrytis* and PEG treatment vs. the single *Botrytis* treatment than accessions containing the Col-0 allele (Figure 5.h). SNP m200389, residing in *NITRILE SPECIFIER PROTEIN 5 (ATNSP5)* gene (At5g48180), the non-Col-0 allele did not show difference in relative WC upon *Botrytis* and PEG stress vs. PEG stress alone, when compared to accessions carrying the Col-0 allele (Supplementary Figure 4.d). SNP m60883 was identified by both univariate and ME GWA-mapping approaches. is from C (Col-0 like) to G (non-Col like) led to a nonsynonymous amino acid substitution from Glu to Gln (A-A position E500Q. The SNP is residing in gene with TAIR ID AT2G13690, and the non-Col-0 allele did not show difference in relative WC upon *Botrytis* and PEG stress vs. PEG stress alone, when compared to accessions carrying the Col-0 allele (Supplementary Figure 4.e)

Amino acid substitution of the significant SNPs in response to combinatorial stress

Using ME and univariate GWA mapping approaches revealed a number of QTLs containing candidate genes that could play a role in the response to combinatorial stresses. To support the relevance of the identified loci, several SNPs were found using both methods (Figure 6). This involves only a few loci though, for the response to *Pieris* and subsequent PEG the QTL on Chr 2 comprising genes with IDs At2g36550 and At2g36560, linked to eight associated

SNPs and the QTL on Chr 3 around gene AT3G45910, linked to one associated SNP. For the response to *Botrytis* and PEG four common SNPs were found in both approaches, covering as many loci, around genes with IDs At1g48670, At1g56280 (*DI19*), At2g13690 and a larger region comprising At5g48120 – At5g48180.
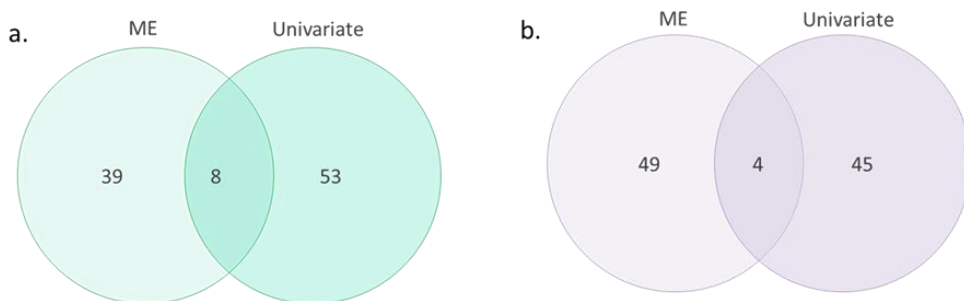
Figure 6. Venn diagram exhibit significant SNPs identified by ME and univariate GWA-mapping methods for traits related to the combined *Pieris* and subsequent PEG treatment response (a), and the *Botrytis* and subsequent PEG treatment response (b).

Although not all polymorphisms will be represented in the SNP markers used for GWAS analysis, some of the identified SNPs could be causal to the allelic variation. Therefore the allelic effect on any coding region was determined for these SNPs. SNP marker m73991 resides in At2g36550, encoding a Haloacid dehalogenase-like hydrolase (HAD) superfamily protein, with the allelic difference causing a non-synonymous amino acid substitution from an Asp to a Lys (A-A position N138K) residue in the non-Col-0 allele. Two additional SNPs, m73993 and m73994, cause non-synonymous changes to the coding region of At2g36560, encoding a protein of unknown function and the expression was very low, substituting Ala to Glu (A-A position A488E) and Glu to Gly (A-A position E420G) amino acids. SNP m111770 is residing in the intergenic region between genes with TAIR IDs At3g45910 and At3g45920, but is in LD (r=0.53) with another SNP causing a Val to Ile (A-A position V44I) substitution in the coding region of At3g45920.

The allelic effect of SNP m33786 on *DI19* gene was showed in Figure 4.e. SNP m60883 is residing in gene with TAIR ID AT2G13690. The coding sequence

substitution of SNP m60883 is from C (Col-0 like) to G (non-Col like) led to a nonsynonymous amino acid substitution from Glu to Gln (A-A position E500Q).

PRX34 and RMG1 play a role in the response of PLA to consecutive biotic and PEG stresses

A number of genes in LD with the significant SNPs have reported roles in response to biotic or abiotic stresses, but not to *Pieris*, *Botrytis*, or PEG. To determine if they play a role in response to these stresses, mutants for seven genes associated with response QTLs were further examined (Table 1). The HAD gene family member At2g36560 (*Pieris* and PEG) and *DI19* (*Botrytis* and PEG) were identified by both ME and univariate GWA-mapping approaches. In total, nine homozygous T-DNA insertion mutants, one for each of these nine candidate genes, were further analysed.

Plants were subjected to the same treatments as the HapMap accessions have been to (Figure 2). The *di19* mutant exhibited small rosette in all conditions when compared to the wild type (Supplementary Figure 5). One-way ANOA was performed to test the significance of projected rosette area expansion of T-DNA insertion mutants to the wild type, it shows that the *prx34* and *bzip25* mutants were more tolerant to *Pieris* herbivory (Figure 7.a) and the *rmg1* mutant was more tolerant to *Botrytis* infection when compared to the wild type (Figure 7.b). Two-way ANOVA was performed to test the significance of the response to PEG treatments compared to wild-type plants. When determining the response to single PEG, the *prx34*, *di19* and *rmg1* mutants were more tolerant than the wild type (Figure 7.c & d). When regarding the combinatorial treatments, the *prx34* mutant was found to be more sensitive to the combination of *Pieris* and subsequent PEG treatment (Figure 7.e); while the *arf4* mutant was more tolerant to the combination of *Botrytis* and subsequent PEG treatment (Figure 7.f).

Table 1. Seven genes associated with response QTLs which were further examined. Gene description obtained from TAIR website (http://www.arabidopsis.org)

Treatment	Gene TAIR IDs	Gene name	Gene description	SNP marker	LD score	-log(p) of linked marker
Pieris & *PEG*	At2g36560	unnamed gene	A paternally expressed imprinted gene; AT-hook domain DNA binding protein	m73994	1	6.28
Pieris & *PEG*	At1g45474	*PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 5 (LHCA5)*	Encodes a component of the light harvesting complex of photosystem I	m27735	1	4.12
Pieris & *PEG*	At3g49120	*PEROXIDASE 34 (PRX34)*	Class III peroxidase Perx34. Expressed in roots, leaves and stems. Located in the cell wall. Involved in cell elongation. Expression activated by light. May play a role in generating H2O2 during defense response	in LD with m114713	0.88	4.44
Pieris & *PEG*	At3g54620	*ARABIDOPSIS THALIANA BASIC LEUCINE ZIPPER 25 (AtbZIP25);*	bZIP transcription factor-like protein	in LD with m118294	0.86	4.55
Pieris & *PEG*	At3g50660	*ATDWF4*	Encodes a 22α hydroxylase whose reaction is a rate-limiting step in brassinosteroid biosynthetic pathway.	in LD with m115956	0.95	4.59
Botrytis & *PEG*	At1g56280	*ARABIDOPSIS THALIANA DROUGHT-INDUCED 19 (ATDI19)*	Encodes a gene whose transcript level in root and leaves increases to progressive drought stress. The increase in transcript level is independent from abscisic acid level. It appears to be a member of plant-specific gene family. It's phosphorylated by AtCPK11 in a Ca(2+)-dependent manner at Thr105 and Ser107 within the AtDI19 bipartite nuclear localization signal.	m33786	1	5.35
Botrytis & *PEG*	At1g56510	*WHITE RUST RESISTANCE 4 (WRR4)*	TIR-NB-LRR protein that confers resistance to four races of *Albugo candida*	in LD with m33786	0.31	5.35
Botrytis & *PEG*	At4g11170	*Resistance methylated gene 1, RMG1*	Disease resistance protein (TIR-NBS-LRR class)	in LD with m137156	0.64	4.05
Botrytis & *PEG*	At5g60450	*AUXIN RESPONSE FACTOR 4 (ARF4)*	Encodes a member of the ARF family of transcription factors which mediate auxin response	in LD with m209212	0.51	4.32
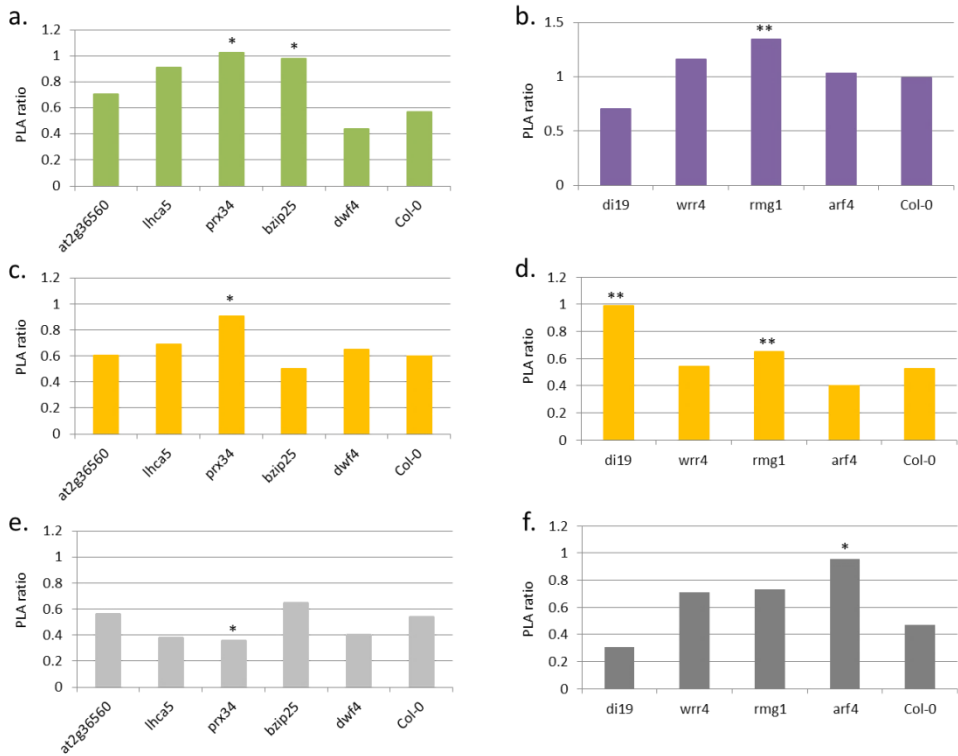
Figure7. Ratios of projected rosette areas (PLA) of T-DNA inerstion mutants *lhca5, prx34, bzip25, dwf4, wrr4, rmg1, arf4, di19* and for the HAD gene family member with TAIR ID At2g36560 subjected to several (a)biotic stress treatments, compared to Col-0 wild type.

a.   Comparing plants treated with *Pieris* to the PLA of the same plants before treated with *Pieris*

b.   Comparing plnats treated with *Botrytis* to the PLA of the same plants before treated with *Botrytis*

c.   Ratio of PLA of the genotypes in figure 7.a after subjected to PEG, compared to the control (yellow).

d.   Ratio of PLA of genotypes in figure 7.b after subjected to PEG, compared to the control (yellow).

e.   Ratio of PLA of Col-0 wild type and the mutant plants after pre-treatment with *Pieris* and subsequent exposure to PEG, compared to plants exposed to PEG only (light grey).

f.   Ratio of PLA of Col-0 wild type and the mutant plants after subjected to pre-treatment with *Botrytis* and subsequent exposed to PEG, compared to plants exposed to PEG only (dark grey).

Two-way ANOVA was used for analysis of differences between mutants and wild type, samples comprised six plants per treatment per line.

The response of the extreme accessions to single and combined stresses

Some accessions that exhibited a phenotype on the extreme ends of the spectrum (either more tolerant or more sensitive) to the combinatorial stress treatments were analysed for expression of the selected candidate genes upon exposure to different stress treatments. This involved four accessions responsive to *Pieris* and PEG and six accessions responsive to *Botrytis* and PEG (table 2).

Table 2. Accessions showed extreme phenotype in response to combinatorial stress from the result of screening Hapmap population

Treatment	CS code	Accessions	Phenotype
Pieris & PEG	CS28201	Da(1)-12	Tolerant
Pieris & PEG	CS76105	Bur-0	Tolerant
Pieris & PEG	CS76124	Duk	Susceptible
Pieris & PEG	CS76156	Kulturen-1	Susceptible
Botrytis & PEG	CS76143	Hovdala-2	Tolerant
Botrytis & PEG	CS76147	In-0	Tolerant
Botrytis & PEG	CS76164	Ler-1	Tolerant
Botrytis & PEG	CS76214	Pro-0	Susceptible
Botrytis & PEG	CS76218	Rennes-1	Susceptible
Botrytis & PEG	CS28743	Sp-0	Susceptible

Different responses to the combinatorial stress was found when compared to the result from screening the Hapmap accessions, Kulturen-1 was tolerant to the *Pieris* & PEG combined stress when compare to single stress (Figure 8.a), Ler-1 was susceptible to *Botrytis* & PEG when compared to single PEG while Rennes-1 and Sp-0 were tolerant to the combinatorial stress (Figure 8.b). No significant correlation between the result from the screening Hapmap population and the re-screen data, showing a variation of the environmental factors between the two times screening. Significant interaction between treatments and accessions were observed on DW (Supplementary Table 12).
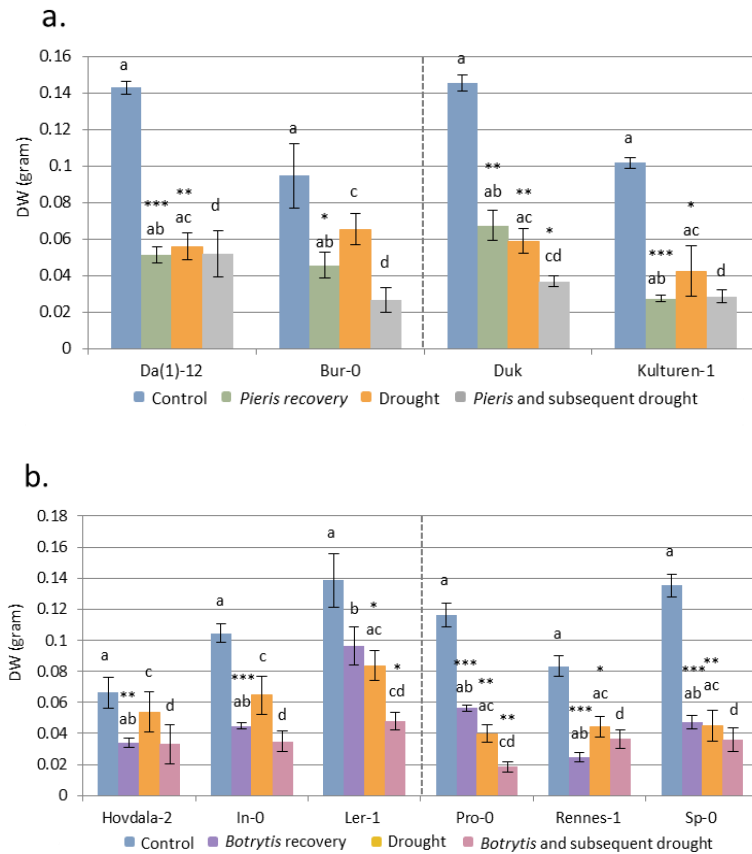
Figure 8. Phenotypes of Arabidopsis accessions Da(1)-12, Bur-0 (more tolerant), Duk and Kulturen-1 (more sensitive), identified as extremes in the response to the *Pieris* and subsequent PEG treatment, and Hovdala-2, In-0, Ler-1 (more tolerant), Pro-0, Rennes-1 and Sp-0 (more sensitive), identified as extremes in the response to the *Botrytis* and subsequent PEG treatment. Vertical dashed line separate accessions were either tolerant or susceptible to combinatorial stress (result from screening the HapMap population).
  a. Rosette dry weights (DW) of accessions grown in control conditions (blue), after *Pieris* pre-treatment (green), after PEG treatment (yellow), and after the combinatorial *Pieris* and PEG treatment (grey).
  b. DW of accessions grown in control conditions (blue), after *Botrytis* pre-treatment (purple), after PEG treatment (yellow), and after the combinatorial *Botrytis* and PEG treatment (lilac).
Two-way ANOVA was used to compare accessions under single *Pieris* and single PEG to the control, or under the combinatorial treatment to the PEG treatment. * indicates mutants with a significant difference under single stress in comparison to the control, or under combinatorial stress in comparison to single PEG ($p < 0.05$, **$p < 0.01$, ***< 0.001). Averages ± SE are shown, n=4.

Variation in candidate gene expression in extreme accessions grown under different stress conditions

The expression of the candidate genes *bZIP25*, *PRX33* (in LD with *PRX34*), *PRX34*, *LHCA5*, *DWF4* was determined in the accessions Duk, Kulturen-1, Da(1)-12, and Bur-0, grown under control, *Pieris*, PEG, and *Pieris* and subsequent PEG treatments (Figure 9). Three-way ANOVA to test the interaction between accessions and treatment was performed for *bZIP25, PRX33,* and *LHCA5* exhibited significant interaction between accessions, *Pieris*, and PEG (Supplementary Table 13). The five genes were downregulated in all accessions grown under PEG and combinatorial stresses. The expression under *Pieris* treatment exhibit contrasting expression direction when compared to the PEG treatment. In general, all genes were down-regulated under single PEG and the combinatorial stress. *bZIP25* is more down-regulated in Kulturen-1 than other accessions under PEG treatment, the expression is less down-regulated in Bur-0 under combinatorial stress when compared to single PEG treatment (Figure 9.a). *PRX33* was less down-regulated in Da(1)-12 than other accessions under PEG treatment, the expression is more down-regulated in Da(1)-12 but less down-regulated in Bur-0 under combinatorial stress in compression to single PEG stress (Figure 9.b). *PRX34* is less down-regulated in Da(1)-12 and Duk than in Bur-0 and Kulturen-1, the expression was more down-regulated under the *Pieris* and PEG combined stress when compared to single PEG (Figure 9.c). *LHCA5* was extreme down-regulated under PEG treatment but less down-regulated under the combinatorial treatment when compared to single PEG (Figure 9.d). *DWF4* is less down-regulated than other accessions under PEG treatment, reduced down-regulation was observed in Bur-0 and Kulturen-1 under *Pieris* combined stress when compared to single PEG treatment (Figure 9.e).

The expression levels of the *DI19*, *RMG1*, *ARF5*, and *WRR4* genes in response to the *Botrytis* treatment and the subsequent PEG conditions was studied in the 6 accessions; Pro-0, Rennes-1, Sp-0, Hovdala-2, In-0, and Ler-1 (Figure 10). Three-way ANOVA to test the interaction between treatments and accessions of *RMG1* and *WRR4* showed significant interaction between accessions, *Botrytis*, and PEG treatments (Supplementary Table 14). Strong

expression of *RMG1* was observed in *Botrytis* infection condition, the expression fold change up to 256 times relative expression fold change in accession Sp-0 (Figure 10.b). Under PEG stress, *DI19* and *WRR4* were up-regulated in all accessions (Figure 10.a & d). variations of *DI19* and *ARF4* were observed among accessions; *DI19* was up-regulated up to 4 times fold change in Hovdala-2 and *ARF4* was up-regulated up to 8 times fold change in the same accession. Under combinatorial stress, all accessions exhibit down-regulation *ARF4*, except in Rennes-1 (Figure 10.c). Variation of *RMG1* expression was found among accessions. The gene is down-regulated in Hovdala-2 but up-regulated in Sp-0 under combinatorial stress when compared to single PEG treatment.
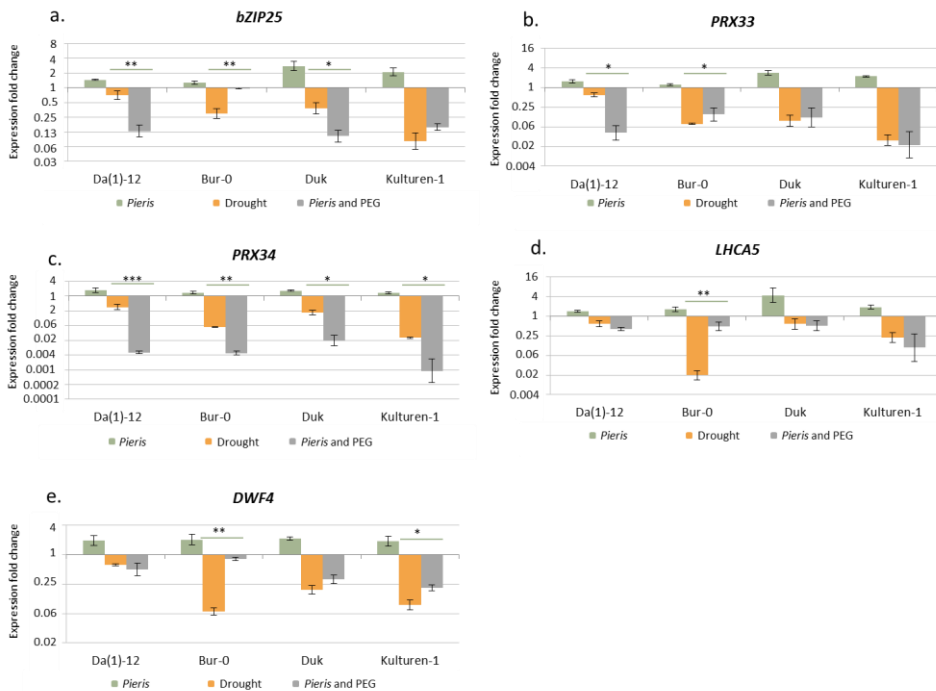
Figure 9. Relative rosette gene expression analysis of *bZIP25* (a.), *PRX33* (b.), *PRX34* (c.), *LHCA5* (d.), and *DWF4* (e.) in four Arabidopsis accessions selected based on extreme phenotypes, after *Pieris* treatment (green), PEG treatment (yellow), and the combinatorial *Pieris* and subsequent PEG treatment (grey), compared to the expression under control, untreated, conditions. Two-way ANOVA was used to compare gene expressions under combinatorial stress to PEG for each accession. Average rel. expression differences observed in treatments compared to control conditions are shown ± SE; n=3. * indicates a significant difference of gene expression under combinatorial stress when compared to the PEG (* $p<0.05$, **$p<0.01$, ***$p<0.001$)
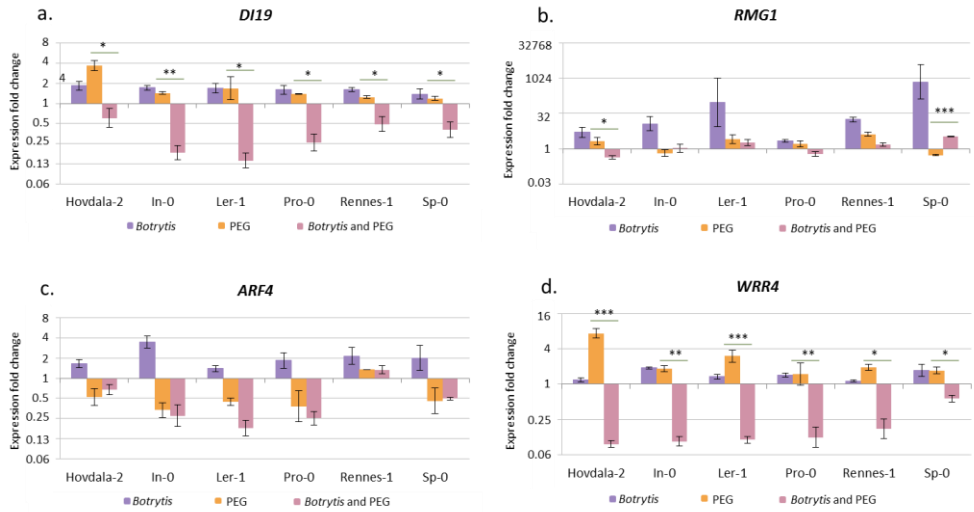
Figure 10. Relative rosette gene expression analysis of *DI19* (a.), *RMG1* (b.), *ARF4* (c.), and *WRR4* (d.) in four Arabidopsis accessions selected based on extreme phenotypes, after *Botrytis* treatment (Purple), PEG treatment (yellow), and the combinatorial *Botrytis* and subsequent PEG treatment (Pink), compared to the expression under control, untreated, conditions. Two-way ANOVA was used to compare gene expressions under combinatorial stress to PEG within each accession. Average rel. expression differences observed in treatments compared to control conditions are shown ± SE; n=3. * indicates a significant difference of gene expression under combinatorial stress when compared to the PEG (* $p<0.05$, **$p<0.01$, ***$p<0.001$)

Discussion

Comparing the PEG osmotic stress treatment with conventional drought stress

PEG8000 was used to simulate drought stress. PEG and conventional drought by soil-drying is known to cause some differences in plant responses, such as for instance shown by differences in proline accumulation and leaf thickness in apple, while the impacts on Relative Water Content (RWC) and chlorophyll content are similar (Kautz et al., 2015). We expected that a PEG treatment would emphasize the osmotic aspects of drought and thus more the physiology related to drought tolerance, rather than plant architectural responses contributing to drought avoidance, like for example adaptations of the root system architecture (El-Soda et al., 2015). The latter would be much less expressed in our rock wool-based growth system anyhow, since the small rock wool cubes do not allow for root drought avoidance. The mean reason to revert to a PEG treatment was related to the difficulties we experienced in obtaining homogeneous drought on peat-based substrates for a large set of plants, which would be needed for a proper GWA analysis of the Arabidopsis HapMap population. We are aware of much more suitable systems, especially designed to screen for plant drought on soil, such as the Phenopsis system (Granier et al., 2006), but logistic issues made us instead decide to use the PEG-mediated osmotic stress. There is also an important advantage of such approach, as we could use this in our rock wool based growth facilities equipped with imaging systems to record plant size and thus accurately measure plant growth (Bac-Molenaar et al., 2016; Flood et al., 2016). Using an ebb-flood system to water the rock wool blocks also allowed us to reproducibly test different PEG-nutrient solution mixtures for the most suitable osmotic potential for screening. In order to verify that the PEG-nutrient solution induced similar, but more controlled, symptoms as observed upon conventional drought stress of plants raised on peat-based mixtures by withholding watering, we examined gene expression of a set of known drought responsive genes in both conditions and their respective controls (Figure 3). The five tested genes are involved in the ABA-dependent and -independent regulatory system for drought responses (Shinozaki and Yamaguchi-Shinozaki, 2007). Since we observed comparable expression profiles

of these drought-responsive genes in response to PEG and soil-drying treatments, we concluded that the PEG8000 induced osmotic stress would be sufficiently suitable to study the Arabidopsis 'drought' response.

Allelic effects on combinatorial stress responses

Previous studies of Arabidopsis natural variation exhibited different effects of allelic variation on plant morphology and stress responses which led to the identification of the gene involved. For instance, GWA mapping of cellular traits identified the *KUK* gene involved in root development regulation and root growth (Meijon et al., 2014). Likewise, different alleles of the *ACCELERATED CELL DEATH 6* (*ACD6*) gene were found to exhibit pleiotropy effects on pathogen resistance and results in plants with small rosettes (Todesco et al., 2010). In general, an allelic effect of a locus can be due to a single bp mutation, such as often found in EMS-mutagenized populations, which can be simple SNPs, for instance when such a SNP causes a non-synonymous amino acid substitution in one of the exons of the gene involved or when it causes an intron-exon splice junction error (Brown, 1996; Guyon-Debast et al., 2010). Both cases are likely to affect the amino acid sequence of the protein encoded by the gene, which can affect its function. For instance, a SNP causing alternative splicing of the *FLOWERING LOCUS C* (*FLC*) antisense transcript *COOLAIR*, resulted in differential expression of *FLC* and altered flowering time in Arabidopsis (Li et al., 2015). Furthermore, natural accessions carrying a single SNP at the *HIGH-AFFINITY K+ TRANSPORTER 1;1* (*AtHKT1;1*) gene affected the expression of the gene leading to a difference in leaf Na^+ accumulation (Baxter et al., 2010).

In this study, we initially identified SNPs as markers for the traits we used to evaluate the response to single and combinatorial stresses. Of the two GWA approaches we employed, the univariate approach yielded more associated SNPs than the ME approach. This could be due to a higher number of false positives in univariate approach, which is known to occur when compared to the ME approach, as the latter also considers the within and the between trait(s) variation (Korte et al., 2012). The traits we measured (FW, DW, WC and PLA) are very general growth traits, likely to be genetically affected by allelic variation at many loci. So, even when heritabilities are high, which was the case for these traits (table 2), the multitude of small-effect QTLs could make it very difficult to

identify these QTLs (Kooke et al., 2016). This is also the reason we used an arbitrary threshold of $-\log(p)=4$ to assign associated SNPs (van Rooijen et al., 2015), instead of the much more conservative Bonferroni corrected threshold ($-\log(p)=6.5$) which is advised (Atwell et al., 2010). If this conservative threshold was used, we would only consider one associated SNP for the univariate GWA approach, m73995, close to gene ID At2g36550, for rosette PLA upon combinatorial *Pieris* and PEG stress (Supplementary Figure 2.a and supplementary Table 8). This same SNP was also identified in the ME GWA approach (Supplementary Figure 3.a and supplementary Table 10). For the response to *Botrytis* and PEG, only the ME GWA approach would have yielded significantly associated SNPs; m64241, close to the *GENE WITH UNSTABLE TRANSCRIPT 15* (At2g18440); and m94602, close to an unnamed gene (At3g22690) involved in chloroplast RNA editing, and in LD with a region containing seven more genes (At3g22670-At3g22740), of which *RNA-DIRECTED DNA METHYLATION 1* (*RDM1*), involved in DNA methylation and *YELLOW SEEDLING 1* (*YS1*), involved in chloroplast RNA editing, have been analysed in some detail (Zhou et al., 2009; Law et al., 2010).

To avoid false positives, we focussed on genes identified in both approaches for further analysis, as we considered those to be less likely to be false positive. The genetically best supported locus comprises two unnamed genes (At2g36550 and At2g36560), supposedly involved in the response to *Pieris* and PEG. There are several SNPs in LD with the main associated SNP, all residing in the At2g36560 gene, which could explain the allelic variation. Unfortunately the KO mutant for this gene did not show any aberrant responses to the stress treatments in comparison to wild-type plants to support it as the most likely candidate underlying the allelic variation. It could be that the Col-0 allele is redundant, but that other accessions carry gain-of-function allelic variants affecting the phenotype. At2g36550 is a member of Haloacid dehalogenase-like hydrolase (HAD) superfamily protein and At2g36560 is a member of AT hook domain DNA-binding protein. The biological functions of the two genes haven't yet to be studied. However, At2g36560 could act as a positive factor for gene expression. For instance, one of the AT hook motif DNA binding protein (PF1)

was found can bind to the promoter site of oat photochromic A3 (*PHYA3*) gene and could has influence on the gene expression (Nieto-Sotelo et al., 1994).

In case of DW measured upon *Botrytis* and subsequent PEG stress, both mapping approaches identified SNP marker m33786 to be associated. This marker resides in an intron of the *DI19* gene, but in LD with this marker there are three more genes: an unnamed gene encoding a CwfJ-like Zn-finger DNA binding protein, that is expressed in response to Cabbage leaf curl virus (CaLcuV) infection (Ascencio-Ibanez et al., 2008) (At1g56290); an unnamed gene which encodes a chaperone DnaJ-domain superfamily protein, expressed in response to oxidative stresses (At1g56300); and an unnamed gene that encodes a ribonuclease H-like superfamily protein (At1g56310). Although no SNP in any of these gene could be identified that would explain the allelic variation, the *DI19* gene is the most likely candidate underlying this QTL, as in our hands, the KO mutant was very small, under all tested conditions (Supplementary Figure 5), and we could not test it properly for response to stress treatments. The expression of *Di19* can be found in seedlings, roots, rosettes, stems, flowers, and siliques (Milla et al., 2006). The gene is up-regulated under drought, but did not respond to ABA (Gosti et al., 1995), it is down-regulated under *Botrytis* infection, and the combination of *Botrytis* and subsequent drought (Coolen et al., 2016). Liu et al. (2013) reported that the *di19* knockout mutant was susceptible to drought and that the constitutive overexpression line was tolerant to drought. The gene encodes a Cys2/His2-type zinc-finger protein that functions as a transcription factor (Liu et al., 2013). The expression of *DI19* can be modulated by Calcium-dependent Protein Kinases CPK3, CPK4, CPK11, and CPK12 via $Ca2^+$-dependent signalling (Milla et al., 2006; Rodriguez Milla et al., 2006). Some of the CPK family proteins were reported to be involved in biotic stress resistances. For instance, CPK1 plays a role in *Botrytis*, *Fusarium oxysporum*, and *Pseudomonas syringae pv.tomato DC3000* (*Pst*) resistance (Coca and San Segundo, 2010), while CPK4, CPK5, CPK6, and CPK11 are involved in *Pst* resistance (Boudsocq et al., 2010), CPK5, CPK6, and CPK11 are involved in *Botrytis* resistance (Gravino et al., 2015), and CPK3 and CPK13 act as positive regulators of *PDF1.2* resistant to *Spodoptera littoralis* caterpillars (Kanchiswamy et al., 2010). Thus, we hypothesize that the down-regulation of *DI19* under *Botrytis* infection is due to the negative regulation by CPKs.

Role of other candidate genes in response to single and combinatorial stresses

Some candidate genes in LD with the significant SNPs have been reported to have a role in the response to stresses, but not yet in the response to *Pieris*, *Botrytis*, and PEG. We therefore identified homozygous T-DNA insertion mutants for as many of these genes and determined their phenotypes in response to treatments.

Mutant *prx34* was found to be more susceptible to the *Pieris* and PEG combinatorial stress (Figure 7). The univariate GWA-mapping on PLA showed another gene, *PRX33* (*AT3G49110*), to be in LD with *PRX34*. Both genes encode cell-wall peroxidases (Valerio et al., 2004). *PRX34* is more abundantly expressed than *PRX33* (Sultana et al., 2015), and the expression of *PRX33* is partially controlled by *PRX34* (Daudi et al., 2012). Knockout mutant *prx34* was found to exhibit reduced expression of both *PRX33* and *PRX34* and reduced 50% MAMP-induced ROS production in apoplastic space (Daudi et al., 2012; O'Brien et al., 2012). The double knock-out mutant *prx33prx34* expressed a number of *Pieris*-responsive genes such as *VSP2*, which were not expressed in the wild type (Liu et al., 2005; Dombrecht et al., 2007; Mammarella et al., 2014), as well as other JA and ET-responsive genes such as *VSP, PR3, PR4* (Mammarella et al., 2014). It is possible that the resistance of the *prx34* mutant to *Pieris* is due to the enhanced expression of *VSP2*. The *prx33prx34* double knockout mutant also showed reduced expression of drought tolerance gene *ETHYLENE RESPONSE FACTOR 1 (ERF1)* (Cheng et al., 2013; Mammarella et al., 2014), which was negatively regulated by *Pieris* (De Vos et al., 2006). This could be the reason why the *prx34* mutant was more sensitive to the consecutive *Pieris* and PEG stress combination instead of more tolerant as expected based on the response to *Pieris* alone.

RMG1 encodes a NB-LRR-TIR protein and the promoter region contains two helitron-related repeats *AtERP4* and *AtERP11*, which are the target by siRNAs and can be heavily methylated in all cytosine sequencing contexts (Yu et al., 2013). It is a primary target of the RNA-directed DNA methylation (RdDM) and the expression can be enhanced by flg22 treatment in the knockout mutant of

gene *Repressor Of Silencing 1* (*ROS1*) (Yu et al., 2013). Thus, the observed phenotype of *rmg1* mutants in response to stresses could be due to the RMG1 involved RdDM. In addition, the body of the defense responsive gene *WRKY22* was found at the 5' part of the open reading frame ORF of *RMG1* (Yu et al., 2013), which suggests a epigenetic control of the two genes under multiple stresses.

When looking at the effect of biotic pre-treatment on plants in response to PEG, we found the *Pieris* pre-treatment has positive effect on *prx34* mutant and the *Botrytis* pre-treatment has positive influence on *rmg1* mutant in response to PEG stress (Figure 7). Comparing candidate genes expression under the combinatorial stress to PEG stress, the expression of *PRX34* is more down-regulated under the combinatorial stress when compared to single PEG stress (Figure 9). Negative effects of *Botrytis* pre-treatment was observed on *DI19* gene expression in response to drought stress (Figure 10). We hypothesised the interaction between biotic and abiotic stress responses can be synergistic, antagonistic, or neutral. In this study, additive effects was found on *arf4* mutant, which did not show significant responses to individual *Botrytis* and PEG treatments but was tolerant to the combination. Antagonistic effect was observed on *prx34* mutant, the mutant was tolerant to *Pieris* and PEG stresses, but was susceptible to the combination of *Pieris* and PEG when compared to the wild type (Figure 7). Neutral effect was observed on *rmg1* mutant, the mutant was tolerant to individual *Botrytis* and PEG treatments, but did not show different phenotype under the combination of *Botrytis* and PEG treatment when compared to the wild type. This suggests it will be impossible to obtain genotypes that would be tolerant to both single and combinatorial stresses. To illustrate this, overexpression of *PRX34* and *ARF4* could result in enhanced tolerance to combinatorial stresses, but this is likely to reduce the tolerance to each of the biotic single stresses.

Conclusion

To conclude, this study used two GWA-mapping approaches and identified a number of candidate genes exhibited allelic variation in the response to combinatorial stress. Genes. To confirm the allelic effect on stress responses, genetic complementation tests to examine the biological function of the different alleles in response to different stress conditions will be needed. We validated the biological function of nine genes, of which seven genes were in LD with the identified associated SNPs, not the gene in which the SNP resides. These genes have reported functions in response to some stresses, in single and combinatorial stress conditions. Antagonistic interaction between *Pieris* and PEG was observed for the *prx34* mutant, and antagonistic interaction between *Botrytis* and PEG for the *rmg1* mutant. The expression of candidate genes in single and combinatorial conditions was measured from Arabidopsis accessions. However, we did not observe a relation between the variation of the phenotypic responses in Arabidopsis natural accessions and the variation in candidate gene expression. A powerful analysing method such as algorithm locally linear embedding graph generator (LEGG) (Van Poecke et al., 2007) could be useful to define relationships between expression profile and phenotypic variances in response to stresses.

Acknowledgement

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Supporting Information

Additional supporting information may be download via link

https://www.dropbox.com/sh/hb95t5dwvxbsc1u/AAB2Z7Q1DPSwCCU-

oaTBBi2Pa?dl=0

CHAPTER 4

Genome wide association mapping of the *Arabidopsis thaliana* growth response to the sequential combination of drought and *Botrytis cinerea* infection

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Abstract

There is an increasing demand to understand the genetics underlying plant response to multiple stresses. Therefore, we designed this work to study the effect of drought pre-treatment on plant response to a subsequent *Botrytis cinerea* infection to identify genes responsible for tolerating this combinatorial stress. Genome-wide association mapping was performed on plant rosette dry weight of 350 *Arabidopsis thaliana* accessions subjected to the combinatorial stress. In addition, Arabidopsis accessions were exposed to a single *Botrytis cinerea* treatment without any pre-treatment. We identified the effect of a number of significant SNPs that were associated with the DW plasticity under stresses, as well as candidate genes that could play a role in the response to the combinatorial stress. Furthermore, we validated the biological functions of six candidate genes under single drought or *Botrytis* stress and the combinatorial stress using T-DNA insertion mutants and the transcriptome data of the most extreme eight accessions. Allelic effect was observed on *MYB46* gene expression under combinatorial stress comparing to single *Botrytis* treatment. The role of *MYB46*, *PAL4* and *HSFA1b* in response to the interaction of drought and *Botrytis* infection was highlighted. We observed antagonistic interaction between drought and *Botrytis* infection, and the effect of drought pre-treatment on subsequent *Botrytis* infection is negative.

Keywords

Introduction

In nature, plants are exposed to various stress that often occur simultaneously or sequentially. When stress factors interfere negatively with plants, this will limit its performance causing a substantial yield reduction (Hirt and Shinozaki, 2004).

Plant responses to biotic and abiotic stresses are often mediated through hormone-signaling pathways. For example, abscisic acid (ABA) plays a major role in the response to abiotic stress such as drought and salinity (Shinozaki and Yamaguchi-Shinozaki, 2007). Jasmonic acid (JA) and ethylene (ET) signaling pathways are essential in reacting to necrotrophic fungi and chewing insects (Vos et al., 2013). Salicylic acid (SA) activates plant defense mechanisms upon the infection with biotrophic fungi (Dodds and Rathjen, 2010; Pieterse et al., 2009). Several additional hormones such as brassinosteroids (BR), auxins (IAA) and cytokinins (CK) are also involved in regulating plant responses to biotic and abiotic stress (Hirt, 2009; Pareek et al., 2010; Pieterse et al., 2012). The interaction between plant response to biotic and abiotic stress can be either synergistic, e.g. positive interactions between ABA and IAA or antagonistic, e.g. negative interactions between JA and SA, ABA and SA, ABA and JA, and between ABA and ET (Yoshioka and Shinozaki, 2009).

The mechanisms by which plants respond to individual biotic or abiotic stress were extensively studied at both molecular and cellular levels. For example, recent comparative transcriptome studies identified unique as well as overlapping genes playing a role in *Arabidopsis thaliana* response to several single biotic and abiotic stress (Narsai et al., 2013; Ramegowda and Senthil-Kumar, 2015; Sewelam et al., 2014; Shaik and Ramakrishna, 2013). In contrast, far less information is known on the plant response to simultaneous or consecutive biotic and/or abiotic stresses. Generally, consecutive stresses trigger the expression of specific multiple stress-responsive genes, indicating a tailored combinatorial stress signaling network (Atkinson and Urwin, 2012; Sewelam et al., 2014). For example, earlier studies (Anderson et al., 2004; Pré et al., 2008; Vos et al., 2013; Yoshioka and Shinozaki, 2009) showed that *Botrytis* infection triggers the expression of defense responsive genes such as

PDF1.2 in the JA-ET signaling pathway through the ERF branch, which interacts negatively with the drought induced ABA responses.

The present study focused on identifying genes underlying *Arabidopsis thaliana* responses to consecutive drought and infection by a necrotrophic fungus, *Botrytis cinerea*. The objectives were to: 1) observe the effect of the prior drought stress on subsequent *Botrytis* infection, 2) study the variation in allelic effect in response to single *Botrytis* and the combinatorial stresses and 3) identify genes that could play a role in the response to the single and combinatorial stresses. To achieve these goals we conducted genome-wide association mapping (GWA-mapping) for the rosette dry weight (DW) of 350 Arabidopsis accessions (Baxter et al., 2010; Li et al., 2010). Our results identified a number of genes exhibited allelic effects on combinatorial stress responses, and reported the new role of several known gene in response to the consecutive drought and *Botrytis* infection.

Materials and Methods

Plant material and growth conditions

For GWA-mapping experiments, the HapMap population (Li et al., 2010) was used. Seeds of the T-DNA insertion mutants (Supplementary Table 1) were ordered from The Nottingham Arabidopsis Stock Centre (NASC) (http://*Arabidopsis*.info/). Seeds of the *HEAT SHOCK TRANCRIPTION FACTOR 1b (HSFA1b)* over-expression lines and its wild-type are described by Bechtold et al (2013).

Seeds were stratified at 4°C for two days in the dark, then sown on river sand and watered with half-strength Hoagland nutrient solution as described by Van Wees et al (2013). Plants were grown in a climate-controlled growth chamber under short day conditions (8 hrs day/16 hrs night) at 21°C, 70% relative humidity and 100 µmol $m^{-2}s^{-1}$ irradiation. Fourteen-day-old seedlings were transferred to individual pots filled with a mixture of river sand and peat (1:1, v/v) and watered three times per week, at the bottom of each pot.

Botrytis spore preparation

Botrytis cinerea strain B05.10 (Staats and Van Kan, 2012) was grown for two weeks at room temperature on half-strength potato dextrose agar plates (PDA; Difco Laboratories) containing 100 µg/ml penicillin and 200 µg/ml streptomycin. Spores were collected, filtered through glass wool and then re-suspended in half-strength potato dextrose broth (PDB; Difco Laboratories) to a final density of 1 x 10^5 spores/ml. Three hours after incubation spores were used for inoculation by applying six 5-µl droplets on six Arabidopsis leaves.

Single and combinatorial stress application

Screening the Hapmap population: For the combinatorial stress experiments, i.e. drought on subsequent *Botrytis,* four-week-old Arabidopsis plants of the Hapmap population were subjected to drought by withholding water for seven days and then re-watered to recover for one day. Thereafter, plants were inoculated with *Botrytis* by pipetting six 5-µl droplets of spore suspension (1×10^5 spores/ml) onto six leaves (one droplet per leaf). For the single *Botrytis*

treatment, 5-week-old plants were inoculated with *Botrytis* as described above. Plants in both treatments were incubated at 100% relative humidity (RH) for 3 days, after which samples were collected (Figure 1.a).

Screening T-DNA mutants: In addition to the two treatments described in the previous paragraph, two mock treatments replaced the *Botrytis* treatment where six 5-µl droplets PDB were applied on six leaves per plants. Two additional treatments were applied in which one treatment involved watering plants for 34 days and the other treatment involved watering plants for 27 days and then subject them to drought for six days (Figure 1.b). After mock or *Botrytis* infection, plants were maintained at 100% RH for three days. For drought treatments, six plants of each mutant/wild-type were used. For the *Botrytis* treatment, 48 leaves (8 plants) of each mutant/wild-type were used. Samples for gene expression were collected twice, directly after drought stress and three days after *Botrytis* treatment.

Screening extreme accessions of the Hapmap population**:** This experiment was carried out with the same design as described in the previous paragraph except that an additional sample for gene expression was collected after one day (at time point 2, Figure 1.b) of *Botrytis* infection treatment.
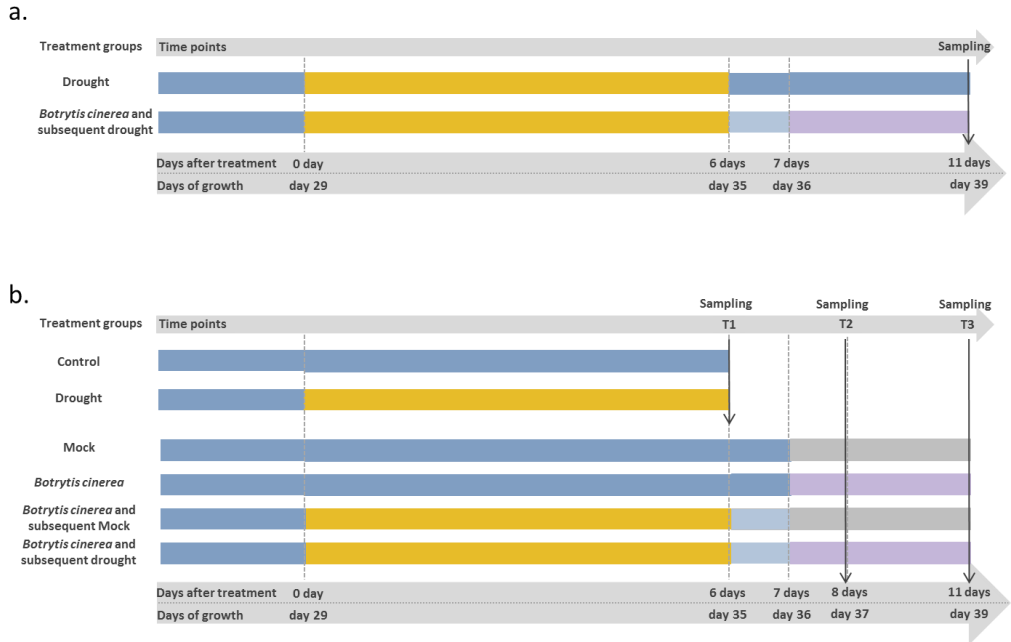
Figure 1. Experimental design for treatment of Arabidopsis plants subjected to drought, *Botrytis cinerea* infection and combinatorial stress. Conditions are indicated in different colors: control (dark blue); drought (yellow); *Botrytis* treatment (purple); rehydration after drought (light blue). Dashed line indicate days after treatment and the growth date. Vertical arrows indicate sampling time point (a.) and sampling time points (T1-T3). a. experimental design for screening Hapmap accessions in response to drought and the consecutive drought and *Botrytis* infection, b. experimental design for T-DNA insertion mutants, wild-type and Arabidopsis accessions in different conditions.

Genome-Wide Associations mapping

GWA-mapping was performed using the scanGLS software as described by Kang et al (2010) and Kruijer et al (2015). In brief, this approach performs generalized least squares (GLS) calculations conditional on variance components estimated in the model without markers. Thereafter, SNPs with minor allele frequency (MAF) lower than 5% were excluded. Marker-trait associated *P*-values at 10^{-4} with type I error was used to evaluate significant SNPs. The proportion of explained phenotypic variance of each significant SNP was the criterion used to identify significant SNPs as described by Sun et al (2010). The information of SNPs in the linkage disequilibrium (LD) with the significant SNPs were identified by the online LD tool (http://dev3.ab.wur.nl/AthaLD), as well as the information of coding sequence substitution and amino acid substitution.

Selection of candidate genes

Gene ontology annotation of selected candidate genes for the quantitative trait loci (QTL) identified after the GWA analysis was found in The Arabidopsis Information Resource (TAIR; www.Arabidopsis.org/tools/bulk/go/index.jsp). Genes characterized by one or more of the following criteria were considered as candidates: 1) Genes known to be responsive to biotic and abiotic stimulus and expressed under abiotic stress and hormonal (JA and ABA) treatments; 2) Genes with reported physiological functions in response to stress conditions and expressed under abiotic stresses and hormonal (JA and ABA) treatments (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). 3) Genes with reported physiological functions in response to stress conditions and responsive to biotic and abiotic stimuli.

Quantitative reverse transcriptase (qRT)-PCR

To determine the expression of the candidate genes in Arabidopsis accessions with an extreme phenotype compared to the whole set of accessions, total RNA was extracted from rosettes following the protocol described by Onate-Sanchez and Vicente-Carbajosa (2008). Thereafter, cDNA was synthesized from 800 ng of total RNA using an iScript cDNA synthesis kit (Bio-Rad) at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. cDNA was diluted ten times to be used in the quantitative reverse transcription PCR (qRT-PCR). Based on the transcriptome data collected from several drought, osmotic and *Botrytis* studies using the Genevestigator web tool http://refgenes.org/rg, unnamed gene *At3G19460* was considered to be the most stable gene expressed across experiments and was selected as reference gene for gene expression studies. The qRT-PCR was performed using iQ SYBR® Green Supermix (cat. no. 170-8885) on a Bio-Rad CFX96 real-time PCR system set at 95°C for 4 minutes, followed by 40 cycles of 95°C for 10 seconds and 55°C for 30 seconds. The primer sequences used to amplify the four investigated genes, i.e. TUBLINE 5 (*TUB5*), PHENYLALANINE AMMONIA-LYASE 4 (*PAL4)*, MYB DOMAIN PROTEIN 46 (*MYB46)* and HEAT SHOCK FACTOR 1b (*HSFA1b),* are provided in Supplementary Table 2 and their expression was measured in eight accessions, i.e. Est-1 (CS76127), Sei-0 (CS28729), Kulturen-1 (CS76156), RRS-7 (CS28713), (Col-0 (CS76113), Rsch-4 (CS76222), Cnt-1 (CS28160), Can-0

(CS76109). Gene primers were designed at the identical sequence region of the eight accessions.

Statistical analysis

Pearson's correlation coefficient, one-way and 2-way ANOVAs were performed using GenStat for Windows 16th Edition (VSN International Ltd., Hempstead, UK). Three-way ANOVA was performed as described by (Sokal RR, 1981). Student's t-test was used to compare rosette dry weight (DW) and lesion diameter of plants grown under the combinatorial stress with those grown under the single *Botrytis* stress. For lesion diameter, 48 leaves (eight plants) per accession or line were used, whereas, for DW, five plants per accession or line were used.

Results

Phenotyping the Arabidopsis accessions under drought and the combinatorial stress

To identify genes that are involved in the response to consecutive abiotic and biotic stress the Arabidopsis HapMap population was grown for four weeks under normal, well-watered conditions and then subjected to one week of drought. Thereafter plants were watered and allowed to recover for one day (rehydration), before being infected with *Botrytis*. Rosettes of plants were harvested after three days *Botrytis* treatment and used to determine rosette dry weights (DW) (Figure 1.a). High broad sense Heritabilities (H^2) of DWs under single *Botrytis* treatment (0.69) and the combinatorial stress (0.73) were observed. High Pearson's correlation coefficient (0.81) between the two treatments was found. Two-way ANOVA of DW was used to test the interaction between treatments and accessions. Significant interaction between treatments and accessions was found ($p<0.001$), indicating that there is genetic variation for the drought pre-treatment effect.

Univariate GWA-mapping of DW residuals identified SNPs associated with phenotypic plasticity

GWA-mapping using the DW residuals that was calculated from a regression analysis of DW under consecutive drought and *Botrytis* to DW under single *Botrytis* treatment, and revealed 34 significantly associated SNPs (Figure 2 and Table 1) that are located in or very close to 30 genes.
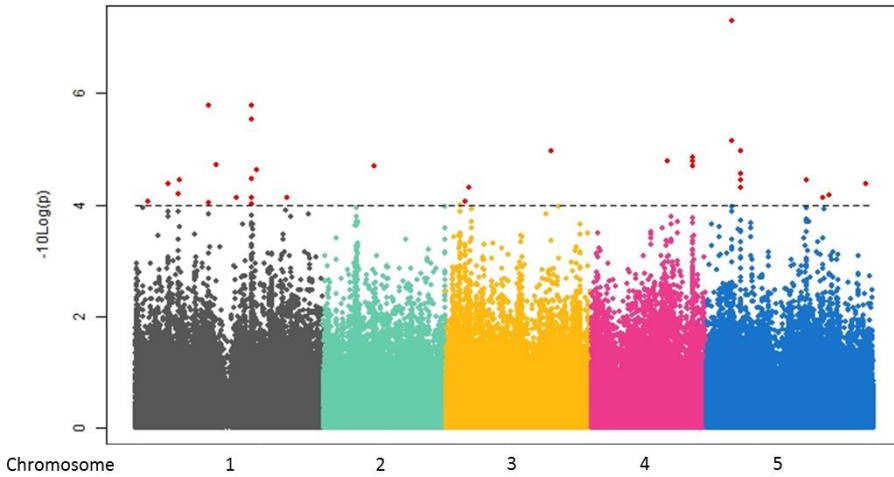
Figure 2. univariate GWA-mapping on DW for consecutive drought and *Botrytis* stress responses identified 34 significant SNPs (-log(p)>4) (red) that located on five chromosomes (black, green, yellow, red, blue).

SNP m168340 exhibited strong association with –log(*p*) score at 7.29 (Figure 2 and Table 1). The non-Col like allele of SNP m168340 presented strong effect on combinatorial stress responses and explained 8.06% genetic variation of accessions in response to the combinatorial stress. This SNP is not located or very close to any gene, but when looking at the Linkage Disequilibrium (LD) of the SNP, a large locus (150kb) including 74 SNPs that are associated to 16 genes was found. Another four SNPs exhibited higher –log(*p*) score (>5) than the other SNPs (Table 1). SNP m168339 is in LD with m16340 and is present at the same locus. SNP m20853 is located at the 2.2 kb upstream region of *MULTIPLE ORGANELLAR RNA EDITING FACTOR 5 (MOREF5*; AT1G32580), which acts as a cofactor of pentatricopeptide repeat proteins (PPR proteins) for RNA editing in plastids and mitochondria in flowering plants (Zehrmann et al., 2015). The synonymous non-Col like allele of the SNP explained 5.82% phenotypic variance. Two SNPs m370070 and m370071 are located at the exon of an unnamed gene AT1G50650 with unknown molecular function. The non-Col like alleles of the two SNPs explained respectively 5.78% and 6.03% phenotypic variations of accessions under the combinatorial stress. The synonymous substitution of SNP m370070 is from G (Col-0 like) to T (non-Col like). The nonsynonymous substitution of SNP m370071 is from A (Col-0 like) to G (non-

Col like) and causes an isoleucine to threonine change, but the on effect on combinatorial stress responses was found when comparing the non-Col allele to Col-0 allele.

SNPs such as m157533, m157535, and m151030 exhibited large allelic effects on combinatorial stress responses among the 34 significant SNPs and displayed high $-\log(p)$ score ($\sim$ 4.8; Table 1). SNPs m157533 and m157535 are associated with unnamed gene AT4G34400; and another SNP m157515 (4.69) was found in LD with m15735 and is associated with gene *ARIADNE 1 (ARI1*; AT4G34370), which could interact with the ubiquitin-conjugating (E2) enzymes for ubiquitination (Mladek et al., 2003). The coding sequence substitution of SNPs m157533 and m151030 were synonymous, while SNP m157535 in *ARI1* displayed a nonsynonymous substitution from G (Col-0 like) to A (non-Col like) leading to an amino acid substitution from aspartic acid to asparagine. We found that the amino acid substitution in *ARI1* has a negative effect on combinatorial stress responses as reduced DW ratio was observed under the combinatorial stress when compared to *Botrytis* infection (Figure 3.a). The SNP m151030 present in the 5th exon of the *3XHIGH MOBILITY GROUP-BOX2 (3XHMG-Box2*; AT4G23800) gene showed a synonymous substitution and is unlikely to cause an effect on the protein function. However, another SNP is tightly linked (LD =0.98) to SNP m151030 and displayed a nonsynonymous substitution from G to C resulting in a serine to threonine change. The non-Col allele of the SNP exhibited negative effects on combinatorial stress tolerance in comparison to the Col-0 like allele (Figure 3.b) Gene *PROTEIN ARGININE METHYLTRANSFERASE 4A (PRMT4A*; AT5G49020; Table 1) encodes a type I protein arginine methyltransferase which plays a role in regulating flowering time through the FLOWERING LOCUS C (FLC)-dependent pathway (Niu et al., 2008). The SNP m201187 present in *PRMT4A* displayed a $-\log(p)$ score of 4.12 and was a sequence substitution from G to T causing a valine to phenylalanine substitution. Plants with this genotype can cope less with combined stress when compared with plants that contain the Col-0 like allele (Figure 3.c).
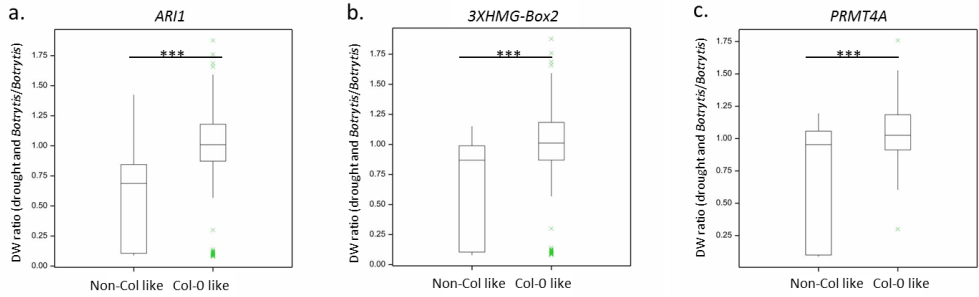
Figure 3. Boxplots of the combination of two types allele (Col-0 like and non-Col like) of SNPs associated with gene ARI1 (a), 3XHMG-Box2 (b) and PRMT4A (c). Student's t-test was used to compare the differences of the Col-0 like and non-Col like alleles of each gene. *** indicates significant differences (*p*<0.0001)

Table 1. GWA mapping identified significant (-log(p)>4) SNPs in response to the drought and subsequent *Botrytis* infection. SNPs that were described in the text were highlighted in grey, dark grey and black color.

marker	chromosome	position (bp)	p-value	-log(p)	associated gene 1	associated gene2	MAF	effect.size	% of variation
m11959	1	7004146	6.32E-05	4.2	AT1G20200	NA	0.32	0.552947	6.2
m12415	1	7218120	3.56E-05	4.45	AT1G20780	NA	0.07	-0.8422024	4.55
m20853	1	11781846	1.62E-06	5.79	AT1G32580	NA	0.11	-0.7981752	5.82
m20874	1	11788214	9.07E-05	4.04	AT1G32583	NA	0.08	-0.7474592	3.85
m23257	1	13002595	1.91E-05	4.72	AT1G35370	NA	0.14	-0.6492136	4.64
m26205	1	16240507	7.34E-05	4.13	AT1G43150	NA	0.37	-0.4527572	4.51
m30770	1	18764103	2.92E-06	5.54	AT1G50650	NA	0.12	-0.7608652	5.78
m30771	1	18764176	1.68E-06	5.78	AT1G50650	NA	0.14	-0.7562738	6.3
m30821	1	18796993	9.59E-05	4.02	AT1G50730	NA	0.14	-0.6346076	4.52
m30823	1	18798064	7.38E-05	4.13	AT1G50730	NA	0.13	-0.6624638	4.66
m30885	1	18861784	7.63E-05	4.12	AT1G50880	NA	0.13	-0.647296	4.36
m30888	1	18863001	3.43E-05	4.46	AT1G50890	NA	0.14	-0.6437446	4.81
m32080	1	19685632	2.39E-05	4.62	AT1G52860	AT1G52870	0.07	-0.8903436	4.9
m3341	1	2080758	8.91E-05	4.05	AT1G06770	NA	0.26	-0.4703418	4.02
m41895	1	24400469	7.48E-05	4.13	AT1G65620	NA	0.06	-0.8847854	4.08
m8980	1	5322833	4.31E-05	4.37	AT1G15490	NA	0.26	-0.5382988	5.19
m64561	2	8155673	2.02E-05	4.7	AT2G18820	NA	0.73	-0.5275732	5.1
m112024	3	16999950	1.09E-05	4.96	AT3G46260	NA	0.1	-0.7799526	5.29
m85730	3	3164703	8.81E-05	4.05	AT3G10230	NA	0.24	-0.4892898	4.16
m86747	3	3684957	5.02E-05	4.3	AT3G11670	NA	0.18	-0.5754214	4.59
m151030	4	12391936	1.60E-05	4.79	AT4G23800	NA	0.06	-0.9443776	5.08
m157515	4	16435607	2.04E-05	4.69	AT4G34370	NA	0.06	-0.9135122	4.55
m157533	4	16446260	1.61E-05	4.79	AT4G34400	NA	0.07	-0.9139914	4.96
m157535	4	16447184	1.44E-05	4.84	AT4G34400	NA	0.06	-1.0156078	5.13

m168339	5	4162238	7.20E-06	5.14	NA	NA	0.08	-0.8903852	5.46
m168340	5	4162436	5.14E-08	7.29	NA	NA	0.09	-1.0165636	8.06
m170625	5	5523616	2.75E-05	4.56	AT5G16790	NA	0.24	-0.549152	5.19
m170626	5	5523865	1.08E-05	4.96	AT5G16790	NA	0.18	-0.6298396	5.5
m170627	5	5524512	4.81E-05	4.32	AT5G16800	NA	0.24	-0.533261	4.86
m170665	5	5548843	3.69E-05	4.43	AT5G16880	AT5G16870	0.18	-0.6072566	5.04
m191488	5	16143599	3.66E-05	4.44	AT5G40360	NA	0.93	-0.8864544	5.04
m198864	5	18767384	7.60E-05	4.12	AT5G46270	NA	0.52	-0.4134298	4.01
m201187	5	19871464	6.69E-05	4.17	AT5G49020	NA	0.07	-0.8438792	4.23
m211864	5	25843861	4.22E-05	4.37	AT5G64660	NA	0.11	-0.6807628	4.23

MAF= minor allele frequency. Gene1 and gene2 = genes that associated with corresponding SNPs. NA = no other associated genes.

Effect size in minus indicates the effect size of non-Col-0 allele of the corresponding SNP. % of variation = percentage of explained phenotypic variation.

Genes play a role in response to combinatorial stress

Another 191 candidate genes were in linkage disequilibrium (LD > 0.3) with the associated significant SNPs, increasing the total number to 221 candidate genes that could explain the allelic variance revealed by challenging the population with the single and combinatorial stresses (Supplementary Table 3). Some candidate genes were reported to play a role in response to biotic, abiotic or plant hormone treatments, but none were reported a role in response to both drought and *Botrytis* infection. To study the interaction of drought and *Botrytis* infection, six candidate genes: *DREB2A-INTERACTING PROTEIN 1 (DRIP1*; AT1G06770), *ARMADILLO BTB PROTEIN 1 (ABAP1*; AT5G13060), *TUBULIN BETA-5 CHAIN (TUB5*; AT1G20010), *PHENYLALANINE AMMONIA-LYASE 4 (PAL4*; AT3G10340), *MYB DOMAIN PROTEIN 46 (MYB46*; AT5G12870) and *HEAT SHOCK FACTOR 1B (HSFA1b)* (AT5G16820), were selected based on available homozygous T-DNA insertion mutants. *drip1, tub5* and *pal4* mutants were tolerant to drought and *myb46, abap1* and *hsfa1b* were susceptible to drought when compared to the wild-type (Figure 4.a). Under *Botrytis* infection, *pal4* mutant was less infected when compared to the wild-type (Figure 4.b). Comparing the DW of plants under combinatorial stress to *Botrytis* infection without pre-treatment, *pal4* and *hsfa1b* exhibited reduced DW when compared to the wild-type (Figure 4.c). When comparing the lesion diameter under combinatorial stress to *Botrytis* infection, *tub5* and *myb46* mutants exhibited less spread lesion diameter than the wild-type (Figure 4.d) and *hsfa1b* displayed larger spread lesion diameter than the wild-type.
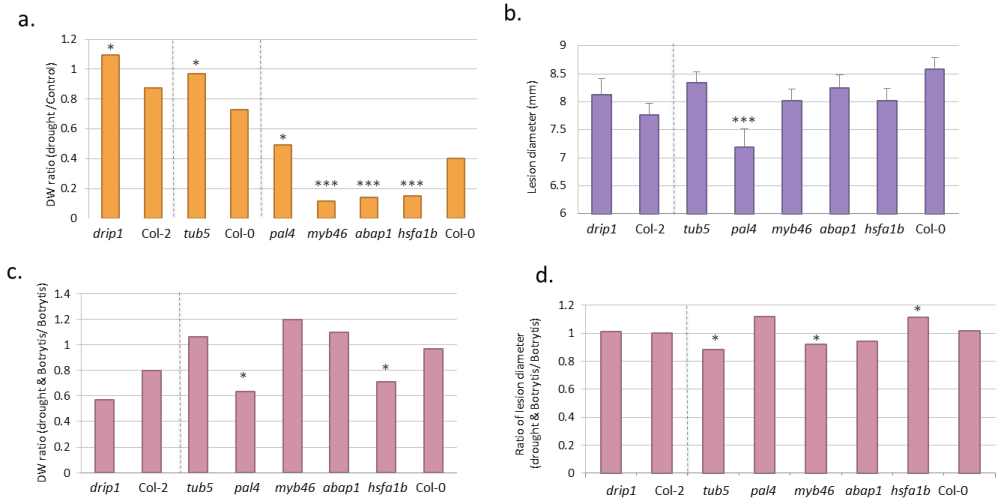
Figure 4. Phenotype of T-DNA insertion mutants of *genes DRIP1, TUB4, PAL4, MYB46, ABAP1* and *HSFA1B,* in response to drought (yellow, a.), in response to *Botrytis* infection (purple, b), in response to the combinatorial stress (pink, c & d). Dashed line indicates Col-2 is the wild-type for drip1 mutant.
a. Ratio of rosette dry weight (DW) of the wild-types (Col-0, Col-2) and the mutant plants in response to drought, when compared to the control.
b. Lesion diameter (mm) of these genotypes under *Botrytis* treatment.
c. Ratio of DW of these genotypes under combinatorial stress, when compared to *Botrytis* without pre-treatment.
d. Ratio of lesion diameter of these genotypes under *Botrytis* treatment.
Two-way ANOVA was used for all figures. Standard error for DW was calculated from at least eight plants, standard error for lesion diameter was calculated from at least 48 leaf replicates (6 leaves per plant x 8 biological repeat). * indicates mutants with a significant difference when compared to Col-0 wild type (* $p<0.05$, ***$p<0.001$)

Two overexpression lines *HSFA1bOX*1 (WS-2 background) and *HSFA1bOX*2 (Col-0 background) were used to observe the effect on *Botrytis* and the combinatorial stress responses. Plants that overexpressed *HSFA1B* were more tolerant to drought than wild-types (Figure 5.a). Under *Botrytis* infection, reduced lesion diameter was observed from the two overexpression lines when compared to the wild-types (Figure 5.b). We found *HSFA1bOX2* is more tolerant to the combinatorial stress when compared to the wild-type (Figure 5.c) and only slightly more tolerance was observed in *HSFA1bOX1*, though not significant. Comparing the lesion diameter under combinatorial stress to single *Botrytis* infection, the two overexpression lines were more sensitive to *Botrytis* when pre-treated with drought than the wild-types (Figure 5.d). Less spread lesion

diameter of the two overexpression lines was found when compared to the wild-types under combinatorial stress (Figure 5.e).
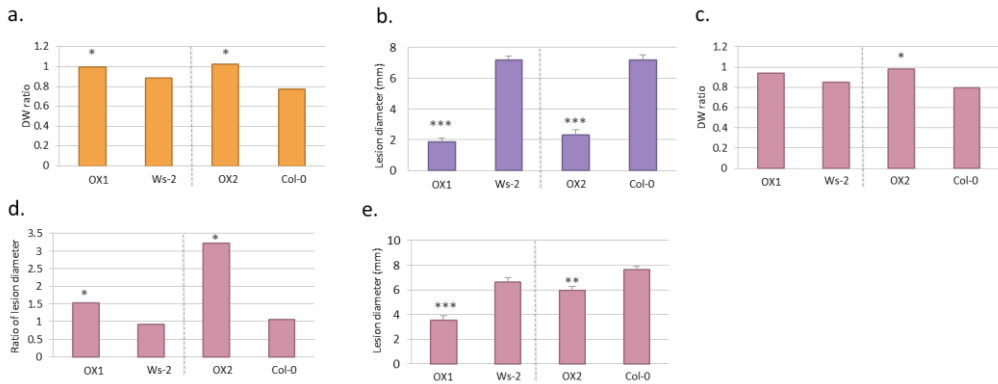
Figure 5. Phenotype of HSFA1b overexpression lines and the wild types in response to drought (yellow, a.), in response to *Botrytis* infection (purple, b), in response to the combinatorial stress (pink, c, d & e), Dashed line indicates the HSFA1b overexpression line 1 (OX1) and the wild-type Ws-2; HSFA1b overexpression line 2 (OX2) and the wild-type Col-0.
a. Ratio of rosette dry weight (DW) of the two wild-types (Ws-2, Col-0) and the two overexpression lines (OX1, OX2) in response to drought when compared to the control.
b. Lesion diameter of these genotypes under *Botrytis* treatment.
c. Ratio of DW of these genotypes under combinatorial stress, when compared to *Botrytis* without pre-treatment.
d. Ratio of lesion diameter of these genotypes under combinatorial stress, when compared to *Botrytis* without pre-treatment.
e. Lesion diameter of these genotypes under combinatorial stress.
Two-way ANOVA was used for all figures. Standard error for DW was calculated from at least eight plants, standard error for lesion diameter was calculated from at least 72 leaf replicates (6 leaves per plant x 12 plants). * indicates mutants with a significant difference when compared to Col-0 wild type (* $p<0.05$, **<0.01,***$p<0.001$).

To observe the effect of drought pre-treatment on gene expression in sequential *Botrytis* infection condition, the relative expression fold change of genes *TUB5*, *PAL4*, *MYB46* and *HSFA1b* were measured in eight Arabidopsis accessions that either were susceptible (Est-1, CS76127; Sei-0, CS28729; Kulturen-1, CS76156; or RRS-7, CS28713) or tolerant (Col-0, CS76113; Rsch-4, CS76222; Cnt-1, CS28160; or Can-0, CS76109) to the combinatorial stress treatment in comparison to *Botrytis* stress alone (phenotype result was from the screening of the Hapmap accessions in the two conditions). *TUB5* was down-regulated under drought but up-regulated under *Botrytis* infection in all accessions (Figure 6.a). We found drought pre-treatment enhance the down-regulation of *TUB5* in sequential *Botrytis* infection condition. *PAL4* was down-regulated under drought but up-regulated under *Botrytis* treatment (Figure 6.b).

The gene was up-regulated under combinatorial stress but the expression fold change was reduced in all accessions when compared to the single *Botrytis* treatment. *MYB46* was up-regulated in all conditions, but the expression fold change was reduced in some accessions under combinatorial stress in comparison to the single *Botrytis* treatment (Figure 6.c). Variations of *HSFA1b* gene expression among accessions in stress conditions were observed (Figure 6.d). No significant effect of the interaction between drought and *Botrytis* on *HSFA1b* expression was observed.
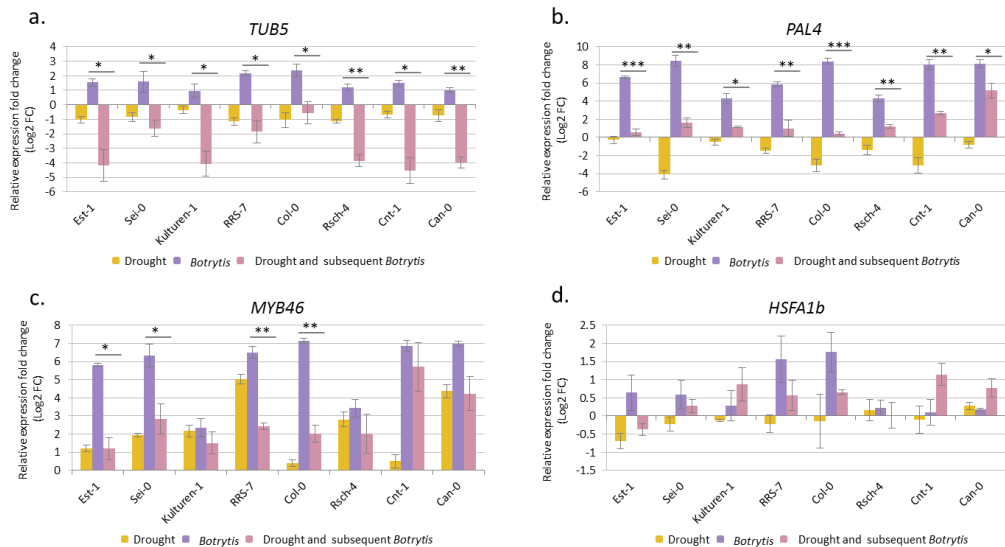
Figure 6. Relative expression fold change at Log2 scale of *TUB5* (a.), *PAL4* (b.), *MYB46* (c.) and *HSFA1b* (d.) expression in eight Arabidopsis accessions after exposure to drought (yellow), after *Botrytis* infection (purple) and after exposure to drought and followed by *Botrytis* infection (pink), compared to control. Log2 scale at 1, 2, 3, 4, 5, 6, 7, 8 = actual expression fold change of 2, 4, 6, 8, 16, 32, 64, 128, 256 times. In all figures, two-way ANOVA was used to compare accession under combinatorial stress to single *Botrytis* stress. Standard error was calculated from 3 plants per accession. * indicates mutants with a significant difference when compared to drought (* $p<0.05$, **$p<0.01$, ***<0.001)

Discussion

Mapping significant SNPs associated with drought, Botrytis and combinatorial stress

This study uncovers the genes for which there is natural allelic variation affecting the Arabidopsis response to the single and combinatorial response to drought and *Botrytis* exposure. GWA-mapping revealed 221 candidate genes that could have allelic effects on *Botrytis* and the combinatorial stress responses. High heritability was found under both conditions, indicating that the variation of accessions in responses to stresses is due to its genetic variation. However, each identified individual QTLs explained only small genetic variances (Table 1). It is possible that a GWAs analysis on a complex trait, such as DW, could result more QTLs but with less effect size, while a simple trait could result less QTLs but with large effect size (Louthan and Kay, 2011). Several studies combined association mapping with linkage mapping and identified flowering time associated QTLs (Brachi et al., 2010), Hyaloperonospora arabidopsidis ex parasitica (Hpa) resistance genes (Nemri et al., 2010), endoreduplication related genes (Sterken et al., 2012) and shoot regeneration genes (Motte et al., 2014). Thus, to confirm the loci associated with combinatorial stress response follow-up studies of QTL analysis using parental populations will be needed.

We observed nonsynonymous amino acid substitution caused by the non-Col alleles of SNPs on gene *ARI1, 3XHMG-Box2* and *PRMT4A* and observed negative effects of the non-Col like alleles in response to the combinatorial stress when compared to the Col-0 like alleles. This shows that the three genes could be causative genes with allelic effects on the combinatorial stress responses. *PRMT4A* is involved in the FLOWERING LOCUS C (FLC)-dependent pathway, but has a redundant function with *PRMT4B* (AT3G06930) because only the *prmt4aprmt4b* double knockout mutant exhibited enhanced expression of *FLC* (Niu et al., 2008). The GWAs analysis identified *PRMT4A* as the candidate gene for combinatorial stress but not PRMT4B, showing a role of PRMT4A to combinational stress response.

Two SNPs m370070 and m370071 associated to gene AT1G50650 exhibited nonsynonymous amino acid substitution but did not affect the combinatorial stress responses. This shows that the two SNPs itself are not the causal SNPs but other SNPs in LD or the two SNPs in combination with other SNPs in LD could be casual SNPs.

Although we did not validate the allelic effects of candidate genes *TUB5*, *PAL4*, *MYB46* and *HSFA1b* on stress responses, the mutants exhibited different phenotype in response to combinatorial stress when compared to the wild-type. This shows that the allelic variation of the four genes could contribute to the genetic variance to combinatorial stress responses. In this study, allelic effects on gene expression was observed for *MYB46.* The expression of *MYB46* was reduced by the drought pre-treatment in most tolerant accessions in sequential *Botrytis* condition, but not in susceptible accessions (except in Col-0) (Figure 5.c). Other genes such as DRIP1 and *SUPERSENSITIVE TO ABA AND DROUGHT 1 (SAD1, At5g48870)*, which are two candidate gene in LD with the significant SNPs, could show the effect of allele variation in stress responses. DRIP1 can interact with DREB2A (*DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A*) hereby inhibiting DREB2A function by marking the protein for proteasomal degradation via ubiquitination (Qin et al., 2008). Recently, similar negative regulation of DREB2A by DRIP1 was reported in cowpea (Sadhukhan et al., 2014) indicating that this part of the drought response in plants is conserved and likely to be tightly controlled. We showed that loss of *DRIP1* resulted in a tolerance to drought stress when compared to wild-type (Figure 3.a). *SAD1* is a multifunctional protein involved in mRNA splicing, export and degradation (Xiong et al., 2001). The *sad1* mutant is impaired in regulation of the ABA-dependent stress responsive genes (Xiong et al., 2001) and has an early flowering phenotype (Perez-Santangelo et al., 2014), a common drought escape strategy (Franks, 2011; Sherrard and Maherali, 2006). Moreover, the finding of *SAD1* shows that there is possibly extensive degradation at the mRNA level (Golisz et al., 2013) and can affect the protein level during stress. Thus, the allele effects of *SAD1* on mRNA degradation is an area interesting to explore. To confirm the effects of the allele variations on combinatorial stress responses, complementation tests to validate the biological role of the natural alleles of the described genes are necessary.

The role of candidate genes in response to drought and Botrytis stresses

The knockout mutants of candidate genes *MYB46, PAL4* and *TUB5* exhibited different phenotypes compared to wild type and the genes displayed differential gene expression under the combinatorial stress when compared to the single *Botrytis* treatment. Antagonistic interaction between drought and *Botrytis* was for pal4 mutant, and the same effect was found on *TUB5* gene expression. Negative effect of drought pre-treatment was observed in *hsfa1b*, *tub5* and *myb46* mutants in sequential *Botrytis* infection. The negative effect of drought pre-treatment was observed on the expression of *PAL4* and *MYB46* as well.

The *MYB46* transcription factor regulates the expression of many secondary wall biosynthesis genes such as cellulose synthases genes (*CESA4, CESA7, CESA8)* and activates MYB family and NAC family transcription factors, which are involved in lignin and secondary wall biosynthesis (Ko et al., 2014). *CESA8* is also a drought and osmotic tolerant gene (Chen et al., 2005). Thus, the susceptible phenotype of *myb46* to drought stress could be due to less expression of *CESA8*. With regard to the *Botrytis* stress, one study reported that the *myb46* mutant is tolerant to *Botrytis* infection because the *Botrytis* defense responsive gene *PDF1.2* is highly expressed in the mutant and that led to an enhanced tolerance to *Botrytis* stress (Ramirez et al., 2011). However, in the present study, we did not observe the same phenotype of *myb46* mutant in response to *Botrytis* infection when compared to the wild type. Downstream of MYB46 are MYB58 and MYB63 that are involved in regulating lignin biosynthesis through regulating the expression of several monolignol biosynthesis genes including *PAL1* and *PAL4* (Kim et al., 2014; Ko et al., 2014; Ko et al., 2009; Zhou et al., 2009). *PAL4* is a member of the Phenylalanine ammonia-lyase (PAL) gene family that contains four genes *PAL1-PAL4* that respond to various stresses such as pathogens, cold, wounding and UV (Huang et al., 2010). *PAL* genes are involved in salicylic acid (SA) accumulation, the knockout mutants of *PAL* genes exhibited 75% reduction of SA when compared to the wild type (Dempsey et al., 2011). SA can antagonize JA (Yoshioka and Shinozaki, 2009). Thus, we hypothesis that the increased tolerance to *Botrytis* is due to reduced SA concentration in pal4 mutant, which led to increased JA accumulation.

Two SNPs were located at the exon of *HSFA1b* and were in LD (LD>0.46) with significant SNP m170625 (-log(*p*)=4.56). The non-Col like alleles of the two SNPs led to nonsynonymous amino acid substitution. However, we did not observe significant effects of the non-Col like alleles (neither individual nor in combination with each other) on combinatorial stress response, when compared to the Col-0 like alleles. This is showing that the two alleles are not the causal alleles of HSFA1b. However, the HSFA1b gene is the most likely candidate underlying this QTL, as in our hands, the KO mutant was susceptible to drought and drought + *Botrytis* and the overexpression lines were tolerant to both stresses. *HSFA1b* belong to the HEAT SHOCK TRANSCRIPTION FACTOR (HSF) family genes and is regulated by NAC019 and flowers of *nac019* plants are hypersensitive to drought stress (Duc et al., 2016; Sakuraba et al., 2015). Bechtold et al (2013) showed that overexpression HSFA1b gene led to up-regulation of more than 500 genes, some of them were drought and *Botrytis* responsive genes. Ethylene- and JA-related genes *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6/9 (ERF6/9), JSMONATE-ZIM-DOMAIN PROTEIN 1/5/7 (JAZ1/5/7); Botrytis* defense responsive genes *BCL-2-ASSOCIATED ATHANOGENE 6 (BAG6), OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59);* and drought responsive genes such as *DRE/CRT-BINDING PROTEIN 2B (DREB2b), EARLY RESPONSIVE TO DEHYDRATION 5 (ERD5),* and *RESPONSIVE TO DESSICATION 29B (RD29B)* were up-regulated. This is showing that *HSFA1b* is play a role in response to *Botrytis* and drought stress.

Conclusion

In this study, the univariate GWA-mapping identified a number of SNPs for combinatorial stress responses but the each SNP only explained small proportion of phenotypic variance . Follow-up analysis such as QTL mapping with bi-parental population would be helpful to reduce false positives and to identify QTLs with large effect. A set of 221 candidate genes associated with the significant SNPs were identified and some exhibited allelic effects on stress responses. Follow-up experiment such as complementation tests to validate the biological role of the natural alleles will be needed. Antagonistic interaction between drought and *Botrytis* was observed and we found the negative effect of drought pre-treatment on mutants performance and gene expression during subsequent *Botrytis* infection. We did not observe any mutants were tolerant to the combinatorial stress, as well as to the two single stresses. The present study provides an insights into plant response mechanisms triggered by sequential drought and *Botrytis* infection. We highlighted some candidate genes as targets for breeding by allele mining aimed at ultimate improvement of crop tolerance to relevant stress factor combinations.

Acknowledgements

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Supporting Information

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CHAPTER 5

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 stress conditions that often occur simultaneously or in sequence. To investigate how plants cope with multi-stress conditions, we analyzed the dynamics of whole-transcriptome profiles of *Arabidopsis thaliana* exposed to six sequential double stresses inflicted by combinations of (1) infection by the fungus *Botrytis cinerea,* (2) herbivory by *Pieris rapae*, and (3) drought stress. Each of these stresses induced specific expression profiles over time, in which one third of all differentially expressed genes was shared by at least two single stresses. Of these, 394 genes were differentially expressed during all three stress conditions, albeit often in opposite directions. When two stresses were applied in sequence, plants displayed transcriptome profiles that were very similar to the second stress, irrespective of the nature of the first stress. Nevertheless, significant first-stress-signatures could be identified in the sequential stress profiles. Bioinformatic analysis of the dynamics of co-expressed gene clusters highlighted specific clusters and biological processes of which the timing of activation or repression was altered by a prior stress. The first-stress-signatures in second stress transcriptional profiles were remarkably often related to responses to phytohormones, strengthening the notion that hormones are global modulators of interactions between different types of stress. Because prior stresses can affect the level of tolerance against a subsequent stress (e.g. prior herbivory strongly affected *B. cinerea* resistance), the first-stress-signatures can provide important leads for the identification of molecular players that are decisive in the interactions between stress response pathways.

Keywords:

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 (Broekgaarden *et al.* 2015, Giron *et al.* 2013, Kazan and Lyons 2014, O'Brien and Benková 2013, Pieterse *et al.* 2012, Robert-Seilaniantz *et al.* 2011). 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 and 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 (Verhage *et al.* 2011, Vos *et al.* 2013b, Yasuda *et al.* 2008). Antagonistic and synergistic interactions between hormonal signal-transduction pathways are 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, Reymond and Farmer 1998, Robert-Seilaniantz *et al.* 2011, Vos *et al.* 2015, Vos *et al.* 2013a).

In natural and agricultural settings, plants often have to cope with multiple stress conditions simultaneously. 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 (Appel *et al.* 2014, Atkinson and Urwin 2012, Rejeb *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 (Atkinson *et al.* 2013, De Vos *et al.* 2006, Kissoudis *et al.* 2014,

Mohr and Cahill 2003, Prasch and Sonnewald 2013, Ramegowda and Senthil-Kumar 2015, Rasmussen *et al.* 2013, Rivero *et al.* 2014, Santino *et al.* 2013, Sewelam *et al.* 2014, Sham *et al.* 2015, Stam *et al.* 2014, Suzuki *et al.* 2014, Van Oosten *et al.* 2008). From these studies, the picture emerged that different stress signaling pathways are interconnected in a network that is under control of key regulators such as MAP kinases, transcription factors and the above-mentioned stress-related hormones (Caarls *et al.* 2015, Fujita *et al.* 2006, Pieterse *et al.* 2009, Rejeb *et al.* 2014, Robert-Seilaniantz *et al.* 2011). In order to gain insight in the complexity of the plant response to combinatorial stresses, several recent studies investigated changes in the transcriptome of *Arabidopsis thaliana* (hereafter called Arabidopsis) in response to simultaneous exposure to abiotic and biotic stresses (Atkinson *et al.* 2013, Prasch and Sonnewald 2013, Ramegowda and Senthil-Kumar 2015, Rasmussen *et al.* 2013, Sham *et al.* 2015, Suzuki *et al.* 2014). 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 on the other may primarily have an effect on the timing of the response to the second stress, causing the detection of large transcriptional differences in combinatorial stresses at one time point, while over time these differences may be much smaller or are the result of a shift in the phasing of the expression profiles.

In order to gain detailed insight into how plants cope with multiple stresses, we here investigated 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 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 are 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 (Birkenbihl *et al.* 2012, Clauw *et al.* 2015, De Vos *et al.* 2005, Ferrari *et al.* 2007, Huang *et al.* 2008, Rehrig *et al.* 2014, Reymond *et al.* 2004, Reymond *et al.* 2000, Rowe *et al.* 2010, Windram *et al.* 2012), but their dynamic behavior during multi-stress conditions is largely 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 (Laluk and Mengiste 2010, Van Kan 2006). 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* (Diaz *et al.* 2002, El Oirdi *et al.* 2011, Geraats *et al.* 2002, Rowe *et al.* 2010, Thomma *et al.* 1998, Thomma *et al.* 1999), while ABA and SA can have a negative effect on *B. cinerea* resistance (El Oirdi *et al.* 2011, Liu *et al.* 2015, Vos *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 (Agrawal 2011, Cyr and Pace 1993, 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 crucifers as chemical defenses (Hopkins *et al.* 2009). Arabidopsis and other plants activate additional defense responses that reduce the performance of 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 and Jander 2008, Vos *et al.* 2013b). 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 and Reymond 2007,

Howe and Jander 2008, Soler *et al.* 2013, Vos *et al.* 2013b). SA is reported to inhibit the JA-dependent defense pathway that is induced by *P. rapae* feeding (Koornneef *et al.* 2008).

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 and 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 and 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, Huang *et al.* 2008, Riera *et al.* 2005, Shinozaki *et al.* 2003).

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 adapting its transcriptome to respond to the second stress. Over time, this second stress-induced 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.

Material &Methods

Plant cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 were sown in cultivation containers filled with autoclaved river sand. Sand was supplied with half-strength Hoagland solution containing Sequestreen as described (Van Wees *et al.* 2013). In order to attain 100% relative humidity (RH) for germination, cultivation containers were enclosed in a tray with water covered with a transparent lid. Seeds were stratified for two days at 4°C in the dark to ensure a homogeneous germination after which the tray was moved to a growth chamber (t=0) with an 8-h day/16-h night rhythm, a temperature of 21°C, and a light intensity of 100 µmol/m^2/s. After 10 d, the lids of the trays were slightly opened and gradually removed over a 4-d period to adjust to the 70% RH present in the growth chamber. Fourteen-day-old seedlings were transplanted to individual pots containing a mixture of river sand and potting soil (1:1 (v:v)). Pots were supplied with water from the bottom up three times per week. At an age of 3 weeks the plants were supplied once with half-strength Hoagland solution.

Rearing of P. rapae and treatment with P. rapae caterpillars

Pieris rapae 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 (nectar) food. When flowers were scarce, additional food (solution of 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. After starving for 1 h, first-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described (Van Wees *et al.* 2013).

Cultivation of B. cinerea and treatment with B. cinerea spores

Botrytis cinerea strain B05.10 (Staats and 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, filtered through glass wool, and re-suspended in half-strength Potato Dextrose Broth (PDB; Difco Laboratories) to a final density of 1×10^5 spores/ml. After a 3-h incubation period, the spores were used for inoculation by applying 5-µl droplets on Arabidopsis leaves as described (Van Wees *et al.* 2013). For the RNA-Seq analysis, 4 droplets were applied on a single leaf, while for disease resistance assays, a single droplet was administered to the leaf.

Single and sequential double stress treatments

Single and sequential double stress treatments were applied according to the schedule shown in Fig. 1. Developmental leaf number 8 was treated with the second stress and harvested for RNA-Seq analysis. Individual leaves were numbered from oldest to youngest. For single and sequential double stress treatments in which *B. cinerea* was the second stress, developmental leaf number 8 of 5-week-old plants was inoculated with *B. cinerea* by pipetting four 5-µl droplets of spore suspension (1×10^5 spores/ml) onto the leaf. Plants were kept at 100% RH for the remaining time period. Mock-treated plants received droplets of PDB and were kept at 100% RH. Pre-treatment with drought was started when plants were 4 weeks old by withholding water for 7 d, after which plants were re-watered and allowed to recover for 1 d before plants were inoculated with *B. cinerea*. *P. rapae* pre-treatment was started 1 d prior to *B. cinerea* inoculation by placing a single *P. rapae* L1 caterpillar on leaf 7 and allowing it to feed for 1 d. Only plants of which leaf number 8 was undamaged 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 in which *P. rapae* herbivory was the second stress, two *P. rapae* L1 larvae were transferred to developmental leaf number 8 of 5-week-old plants. Pre-treatment with drought was achieved as described above for the *B. cinerea* experimental set-up. *B. cinerea* pre-treatment was performed 1 d prior to introduction of *P. rapae* by inoculating leaves 6 and 7 with one 5-µl droplet of *B. cinerea* spore suspension (1×10^5 spores/ml) per leaf and placing the plants at 100% RH for 1 d. A mock treatment for the *B. cinerea* pre-treatment was included by placing droplets with

PDB on the leaves and keeping the plants at 100% RH for 1 d. 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 in which drought was 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 1 d. *B. cinerea* pre-treatment was performed at the beginning of day 1 of the drought period by inoculating leaves 6 and 7 with one 5-μl droplet of *B. cinerea* spore suspension (1×10^5 spores/ml) per leaf and placing the plants at 100% RH for 1 d. A mock treatment for the *B. cinerea* pre-treatment was included by placing droplets with PDB on the leaves and keeping the plants at 100% RH for 1 d. *P. rapae* pre-treatment was performed at the same time as the *B. cinerea* pre-treatment by placing one *P. rapae* caterpillar on leaf 7 and allowing it to feed on the plant for 1 d. Only plants of which leaf number 8 was undamaged were used to harvest leaf 8. 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 1 d 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 PDB and placed at 100% RH for 1 d. For all treatments without *B. cinerea,* controls consisted of untreated plants. 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, with time having four levels (four time points analyzed per stress combination) and treatment having five levels (control, mock, two different first stresses per sequential stress, and one single stress). 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. 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 the Plant RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. All samples were treated with DNase I on column using the Qiagen RNase-Free DNase Set. Quality of RNA was checked by determining the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabChip. For the library preparation samples with a RIN value ≥6 were used. The samples were processed 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, 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.

Processing of raw sequencing data, alignment of the RNA-Seq data to the Arabidopsis genome, and downstream processing was performed as described (Van Verk *et al.* 2013). RNA-Seq reads were aligned to the Arabidopsis genome (TAIR version 10) using TopHat v2.0.4 (Trapnell *et al.* 2009) with parameters: 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', 'read-mismatches 3', 'G', 'min-intron-

length 40', 'max-intron-length 2000'. Gene expression levels were calculated by counting the number of mapped reads per annotated gene model using HTSeq-count v0.5.3p9 (Anders *et al.* 2014). For downstream analyses, raw read counts were normalized for between sample differences in sequencing depth (Love *et al.* 2014). Differential gene expression was calculated using DESeq2 (Love *et al.* 2014) for all stress treatments and time points relative to the appropriate non-stress-treated control/mock treatment that was cultivated and harvested in exactly the same way as the stress-treated samples. The raw *P. rapae* RNA-Seq data have been used in a previous study (Davila Olivas *et al.* 2016). In the study of Davila Olivas *et al.*, the raw *P. rapae* RNA-Seq reads were analyzed independently of this study with the specific goal to identify *P. rapae*-responsive genes that are affected by prior drought stress or *B. cinerea* infection and possibly link them to effects on changes in *P. rapae* resistance. In the present study, the raw *P. rapae* RNA-Seq reads were used in the larger framework of analyzing global dynamics of gene expression profiles during multiple combinatorial stress conditions in which also *B. cinerea* and drought stress were analyzed as second stresses. All raw RNA-Seq read data are deposited in the NCBI Short Read Archive (http:// www.ncbi.nlm.nih.gov/sra/) under the BioProject accession code PRJNA315516.

Gene ontology analysis

To identify enrichment of gene ontology (GO)-terms in the different sets of DEGs, 'Go term finder' (Boyle *et al.* 2004) analysis was performed using an *A. thaliana* gene association file downloaded from ftp.geneontology.org on May 2nd 2013. The default background set was used (all 30504 transcripts in the database that have GO annotations). GO term finder tests for over-representation of GO categories using the hypergeometric distribution and false discovery rate for multiple testing (*P*-value ≤0.05). Figures showing heatmaps of *P*-values were generated using the R package (version 3.2.1).

Clustering

Hierarchical clustering of the core set of single stress DEGs was performed on $\log_2$ 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 optimized in the range 1 to 10 using the Bayesian information criterion.

Wigwams analysis

To identify modules of co-expressed genes across single and sequential stresses the Wigwams algorithm was applied (Polanski *et al.* 2014), using $\log_2$ transformed expression values for the DEGs of each single stress across their respective sequential double stresses. For each main treatment, Wigwams was run with default arguments to partition genes into modules that indicate co-expression in subsets of the relevant main treatments.

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 single and sequential stress was compared at each time point to a non-treated control (for treatments not involving *B. cinerea*) or a mock-treated control (same 100% relative humidity conditions as *B. cinerea* treatments) that was harvested at the same time as the stress treatment. For the study of *B. cinerea* stress, a time span between 6 and 24 h after inoculation with a 5-µl droplet of 5×10^5 spores/ml was chosen, 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 (Vos *et al.* 2015, Windram *et al.* 2012). For the study of *P. rapae* stress, we chose a time span between 3 and 24 h after infestation by larvae of stage L1 because previous studies demonstrated that this would yield a maximal dynamic range of transcriptional responses (De Vos *et al.* 2005, Reymond *et al.* 2004, Reymond *et al.* 2000, Verhage *et al.* 2011). 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 colored than the watered control plants, a phenotype that progressed further on day 6 and 7 when they were at the point of wilting. 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 this recovery response after drought stress is interesting by itself, and at this time point the sequential treatment with *B. cinerea* and *P. rapae* was executed and thus could function as

a reference treatement. Prior to applying the second stress, further development of the first stress was stopped by changing the 100% relative humidity condition to 70% (first stress *B. cinerea*), by removing the caterpillar (first stress *P. rapae*), or by re-watering the plants (first stress drought). Developmental leaf number 8 was used for applying *B. cinerea* or *P. rapae* 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 and 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).
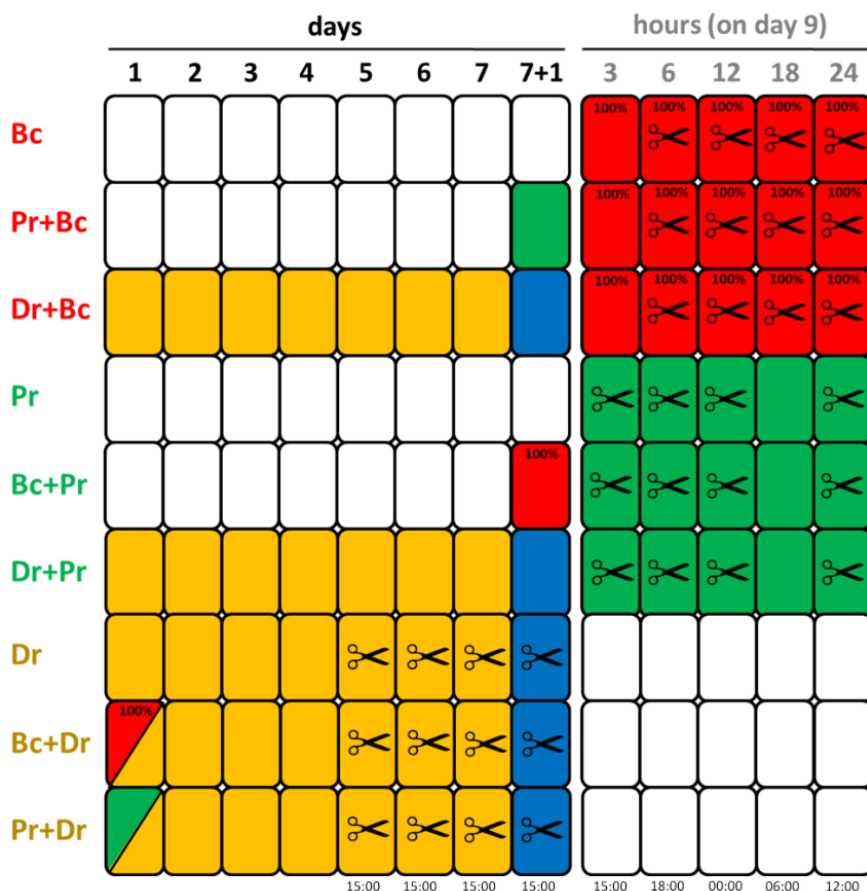
Figure 1. Experimental schedule of treatments and harvests for RNA-Seq time series of single and sequential double stresses. The schedule shows the timing of treatments and time points of harvest for the three main treatments, *B. cinerea* (Bc, red), *P. rapae* (Pr, green) and Drought (Dr, yellow), and the respective pre-treatments. Each single and sequential double stress sample had a mock/control (not visualized) that was harvested at the same time point as the stress treatment. Mock-treated plants were cultivated under the same conditions as their respective *B. cinerea*-treated plants (same periods of 100% RH). Untreated control plants were cultivated under the same conditions as their respective *P. rapae*- and/or drought-treated plants. First stresses were stopped by either lowering relative humidity from 100% to 70% (after 1 d in case of Bc pre-treatment), removing caterpillars from plants (after 1 d), or re-watering after a 7-d period of drought (7+1; blue). In case the second stress was drought, the pre-treatments with *B. cinerea* and *P. rapae* were performed 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 plants were harvested.

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 these 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 an additional threshold level of at least 2-fold change (-1> $\log_2$ >1) in comparison to the respective control (Supporting Table S1). 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 activated or repressed during the different single stress conditions (Fig. 2). For responses to *B. cinerea* (total 2076 unique DEGs) and *P. rapae* (total 3952 unique DEGs)*,* a strong increase in the number of activated genes is observed over time, while relatively few genes are repressed. Upon exposure to drought stress (total 4032 unique DEGs for the first three time points, plus 482 additional unique DEGs for the 1 d after re-watering time point), relatively more genes become repressed than activated. A prior stress did not dramatically change the number of DEGs relative to the single stresses (Fig. 2). Clustering the union of DEGs of the single stress sets (total 7355 unique DEGs), and subsequent Gene Ontology (GO) analysis (Boyle *et al.* 2004) of overrepresented biological processes in each cluster highlights the differentially regulated biological processes during the plant response to the single stresses and uncovers similarities and contrasts between the different stress responses (Fig. 3; Supporting Table S2).
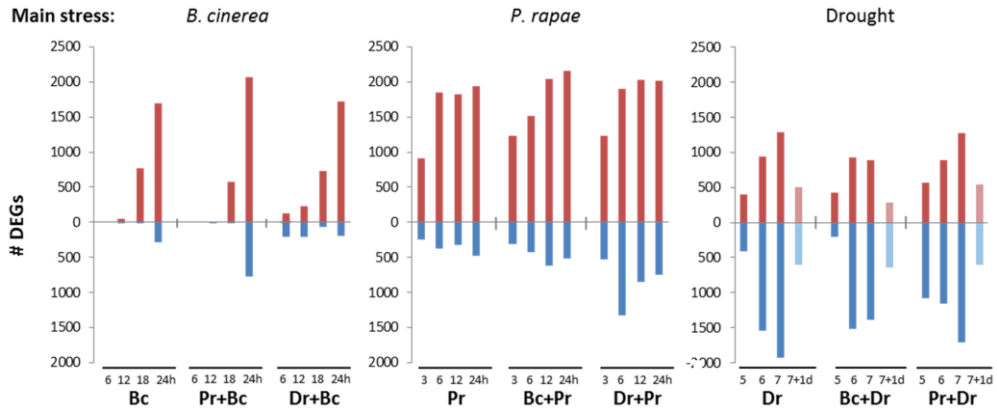
Figure 2. Numbers of DEGs at different time points in single and sequential stress responses. Graphs show the number of activated (red bars) and repressed (blue bars) differentially expressed genes (DEGs) for all single stresses and their corresponding sequential double stresses at different time points after treatment (FDR <0.05; >2-fold). The one day after rewatering time point of the drought treatments is indicated as "7+1d". Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr+Bc, Dr+Bc, Bc+Pr, Dr+Pr, Bc+Dr, and Pr+Dr, respective sequential double stresses.
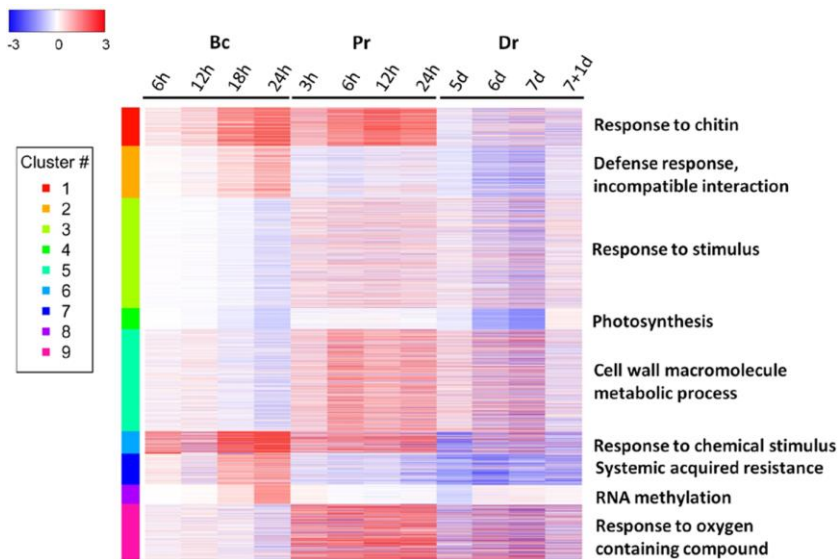

Figure 3. Clustering of the single stress DEGs. Heatmap showing the expression patterns of the union of differentially expressed genes (DEGs) in the three single stresses at different time points after induction (total 7173 unique genes). DEGs were clustered using Mclust yielding 9 gene clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Supporting Table S2). Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought. For drought stress, the time point one day after re-watering (7+1d) was included in the cluster analysis. Blue-red color key for change in gene expression level: -3 > $\log_2$ fold change > 3.

Core DEGs that are shared between the single stress responses

To investigate to what extent genes and biological processes are shared between the three single stress responses, we compared their DEGs. Figure 4a shows that there is a large overlap between the DEGs of the single stress responses, ranging from 1716 genes shared between the drought and *P. rapae* sets, to 788 genes between the drought and *B. cinerea* sets, and 777 genes between the *P. rapae* and *B. cinerea* sets. Of all 7173 DEGs (excluding the 1 d after re-watering time point), 2493 DEGs (35%) are shared with one or both of the other stresses. A core set of 394 DEGs (5%) was differentially expressed in response to all three single stresses, and clustered into 12 co-expressed gene clusters (Fig. 4b). Among this core set of shared DEGs are several well-characterized hormone-responsive marker genes, including *LOX2* (At3g45140), *JAZ7* (At2g34600), and *JAZ8* (At1g30135) (JA responsive), *PDF1.2* (At5g44420) and *ORA59* (At1g06160) (JA/ET responsive), *PR4* (At3g04720), *ERF5* (At5g47230), *ERF6* (At4g17490), and *ACS2* (At1g01480) (ET responsive), *Rap2.6L* (At5g13330), and *HAI1* (At5g59220) (ABA responsive), and *PR-1* (At2g14610), *PR-5* (At1g75040), and *FRK1* (At2g19190) (SA responsive) (Supporting Table S1 for details of their expression patterns). In Fig. 4c the expression patterns of well-characterized marker genes of the response to *B. cinerea* (*PDF1.2*; At5g44420), *P. rapae* (*LOX2*; At3g45140), and drought stress (*RAD18*; At5g66400) are depicted, confirming that the different stress treatments resulted in the expected response. When looking at the co-expressed gene clusters, only the genes of cluster 1 (top GO terms related to "response to oxygen-containing compound", "response to JA" and "response to wounding"; Supporting Table S3) are regulated in the same direction (activated) during all three individual stress conditions (Fig. 4b). All other gene clusters behave clearly different in response to the three single stresses and are often regulated in opposite directions (Fig. 4b). For example cluster 8 (top GO terms related to "response to other organism", "defense response" and "immune system process") is activated by *B. cinerea*, but repressed by *P. rapae* and drought. Conversely, cluster 10 (top GO terms "multidimensional cell growth", "response to light stimulus", and "cell wall organization") is activated by *P. rapae,* but repressed by *B. cinerea* and drought. The fact that there is an overlap in the expression of genes under all three single stresses, whether in the same or in

opposite directions, suggests that these genes or their regulators may act as a point of convergence if plants were to experience these stresses in combination.
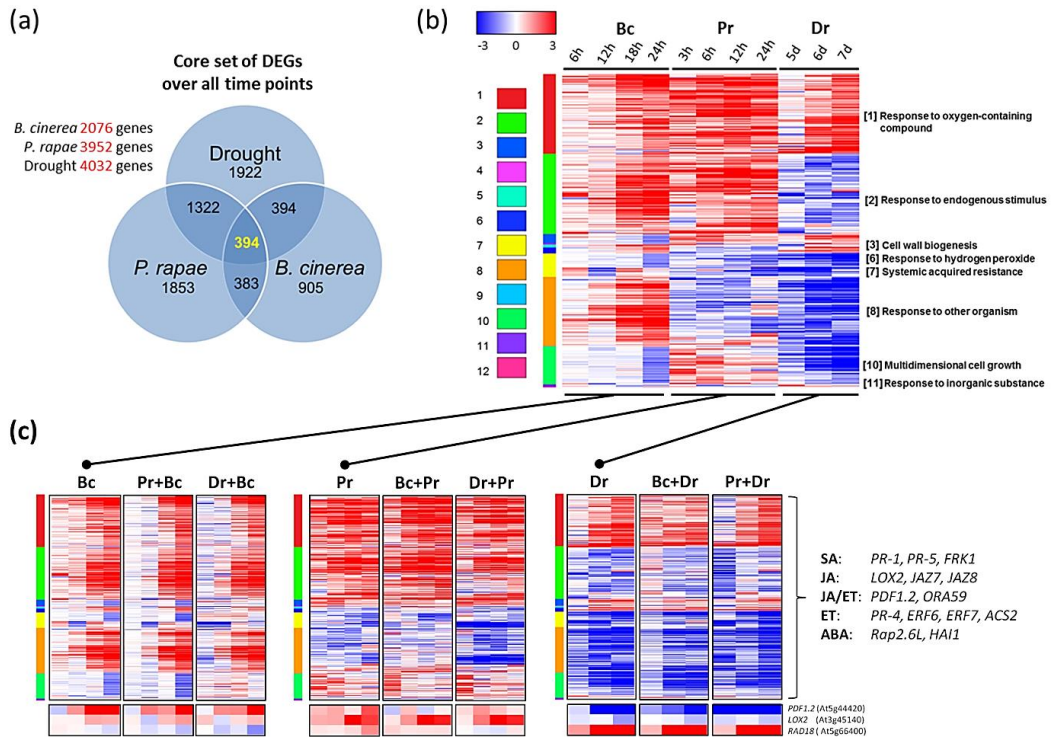

Figure 4. Shared DEGs between the single stress responses. (a) Venn diagram showing the overlap between the DEGs of each of the single stress responses. The total number of unique DEGs per single stress over all time points is shown in red (full data set in Supporting Table S1). (b) Hierarchical clustering of the 394 core DEGs that are shared between the three single stresses (Cosine similarity metric; 12 clusters are color coded in the square boxes on the left). On the right side, the most significant GO term for each cluster (full data set in Supporting Table S3). (c) Comparison of the expression patterns of the 394 core DEGs in response to the single and respective sequential double stresses. Different lanes in (c) reflect the transcription profiles at the time points after treatment as indicated above the lanes in (b). Gene names in the lower right corner represent marker genes of the SA, JA, JA/ET, ET and ABA response pathways that are among the 394 core DEGs. *PDF1.2, LOX2,* and *RAD18,* represent known marker genes for the response to *B. cinerea, P. rapae,* and drought, respectively. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr+Bc, Dr+Bc, Bc+Pr, Dr+Pr, Bc+Dr, and Pr+Dr, respective sequential double stresses. Blue-red color key for change in gene expression level: -3 > log$_2$ fold change > 3.

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* infestation and drought stress on the dynamics of the transcriptome changes that are induced by *B. cinerea* infection, we analyzed the expression patterns over time of all 2076 *B. cinerea*-responsive DEGs. Clustering of this group of genes yielded 10 clusters of co-expressed genes across the *B. cinerea* single and sequential stress data sets. Gene clusters that are activated in response to *B. cinerea* infection are enriched for GO terms such as "response to chitin" (Fig. 5, cluster 3; Supporting Table S4), reflecting recognition of fungal chitin by the plant immune system (Pel and Pieterse 2013), and "response to ET stimulus", reflecting the high level of ET emission that is related to plant responses to *B. cinerea* infection (Broekgaarden *et al.* 2015). In addition, gene clusters that are repressed in response to *B. cinerea* infection are associated with GO terms such as "multidimensional cell growth" (Fig. 5, cluster 9; Supporting Table S4), highlighting the antagonistic relationship between plant growth and defense (Wang and Wang 2014).

In order to identify in greater detail co-regulated genes of which the expression pattern in response to *B. cinerea* infection was affected by 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 gene expression time series and identifies signatures of condition-dependent regulatory mechanisms in co-regulated gene sets. Wigwams identified 35 modules of co-regulated genes in the *B. cinerea* data sets (Supporting Fig. S1). Analysis of these clusters for co-expression revealed gene modules of which the expression patters were clearly affected in one or both of the sequential stress treatments in comparison to the *B. cinerea* treatment alone (examples shown in Fig. 6). These gene modules represent signatures of a previous stress in the *B. cinerea*-induced transcriptome profile, and may thus be functionally related to the effect of the first stress on the outcome of the plant response to *B. cinerea* infection. The genes in these Wigwams modules are given in Supporting Table S5 along with their GO term analysis.
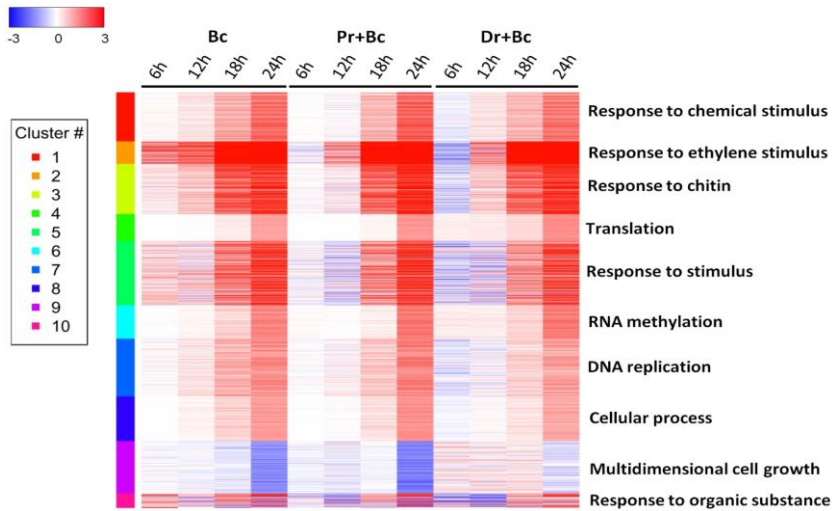
Figure 5. Dynamics of the expression of the _B. cinerea_ set of DEGs during single and sequential double stresses. Heatmap showing the expression pattners of the 2076 _B. cinerea_-responsive DEGs during _B. cinerea_ infection on mock pre-treated (Bc), _P. rapae_ pre-infested (Pr+Bc) or drought pre-treated (Dr+Bc) Arabidopsis plants. The _B. cinerea_-responsive DEGs were clustered using Mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Supporting Table S4). Blue-red color key for change in gene expression level: -3 > $\log_2$ fold change > 3.
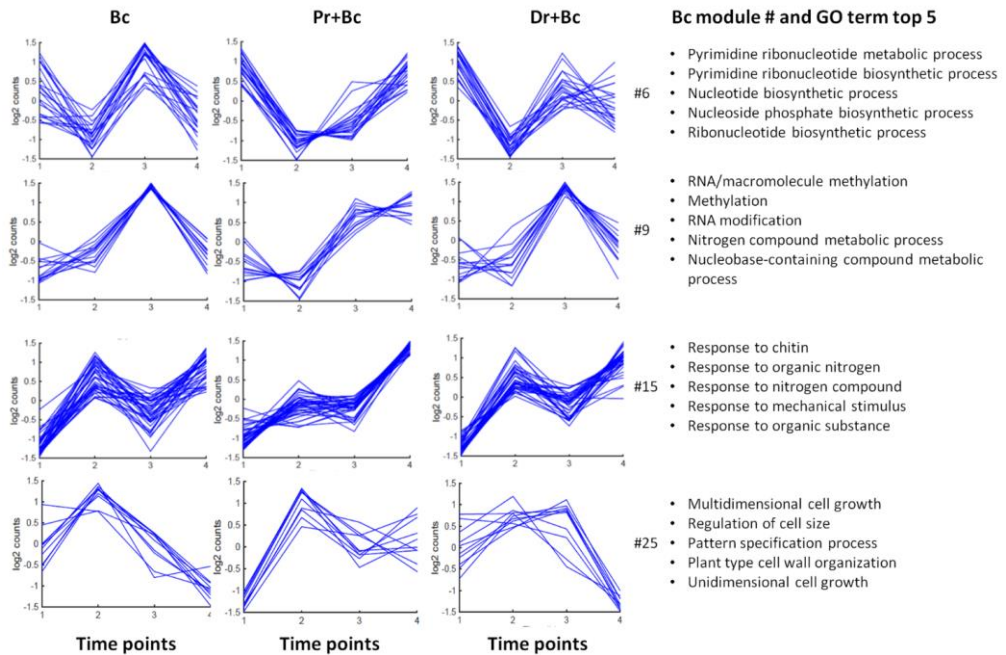
Figure 6. Expression patterns of selected Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *P. rapae-B. cinerea* (Pr+Bc) and drought-*B. cinerea* (Dr+Bc) in comparison to the single stress *B. cinerea* (Bc). The modules represent standardized patterns of differential gene expression over time (log$_2$ 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* inoculation. The top 5 of GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Supporting Table S5).

Among the *B. cinerea*-responsive Wigwams modules of which the co-expression pattern is different when plants were previously exposed to herbivory or drought stress, are gene modules with GO term enrichments for rather general plant processes such as nucleoside biosynthesis and metabolism (modules 6 and 9), and cell growth (module 25), but also modules related to more specific plant processes, such as response to chitin and nitrogen (module 15). Functional analysis of underlying candidate genes should reveal their importance for effects on the outcome of the second stress response.

Further knowledge about the biological processes that are affected when *B. cinerea* infection is preceded by either drought stress or herbivory can be gained by analyzing the phasing of gene expression under the different single and

sequential stress conditions. We did this by analyzing at which time point a GO term becomes significantly overrepresented in the *B. cinerea*-related DEG set. For this, we clustered all *B. cinerea*-responsive DEGs according to their time point of first differential expression, divided them over activated and repressed 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 related to responses to ET, fungus, chitin, SA, and oxygen-containing compound, or to processes such as systemic acquired resistance, respiratory burst, and defense appear early in the activated gene set, reflecting the importance of these processes in the plant response to this necrotrophic pathogen. Prior infestation with *P. rapae* clearly delayed the appearance of these GO terms (become visible at 18 hai in Fig. 7), while pre-treatment with drought stress did not have a dramatic effect on the phasing of the activated genes. For the repressed genes in the *B. cinerea* set of DEGs, pre-infestation with *P. rapae* has clearly only minor effects on the GO term phasing. By contrast, pre-treatment with drought stress noticeably affected the phasing of GO terms related to responses to fungus, JA, SA, ABA, chitin, and oxygen-containing compound, and to auxin metabolic process, defense, systemic acquired resistance, and glucosinolate biosynthetic process. Remarkably, 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.
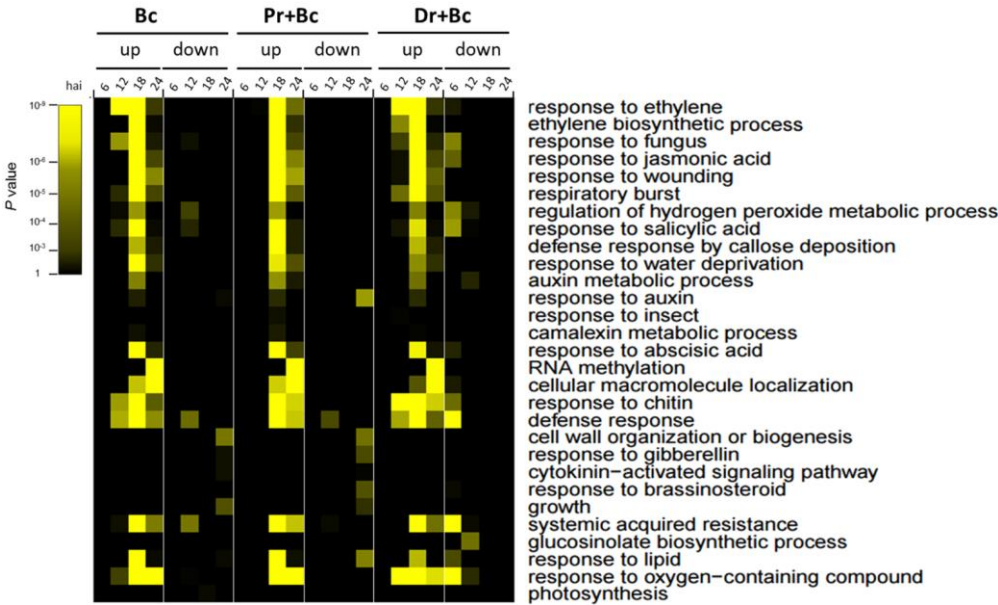
Figure 7. Timing of GO term overrepresentation patterns in *B. cinerea* single and sequential stress data sets. Heatmap represents the strengths of the *P*-values of GO term overrepresentation in the *B. cinerea*-responsive DEG sets (corresponding to the gene lists in Supporting Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P*-values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *B. cinerea* infection.

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

Both herbivory and drought stress imposed a first-stress-signature in the dynamics of the *B. cinerea*-induced transcriptome profiles. Wigwams analysis gained insight into the identity of candidate genes related to these first-stress-signatures (Supporting Fig. S1; Supporting Table S5), whereas analysis of GO term enrichment provided global insight into the biological processes that were affected by the stress pre-treatments (Fig. 7). To investigate whether the two prior stresses affected the resistance level to *B. cinerea* infection we performed disease resistance bioassays. 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. 8).
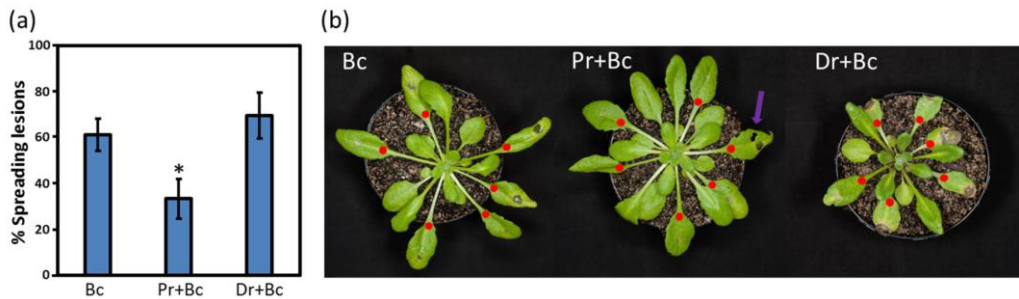

Figure 8. Effect of herbivory and drought stress on resistance of Arabidopsis to *B. cinerea*. (a) Quantification of *B. cinerea* disease symptoms on Arabidopsis accession Col-0 plants (% spreading lesions per plant). On each plant, six leaves were inoculated with one droplet of *B. cinerea* spores. 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; Pr+Bc, *B. cinerea*-inoculated plants that prior to inoculation were exposed to herbivory by *P. rapae* larvae for 24 h; 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. Red dots, *B. cinerea*-inoculated leaves; purple arrow, damage caused by *P. rapae* feeding.

Plants that were exposed to drought stress prior to *B. cinerea* inoculation showed a similar percentage of leaves with spreading lesions (~70%). Interestingly, plants that were exposed to herbivory prior to *B. cinerea* inoculation showed a significantly enhanced 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.

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

The same approach as described above for the *B. cinerea* transcriptome data was taken to investigate the effect of prior *B. cinerea* infection and drought stress on the transcriptional dynamics that are induced by *P. rapae* feeding. Analysis of the global expression profiles of the 3952 *P. rapae*-responsive DEGs yielded 9 clusters of co-expressed genes during single and sequential *P. rapae* stress (Fig. 9).

As expected, *P. rapae* feeding induced many genes related to the GO term "response to JA stimulus" (Fig. 9, cluster 7; Supporting Table S6), reflecting induced defenses that are triggered by herbivory-inflicted wounding (Wasternack 2015). In addition, *P. rapae* feeding repressed SA-related genes associated with GO terms "defense response" and "systemic acquired resistance" (Fig. 9, cluster 3; Supporting Table S6), reflecting the antagonistic relationship between JA- and SA-dependent defenses (Pieterse *et al.* 2012). In analogy with what we observed in the *B. cinerea* data sets, the general gene expression patterns over time overlapped greatly between the responses to *P. rapae* single and sequential double stress treatments, again suggesting that Arabidopsis is capable of reprogramming its transcriptome to the last stress encountered, thereby overruling the effects of the prior stresses. For instance, while prior drought stress impacted the expression of over 1000 genes in the leaf tissue just prior to the start of the *P. rapae* treatment (Supporting Table S1; Fig. 3 last lane), already from the first time point (3 h) after herbivory this effect was mostly vanished in the *P. rapae*-induced profiles, which readily followed a similar expression pattern as in the *P. rapae* single treatment (Fig. 9). A similar pattern is visible in the core set of 394 DEGs (Fig. 4c, middle panel). Nevertheless, during the sequential stresses first-stress signatures can be detected, e.g. genes in cluster 3 of Fig. 9 and clusters 7 and 8 of Fig. 4c (middle panel) show a weaker repression in the *B. cinerea* pre-treatment and a stronger repression in the drought pre-treatment. In general, these *P. rapae*-related results confirm previous findings (Davila Olivas *et al.* 2016).
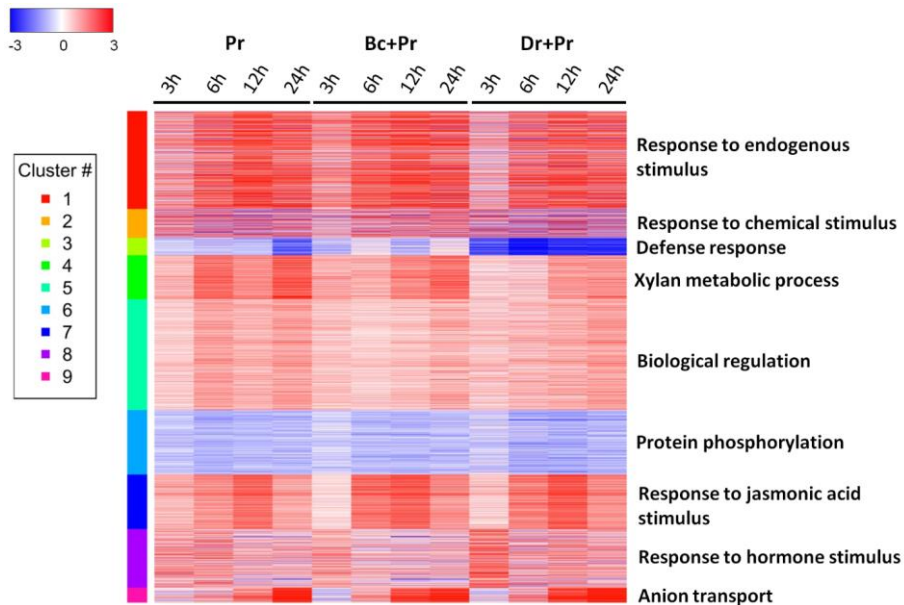
Figure 9. Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the 3952 *P. rapae*-responsive DEGs during feeding of *P. rapae* on control (Pr), *B. cinerea* pre-infected (Bc+Pr), or drought pre-treated (Dr+Pi) Arabidopsis plants. The *P. rapae*-responsive DEGs were clustered using Mclust yielding 9 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Supporting Table S6). Blue-red color key for change in gene expression level: -3 > log$_2$ fold change > 3.

To pinpoint co-regulated genes whose expression pattern in response to herbivory is affected by prior *B. cinerea* infection or drought stress, the set of *P. rapae*-responsive DEGs was analyzed with the Wigwams algorithm. Wigwams identified 93 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 revealed gene modules of which the expression patterns were clearly affected by 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 Supporting Fig. S2). The identities of the genes in the *P. rapae*-related Wigwams gene modules are given in Supporting Table S5 along with their GO term analysis. It is beyond the scope of this paper to discuss the identity of the genes in detail. However, among the *P. rapae*-responsive Wigwams modules of which the co-expression pattern is clearly different when plants were exposed before to *B. cinerea* infection or drought stress, are gene modules with GO term enrichments for xylem, phloem and tissue development

(module 3), cell wall biosynthesis processes (module 45), and responses to JA and wounding (module 69).
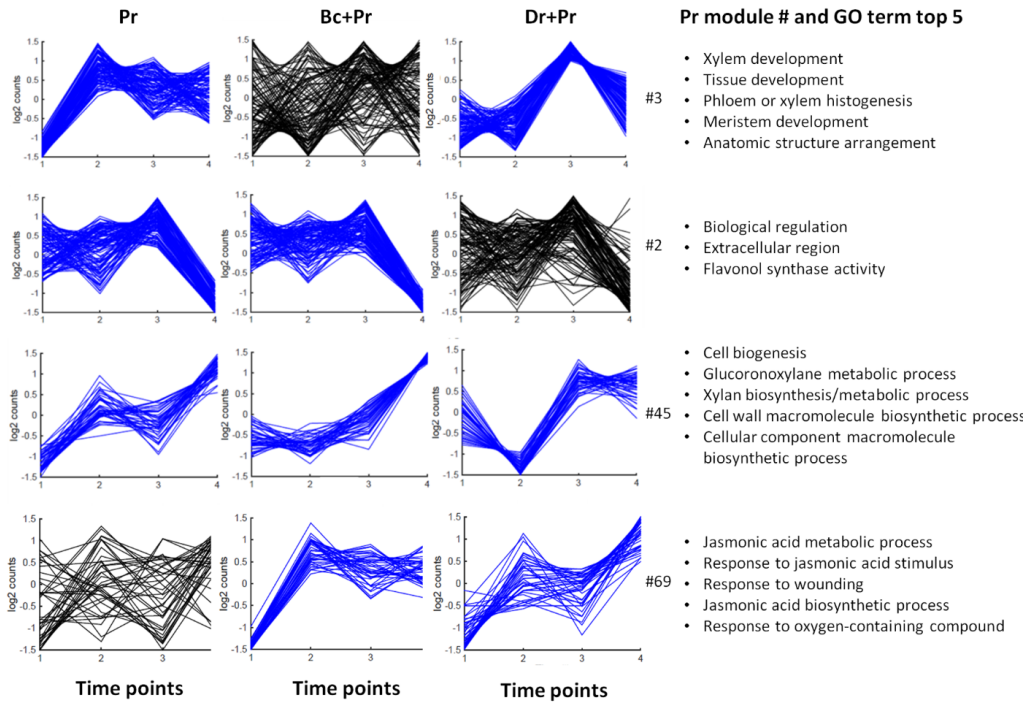
Figure 10. Expression patterns of selected Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea-P. rapae* (Bc+Pr) and drought-*P. rapae* (Dr+Pr) in comparison to the single stress *P. rapae* (Pr). The modules represent standardized patterns of differential gene expression over time (log$_2$ 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. The top 5 of GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Supporting Table S5).

Also for the *P. rapae* data set, phasing of the regulation of biological processes that are associated with herbivory and influenced by prior *B. cinerea* infection or drought stress was determined. Therefore, the level of significance of overrepresentation of the GO terms for the *P. rapae* set of DEGs at their first time of differential expression was assessed. In the *P. rapae* single stress data set, GO terms related to responses to chitin, wounding, JA, ET, SA, auxin, ABA, water deprivation, osmotic stress, and oxygen-containing compound are already highly enriched at 3 h after infestation in the activated set of DEGs (Fig. 11),

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reflecting the importance of these processes in the response of Arabidopsis to herbivory. In many cases, pre-infection with *B. cinerea* strengthened the level of overrepresentation of these GO-terms at different time points after *P. rapae* infestation, while pre-treatment with drought stress often weakened them. In the repressed set of DEGs, drought stress clearly enhanced the overrepresentation of GO terms related to responses to chitin, JA, fungus, ABA, SA, bacterium, and oxygen-containing compound, and to processes such as systemic acquired resistance, defense response to fungus, and negative regulation of programmed cell death, while *B. cinerea* infection had no major effect on the phasing of these GO terms. Overall, these data indicate that *B. cinerea* infection and drought treatment prior to *P. rapae* infestation affects the timing of several defense-related processes, in particular responses to JA, ABA, SA and ET, corroborating the notion that different stresses interact via the hormone-regulated signaling network.
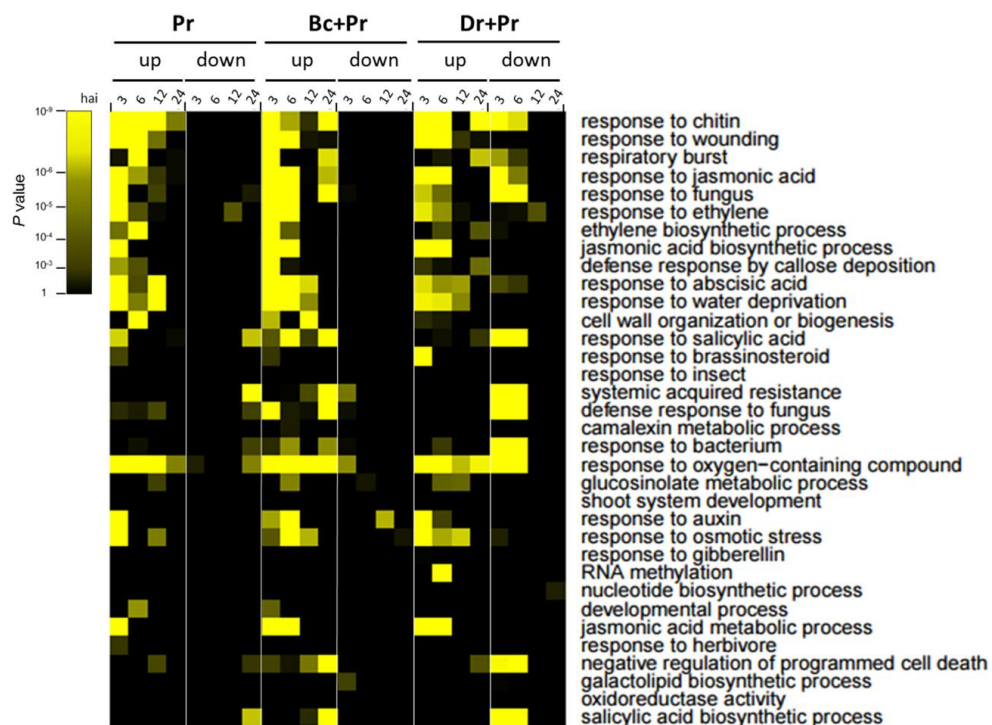
Figure 11. Timing of GO term overrepresentation patterns in *P. rapae* single and sequential double stress data sets. Heatmap represents the strengths of the *P*-values of GO term overrepresentation of *P. rapae*-responsive DEG sets (corresponding to the gene lists in Supporting Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P*-values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *P. rapae* infestation.

Drought data set: effect of B. cinerea infection and herbivory 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 the 4032 drought-responsive DEGs during single and sequential stress with drought as the second stress, which yielded 10 clusters of co-expressed genes (Fig. 12). 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" (Fig. 12, cluster 6; Supporting Table S7) and GO terms related to "response to oxygen-containing compound" (clusters 3 and 10, and cell wall-related processes (cluster 7). Another feature that stands out is the association of drought stress with massive repression of genes, many of which are associated with biological processes such as "photosynthesis" and "defense response" (clusters 1, 2, 5 and 8), reflecting the fact that drought-stressed plants shift their strategy from energy-demanding processes related to growth and immunity 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 for 77% (3106 of the 4032 DEGs; Supporting Table S1) reset towards basal levels within 24 h, demonstrating the plant's ability to swiftly redirect transcriptional programming when drought stress is relieved. Similar to what we observed for the *B. cinerea* and *P. rapae* sequential double stress responses, the gene expression patterns over time in the sequential drought double stress treatments were to a large extent similar to those inflicted by the single drought treatment. In the core set of 394 DEGs it is clear that on the first time point after the start of the drought treatment (5 d), *B. cinerea* and *P. rapae* pre-treatment still had noticeable effects on the drought-induced gene expression profiles (Fig. 4c, right panel; compare the left lanes of Dr, Bc+Dr, and Pr+Dr). However, at the later time points (6 and 7 d) these effects dampened off and the expression patterns became more similar to that of the drought single treatment. Nevertheless, prior stress caused by *B. cinerea* infection or *P. rapae* infestation left first-stress-signatures in the drought-induced transcriptome.
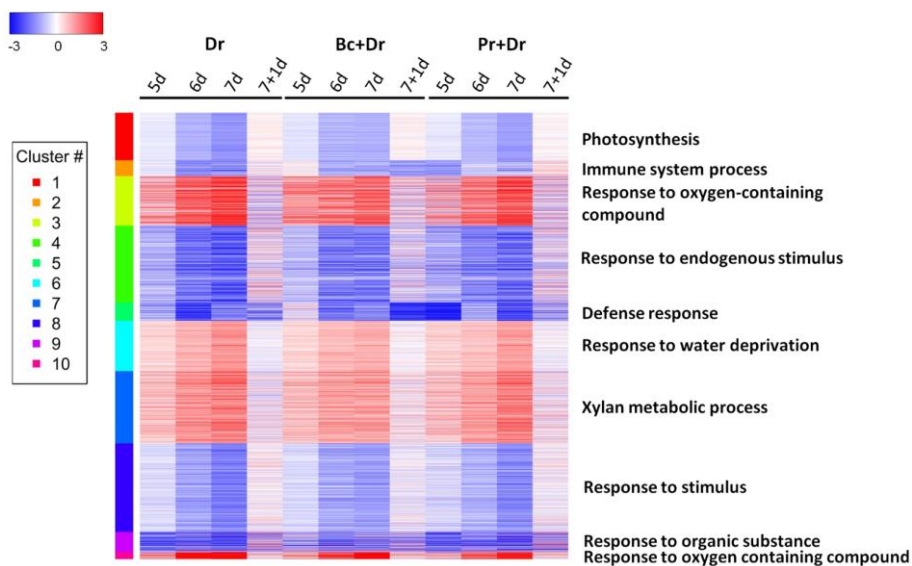
Figure 12. Dynamics of the expression of the drought set of DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the 4032 drought-induced DEGs during a 7-d period of water withhold and 1 d after rewatering on control (Dr), *B. cinerea* pre-infected (Bc+Dr), or *P. rapae* pre-infested (Pr+Dr) Arabidopsis plants. The drought-responsive DEGs were clustered using Mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Supporting Table S7). Blue-red color key for change in gene expression level: $-3 > \log_2$ fold change > 3.

Wigwams analysis of co-regulated genes in the drought data sets identified 72 co-expressed gene modules under the single and sequential double drought stress conditions (examples in Fig. 13; full set in Supporting Fig. S3). The identities of the genes in these Wigwams gene modules are given in Supporting Table S5 along with their GO term analysis. Wigwams modules with clear changes in expression pattern when drought-stressed plants were pre-treated with either *B. cinerea* or *P. rapae* represent genes related to the biological processes such as SA and defense signaling (module 10 and 55), and Nucleosome organisation (module 11). Future analysis of candidate genes in these modules should reveal their importance for the outcome of the combinatorial stress responses.
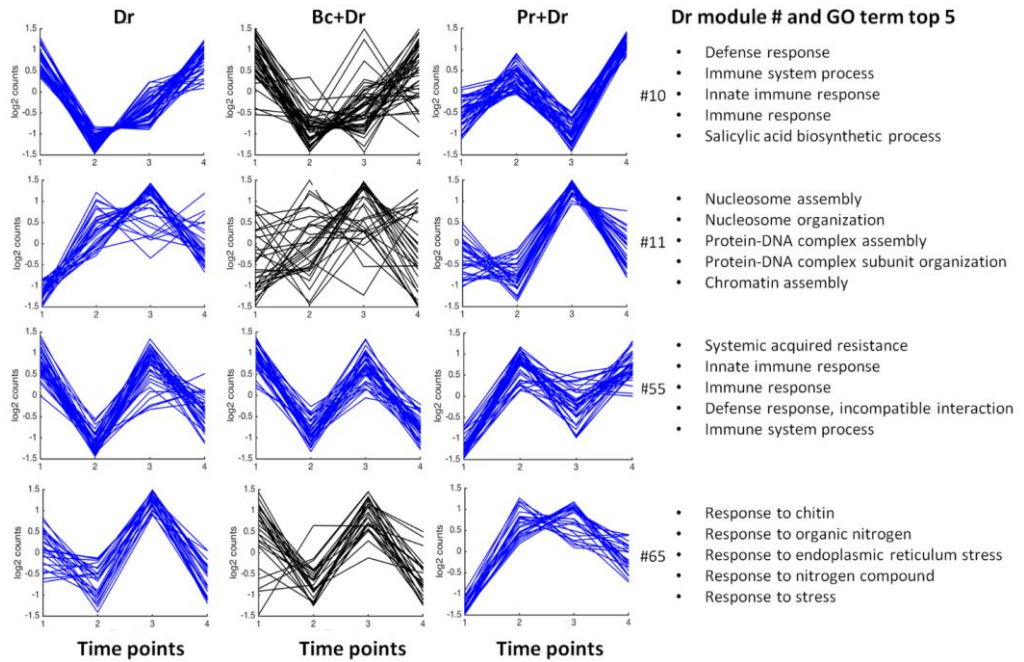
Figure 13. Expression patterns of selected Wigwams modules from the drought set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea*-drought (Bc+Dr) and *P. rapae*-drought (Pr+Dr) in comparison to the single stress drought (Dr). The modules represent standardized patterns of differential gene expression over time (log$_2$ 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. The top 5 of GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Supporting Table S5).

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 related to responses to oxidative stress, water deprivation, osmotic stress, ABA, and oxygen-containing compound, and to processes such as phenylpropanoid biosynthesis, cell wall biogenesis, and lignin metabolism are enriched in the activated gene set at the first day of sampling, while responses to wounding and JA follow somewhat later. In the repressed gene set, GO terms related to responses to JA, SA, chitin, fungus, insect and oxygen-containing compound and to processes such as photosynthesis, shoot system development, systemic acquired resistance, glucosinolate metabolic process, nitrogen compound transport, and respiratory burst are enriched already at the first sampling point. This highlights the biological processes that are engaged or affected during drought stress. Interestingly, pre-infection with *B. cinerea* accelerated the phasing of activated genes associated with biological processes such as responses to chitin, wounding, osmotic stress, ABA, and JA. In the repressed gene set, GO terms related to responses to chitin, fungus, and SA, and to systemic acquired resistance became later enriched than in the single stress data set. When plants were pre-infested with *P. rapae,* the phasing of the drought responsive genes is also clearly affected. Many GO terms in the drought activated gene set become more prominently enriched at later time points. Moreover, in the repressed gene sets GO terms related to responses to water deprivation, osmotic stress, wounding, JA, ABA and ET are highly overrepresented at the first time point of sampling, while this is not the case in the single stress treatment. Like in the *B. cinerea* and *P. rapae* 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.
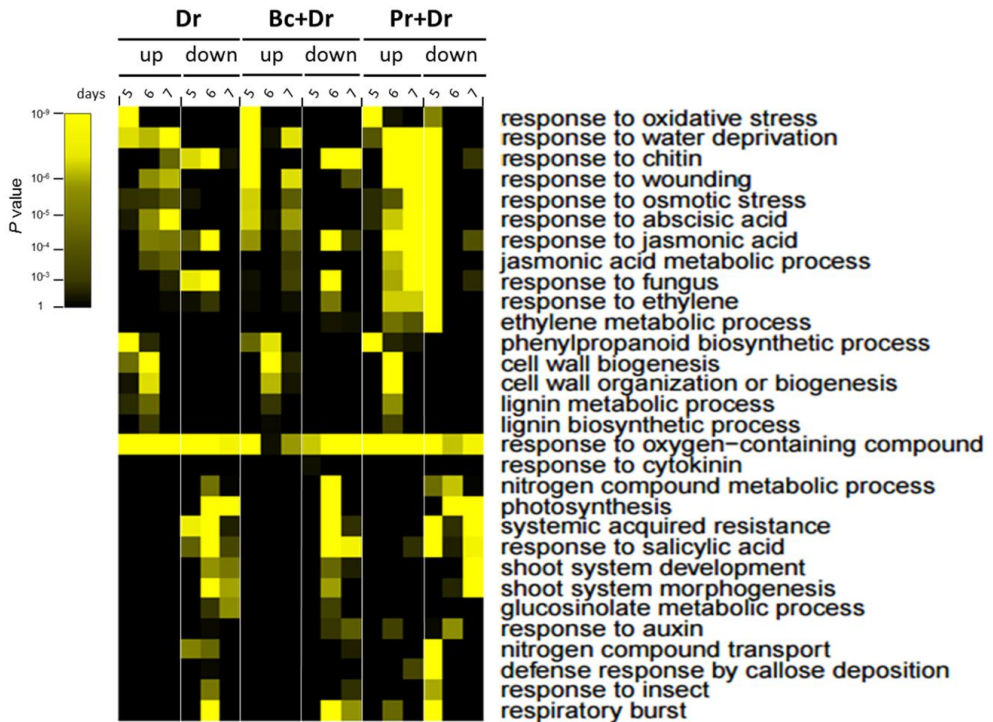
Figure 14. Timing of GO term overrepresentation patterns in drought single and sequential stress data sets. Heatmap represents the *P*-values of GO term overrepresentation of drought-responsive DEG sets (corresponding to the gene lists in Supporting Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index of *P*-values represents level of significance. On the right, overrepresented GO terms. Bc, *B. cinerea*; Pi, *P. rapae*; Dr, drought; days, 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 co-expressed genes related to these first-stress-signatures (Figs. 6, 10 and 13), whereas analysis of GO term enrichment at the onset of gene induction provided global insight into the biological processes that were affected during the time course by the prior stress treatment (Figs. 7, 11 and 14). As an example for the effect of prior stress on the level of plant resistance to a second stress, we showed that the level of infection by *B. cinerea* can be significantly altered when plants were pre-disposed to herbivory (Fig. 8). The bioassays with the other sequential stress treatments showed no strong effects of prior stress treatment on the

performance of the specialist herbivore *P. rapae* (Davila Olivas *et al.* 2016). In future research, we will functionally analyze candidate genes from the first-stress signatures in the second stress profiles to investigate their putative role in a diverse range of multi-stress interactions.

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 to a biotic or abiotic stress 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 we were able to show that 1) on average 35% of the DEGs in a given single stress is also differentially regulated in one or both of the other two single stresses, albeit often in different directions; 2) irrespective of the nature of the first and second stress applied, genes responsive to the second stress rapidly follow a similar pattern as that induced by the second stress alone; 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 the studied stress-induced transcriptomes supporting previous findings (Rejeb *et al.* 2014); and 5) a previous stress can affect the outcome of a response to a sequential second stress, resulting in altered plant resistance.

Transcriptome profiles of different single stress responses show significant overlap

The expression profiles induced by the single stresses *B. cinerea* infection, *P. rapae* infestation, and drought are clearly different in timing and numbers of activated and repressed genes (Fig. 2). Clustering of the profiles of all DEGs (7355) from the three single stress responses shows that, in general, genes related to stress-related processes are overrepresented in the activated gene sets, while genes related to growth become repressed (Fig. 3). This confirms the notion that plants under stress prioritize appropriate adaptive responses over growth (Vos *et al.* 2015, Vos *et al.* 2013a). One third of all DEGs under single stress conditions was also differentially expressed under one or both of the other single stress conditions (Fig. 4a). A set of 394 DEGs was differentially expressed in response to all three single stresses and clustering of their

expression profiles clearly shows that the expression of the genes in the 12 distinguished clusters is often regulated in opposite directions (up or down), depending on the nature of the stress (Fig. 4b). Only one cluster, containing an overrepresentation of genes related to the GO term "response to oxygen-containing compound" shows a general up-regulation of genes under all three single stress conditions, highlighting that production and responsiveness to oxygen-containing compounds are 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 differential stress-type specific up- or down-regulation of genes suggests their positive versus negative function in different adaptive stress responses. When a plant experiences a combination of stresses, this may cause synergistic or antagonistic effects on the level of tolerance to the stresses at hand.

The transcriptome is rewired to the last stress response, but the prior stress leaves first-stress-signatures in the second stress profile

Analysis of the dynamics of both the single and sequential double stress transcriptome profiles showed that the transcriptome profiles of all possible double stress combinations were remarkably similar to those of the last encountered stress if applied individually (Figs. 4c, 5, 9, and 12). In analogy, 1 d after re-watering, the drought-induced transcriptome was largely reset to the non-stressed condition (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 stresses can have dramatic effects on the outcome of the response to a second stress (Rejeb *et al.* 2014).

To identify genes and biological processes that could contribute to the interaction between different stress responses, different types of analyses have been carried out. 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. Using Mclust clustering of the transcriptional profiles of the *B. cinerea*, *P. rapae* and drought sets of DEGs (Figs. 5, 9 and 12) different gene clusters were identified that showed distinct behavior over time during the single and sequential double stress responses. However, in this way relatively small differences between the single and sequential stresses were detected. In order to better pinpoint co-expressed gene clusters that represent first-stress-signatures in the second stress transcriptome profiles, we used the bioinformatics tool Wigwams (Polanski *et al.* 2014). We were able to dissect the transcriptional profiles into modules of co-regulated genes in time across different conditions. This approach highlighted modules whose gene expression patterns differed from the single stress profile in one or both of the sequential double stress profiles (Figs. 6, 10, and 13, and Supporting Figs. S1, S2 and S3). Future studies should reveal the role of candidate genes in these clusters in shaping the outcome of the adaptive stress responses in the sequential dual stress conditions. Moreover, the Wigwams modules of co-regulated genes could aid in dissecting the regulatory circuitry underlying plant responses to combinatorial stresses, e.g. by analyzing the representation of transcription factors binding motifs in the promoters of the gene modules.

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 single versus the sequential double stresses, 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 the different time points provide a landscape of the timing at which 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 (either in activated or repressed DEGs) 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 sequential double 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; El Oirdi *et al.* 2011, Thomma *et al.* 1998, Thomma *et al.* 1999, Vos *et al.* 2015), *P. rapae* (JA and ABA; Bodenhausen and Reymond 2007, De Vos *et al.* 2006, Vos *et al.* 2013b), and drought stress (ABA; Yamaguchi-Shinozaki and Shinozaki 2006). Hence, interactions between the different hormone-controlled signaling pathways may be decisive in the outcome of the adaptive response when two stresses are encountered sequentially.

Effect of prior stress on level of resistance to subsequent stress

Classic examples of interactions between defense pathways are the different forms of induced resistance that are triggered by pathogens, insect herbivores and beneficial microbes as they all 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.* 2006, Howe and Jander 2008, Pieterse *et al.* 2014, Poelman *et al.* 2008, Van Oosten *et al.* 2008, Vos *et al.* 2015). Also for abiotic stresses effects on the level of resistance against other abiotic and biotic stresses are documented (Fujita *et al.* 2006, Rejeb *et al.* 2014). For the combinations of sequential stresses that were investigated here, we found that prior infestation by *P. rapae* caterpillars changed the level of resistance against *B. cinerea* (Fig. 8), even though the global transcriptional profiles induced by *B. cinerea* as single or second stress did not differ dramatically (Fig. 5).

It can thus be concluded that subtle first-stress-signatures in the double stress transcriptional profile may have significant effects on the outcome of the adaptive response to the second stress, although it cannot be excluded that non-transcriptional changes may also contribute to changes in the level of resistance against 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 anti-herbivory defenses (Vos *et al.* 2013b). Since JA-regulated defenses play a major role in resistance against the necrotrophic pathogen *B. cinerea* as well*,* herbivory-induced priming of JA responsiveness may contribute to the enhanced resistance level against *B. cinerea* (Fig. 7; Fig. 8).

This study was aimed at analyzing the dynamics of gene expression patterns in response to a set of single and sequential double stresses. 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.

Acknowledgements

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Supporting Information

Additional supporting information may be download via link

http://onlinelibrary.wiley.com/doi/10.1111/tpj.13167/full

Supplementary Tables

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

Supporting Table S2: Enriched GO terms in gene clusters shown in Figure 3 (Clustering of all DEGs of the single stresses).

Supporting Table S3: Enriched GO terms in gene clusters shown in Figure 4b (Shared core DEGs between the three single stress responses).

Supporting Table S4: Enriched GO terms in gene clusters shown in Figure 5 (Dynamics of the expression of the *B. cinerea* set of DEGs during single and sequential double stresses).

Supporting Table S5: Enriched GO terms in Wigwams modules of *B. cinerea, rapae,* and Drought set of DEGs.

Supporting Table S6: Enriched GO terms in gene clusters shown in Figure 9 (Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses).

Supporting Table S7: Enriched GO terms in gene clusters shown in Figure 12 (Dynamics of the expression of the drought set of DEGs during single and sequential double stresses).

Supporting Figure S1. Expression patterns of all 35 Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions.

Supporting Figure S2. Expression patterns of all 93 Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions.

Supporting Figure S3. Expression patterns of all 72 Wigwams modules from the drought set of DEGs during single and sequential stress conditions.

CHAPTER 6

Opposite interaction effects on the ethylene signalling pathway of two biotic stresses (Botrytis cinerea and Pieris rapae) on subsequent drought stress responses in Arabidopsis thaliana

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Abstract

It is important to understand how plant responses to the combination of biotic and abiotic stresses because plants are exposed to multiple biotic and abiotic stresses that occur either simultaneously or sequentially in the natural environment. The molecular mechanisms underlying plant responses to individual stresses have been studied extensively but less is known about the responses to a combination of stresses. In this study, we performed RNA-seq to discover the transcriptome changes of *Arabidopsis* thaliana to drought and rehydration, in combination with two biotic pre-treatments (necrotrophic fungal infection by *Botrytis cinerea* and herbivory by *Pieris rapae* larvae), in order to gain a deep understanding of the interaction between these combinations of stresses. We found drought stress has the strong influence of secondary messengers and photosynthesis and the one-day sequential rehydration period is insufficient to fully overcome the effects of drought stress on Arabidopsis. Herbivory by *Pieris* has a much higher impact on the transcriptome during sequential drought and rehydration, than the infection with *Botrytis*. The biotic pre-treatment can have strong antagonistic effects on sequential drought responses. Opposite effects were observed on 42 differential expressed genes (DEGs), including ethylene-related genes *ERF5*, *ERF6*, *ERF104* and drought responsive gene *P5CS1*, during the subsequent drought treatment, revealing specific temporal regulation during the sequential drought that is influenced by the biotic pre-treatment factor. Genes with alternative splicing (AS) variants were identified in different stress conditions, but none of them were found among the DEGs, showing a separate regulation of gene expression and AS in response to stresses. Phenotypic differences found for the *mdar3* and *pin7* mutants suggest the *MDAR3* and *PIN7* genes to play an important role in the response to the combinatorial stress.

Keywords

Introduction

Plants growing under natural conditions are often simultaneously or sequentially exposed to various biotic and abiotic factors which may interfere with plant growth and development causing a substantial annual reduction in agricultural production yield (Hirt and Shinozaki, 2004). Strong effects of (a)biotic factors are considered to be imposing plant stress, hence these factors are often referred to as conferring (a)biotic stress, and the tolerance to resist their effect as stress tolerance.

Plants respond to adverse biotic and abiotic conditions primarily through plant hormone-mediated pathways. This includes signalling pathways involving abscisic acid (ABA), which plays an important role in abiotic stress responses to e.g. drought and salt (Shinozaki and Yamaguchi-Shinozaki, 2007); jasmonic acid (JA) and ethylene (ET), which are essential in reacting to necrotrophic fungi and chewing insects; and salicylic acid (SA), which activates plant defence responses upon infection with biotrophic fungi (Pieterse et al., 2009; Dodds and Rathjen, 2010). Other plant hormones such as brassinosteroids (BR)s, auxins (AUX), and cytokinins (CK) are also involved in regulating the responses to biotic and abiotic stresses, but play a minor role (Hirt, 2009; Pareek et al., 2010; Pieterse et al., 2012).

The interaction between biotic and abiotic stress responses, mediated by different plant hormones, can be both synergistic or antagonistic. Negative interactions have been observed between JA and SA, ABA and SA, ABA and JA, and ABA and ET; while positive interactions have been observed between ABA and AUX (Yoshioka and Shinozaki, 2009). Studies in tomato indicate that drought stress leads to increased resistance to *Botrytis cinerea* infection (Audenaert et al., 2002; Achuo et al., 2006). Vice versa, interactions with pathogens and insects can also have a positive impact on plant responses to subsequent drought stress. For instance, pathogen-induced priming has a positive effect on plants in response to drought (Ramegowda and Senthil-Kumar, 2015), and prior infections of the Brome Mosaic Virus (BMV), Cucumber Mosaic Virus (CMV), or Tobacco Mosaic Virus can enhance *Nicotiana benthamiana* tolerance to drought (Xu et al., 2008). On the other hand, prior Turnip Mosaic

Virus (TuMV) infection reduces plant biomass and leaf number under drought stress when compared to control conditions (Prasch and Sonnewald, 2015). *Arabidopsis thaliana* plants exposed to *Pieris rapae* larvae or to the necrotrophic fungus *Botrytis cinerea*, are expected to trigger JA-ET-mediated signalling through different branches within the JA-ET signalling pathway. The *Botrytis* response is regulated through the ETHYLENE RESPONSE FACTOR ('ERF') branch, which is co-regulated by ET, and the *Pieris* response is regulated through the 'MYC' branch, which is co-regulated by ABA (Pré et al., 2008; Vos et al., 2013). These two branches interact with each other antagonistically (Dombrecht et al., 2007). Genes from the two branches can also interact positively or negatively with drought-induced ABA signalling, eg. MYC2 (MYC branch) binds to the promoter of *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) and *ERF1* (ERF branch) and negatively regulates their expression. Both targets are positive regulators of the *Botrytis* defence gene *PDF1.2* (Anderson et al., 2004; Yoshioka and Shinozaki, 2009).

The ways by which plants respond to individual biotic and abiotic stress treatments have been studied extensively. Far less is known regarding plant responses to multiple stresses, either simultaneously or consecutively encountered. Recent studies have identified genes that play a role in response to multiple stresses by comparing transcriptome data from varying biotic and abiotic stresses (Narsai et al., 2013; Shaik and Ramakrishna, 2013; Sewelam et al., 2014; Ramegowda and Senthil-Kumar, 2015). The consecutive combination of exposure to biotic and abiotic stress evidently triggers specific multiple stress-responsive genes, indicating a tailored combinatorial stress signalling network (Atkinson and Urwin, 2012; Sewelam et al., 2014). However, the molecular responses in natural situations, where plants encounter simultaneous or consecutive combinations of biotic and abiotic stress, remain to be elucidated. To gain novel insights into the interaction between biotic and abiotic stress responses we studied the Arabidopsis transcriptome, including alternative RNA splicing, in response to drought as either a single drought-stress-inducing condition or following exposure to two different biotic stresses applied prior to the drought treatment.

The purpose of this study was to: 1) observe the effects of the two prior biotic stresses on the subsequent drought treatment response, and 2) discover the differences of the effects of the two prior biotic stresses on subsequent drought. We identified a large number of differentially expressed genes (DEGs) as well as alternative splicing (AS) events under the two combinatorial treatments with both antagonistic or synergistic effects on plant performance.

Material and Methods

Plant material and growth

Arabidopsis thaliana accession Col-0 seeds were vernalized at 4°C in the dark for 48 h to break seed dormancy. Thereafter, seeds were sown on river sand with half-strength Hoagland nutrient solution containing sequestreen, as described Van Wees et al (2013). Plants were grown in a climate-controlled growth chamber (8 h day/16 h night) set at 21°C and 70% relative humidity. Irradiation was set at 100 µmol m^{-2} s^{-1}. Fourteen-day-old seedlings were transferred to individual pots containing a mixture of river sand and peat (1:1, v:v). Water was supplied to the bottom of each pot three times per week.
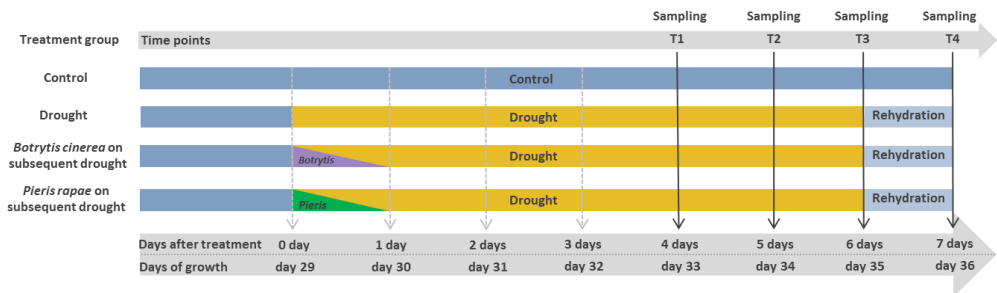
Experimental design

The experiment was carried out in a fully randomized design with two factors: time and treatment. Four time points were selected for measurements, with T1, T2, and T3 after 4, 5, and 6 days of drought treatment, respectively, which started after growing plants for 28 days (Figure 1). Directly after the last time point, plants were re-watered to rehydrate. T4 was set at 24 hrs (1 day) of rehydration. The experiment comprised four experimental groups: control, single drought, *Botrytis* infection followed by drought and *Pieris* exposure followed by drought. Randomized Block Design was used for this experiment. The climate chamber space was divided into three subgroups called blocks. Plants within each block are randomly subjected to different treatments.

Preparation of Pieris rapae and Botrytis cinerea for treatments

Pieris rapae 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 (nectar). When flowers were scarce, additional food (solution of 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. After starving for 1 h, first-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described (Van Wees et al., 2013).

Botrytis cinerea strain B05.10 (Staats and 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, filtered through glass wool, and re-suspended in half-strength Potato Dextrose Broth (PDB; Difco Laboratories) to a final density of 1 x 10^5 spores/ml. After a 3 h incubation period, the spores were used for inoculation by applying two 5-µl droplets on one Arabidopsis leaf as described (Van Wees et al., 2013).

Figure 1. Experimental design for treatment of Arabidopsis plants subjected to drought, *Botrytis cinerea* infection and drought, or *Pieris rapae* herbivory and drought, and control conditions. Conditions are indicated in different colours: control (dark blue); drought (yellow); *Botrytis* pre-treatment (purple); *Pieris* pre-treatment (green) and rehydration after drought (light blue). Vertical arrows indicate sampling time points (T1-T4).

Drought and combinatorial stress treatments

For the single drought treatment, 28-day-old plants of Arabidopsis accessions were withheld from water for 6 days. Afterwards, plants were re-watered and allowed to recover for 1 day. For the combination of *Botrytis* exposure and subsequent drought, a *Botrytis* pre-treatment was performed right after the last watering before the drought treatment, by inoculating leaves 6 and 7 of each plant with two 5-µl droplets of a *Botrytis* spore suspension (1×10^5 spores/ml) per leaf and placing the plants at 100% relative humidity (RH) for 1 day (Figure 1). The next day, the RH was reduced to that of drought-exposed plants. For the combination treatment of *Pieris* and subsequent drought, the *Pieris* pre-treatment was performed at the same time as the *Botrytis* pre-treatment by placing one *Pieris* caterpillar on leaf 7 and allowing it to feed on the plant for 1 day. Plants without receiving any treatment, neither biotic nor abiotic, were used as control. Leaf number 8 was harvested, if undamaged, after

4, 5 and 6 days of withholding water (T1, T2, T3, respectively) and at 1 day after the rehydration treatment (T4)(Figure 1).

The treatments for the T-DNA insertion mutants were the same as described for the accessions. For information on the identity of T-DNA insertion mutants, see supplementary table 1.

RNA extraction and library preparation

RNA was extracted using the Plant RNeasy Plant Mini Kit (Qiagen, cat. no. 74903), according to the manufacturer's instructions. All samples were treated with DNase I on a column using the Qiagen RNase-Free DNase Set (cat. no. 79254). Quality of RNA was evaluated by determining the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabChip. For the library preparation, samples with a RIN value ≥6 were used. The samples were processed 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 total RNA using 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.

RNAseq analysis, Differential expressed genes, Alternative RNA splicing

Samples were sequenced on an Illumina Hi-Seq 2000 sequencing machine randomly assigned to seven lanes of three Illumina flow cells. Sequence reads were quality trimmed and mapped to the *Arabidopsis thaliana* genome version 'TAIR 10' (www.arabidopsis.org) using TopHat with parameters: -N 3 --min-intron-length 50 --max-intron-length 5000 -g 1 -M -p 4 -G --read-edit-dist 3 --library-type (Trapnell et al., 2009). The number of reads mapped to each annotated Arabidopsis gene (TAIR10) was determined using HTSeq count

(Anders et al., 2015). Read counts were subjected to a regularized log transformation, implemented in DESeq2 (Love et al., 2014).

Differentially expressed genes (DEGs) were computed per time point using the DESeq2 Bioconductor package in R (Anders and Huber, 2010; Love et al., 2014). To determine DEGs under drought treatment, we compared drought with control. To determine DEGs under the combinational stresses, we compared each combinatorial treatment to the single drought treatment. To determine DEGs under rehydration treatment, we compared the rehydration to the control.

To determine alternative RNA splicing, putative full-length transcripts were assembled for each RNAseq sample using cufflinks package (Trapnell et al., 2012). The full transcriptome was obtained by merging the individual assemblies using cuffmerge from the cufflinks package. Custom python scripts were used for detecting the following splicing events: intron retention, alternative donor site, alternative acceptor sites, exon skipping and mutually exclusive exons (Davila Olivas et al., 2016).

Mixture of Isoforms Probalistic model (MISO) was used for quantifying alternative splicing events in each individual sample and the pooled replicates for each condition (Katz et al., 2010). For each sample (excluding pooled datasets), those alternative splicing events that were not supported by at least 20 isoform-specific reads were discarded. Differential splicing events were detected in 2 steps:

1. The alternative splicing (AS) events for each pairwise condition were analysed as follows: All events for which either of the two conditions lacked more than one replicate value were discarded. It was required that the values of the replicates in each condition were closer to each other than to any other replicate value in the other condition.
2. For each splicing event that passed the filtering, we tested whether the corresponding values in the pooled set were significantly different from each other. The significance in MISO is provided as Bayes factors, which describe how much more likely the event is differentially regulated than

not. Splicing events with a difference of at least 0.05 and a Bayes factor of ≥4 were considered as significant events.

Enriched gene ontology, metabolic pathway, transcription factors analysis

Enriched gene ontology (GO), metabolic pathways, and transcription factors (TFs) were identified using the online tool PlantGSEA (http://structuralbiology.cau.edu.cn/PlantGSEA/) (Yi et al., 2013). In all cases, we used the following parameters: hypergeometric test with Benjamini-Hochberg FDR adjustment, α = 0.05. As background, we used only genes that were expressed in at least one sample out of all samples that were analysed.

Statistical analysis

One-way ANOVA was used to compare the areas of each leaf consumed by *Pieris* in the *Pieris* pre-treatment. Two-way ANOVA was used to compare plants in response to drought (compared to control) and to compare plants in response to the combination of *Pieris* on subsequent drought (compared to drought). In all cases *p*-values < 0.05 were considered as significant.

Real-time quantitative reverse transcriptase (qRT)-PCR

1 µg total RNA was used for reverse transcription by iScript cDNA synthesis kit (Bio-Rad); 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. cDNA was diluted ten times for Quantitative Reverse Transcriptase-PCR (qRT-PCR) use. The At3g15930 (*PCMP-E51*) gene was used as the reference gene upon selection through the "References" web tool (http://refgenes.org/rg) (forward primer sequence 5'-ACCAAACCCAACATTCCAAA-3' and the reverse primer sequence 3'-ACACAACCACGTCTGGTTCA-5'). The qRT-PCRs were performed using iQ SYBR® Green Supermix (Bio-Rad, cat. no. 170-8885) on a Bio-Rad CFX96 real-time PCR system, set at 95 °C for 4 minutes, followed by 40 cycles of 95 °C for 10 seconds and 55 °C for 30 seconds. The primers of the *PIN-FORMED 7* (*PIN7*) gene and the two AS variants are shown in Supplementary Table 12.

Results

Differentially expressed genes by drought and subsequent rehydration

A time-course experiment was performed using Arabidopsis*A. thaliana* plants which were separately exposed to two biotic stresses: fungal infection by *Botrytis cinerea* and herbivory by *Pieris rapae* caterpillars, followed by drought ("*Botrytis* and subsequent drought" and "*Pieris* on subsequent drought") (Figure 1). In addition to the combinatorial treatment, plants were subjected to a single drought treatment without a pre-treatment ("drought"). Samples were harvested after 4 (T1), 5 (T2) and 6 days of drought (T3) and one day later, meaning after 24 hrs of rehydration(T4) (Figure 1). Gene expression was determined using RNA-seq.
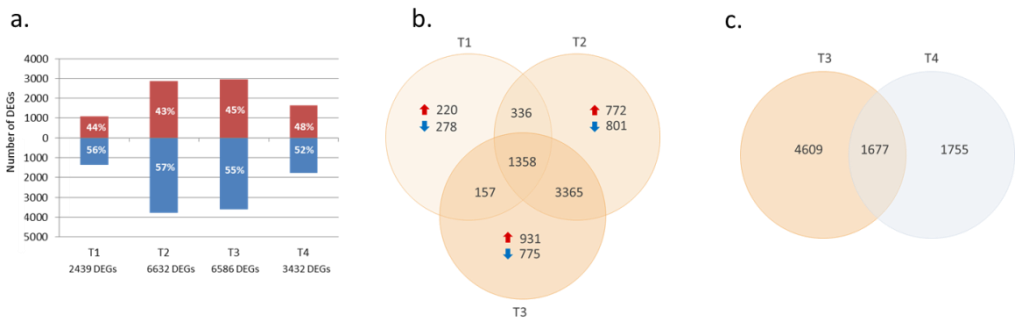
Figure 2. Bar plot (a.) showing the number of up- and down-regulated differentially expressed genes (DEG) at four time points, after (T1), 5 (T2) and 6 days of drought (T3), and after one-day sequential rehydration (T4). The Y axis represents the number of DEGs and the X axis represents time points and the total number of DEGs. The ratio of up- (red) or down-regulated (blue) genes are indicated. Venn diagram showing the numbers DEGs of Arabidopsis Col-0 (b.) at three time points , after 4 (T1), 5 (T2) and 6 days of drought (T3), when compared to well-watered control plants at each time point, the numbers of up- or down-regulated genes are indicated with blue and red arrows. Or (c.) at two time points, after 6 days of drought (T3) and after one day rehydration (T4), when compared to well-watered control plants at each time point.

A total of 8993 differentially expressed genes (DEGs) were identified when plants exposed to drought at time points T1, T2 and T3 were compared with plants grown on control conditions (Figure 2.a; Supplementary Table 2). These expression differences illustrate the effects of drought on gene expression. A total of 2349 DEGs were identified at T1, 6632 DEGs at T2 and 6586 DEGs at T3, which are all one day apart in the drought response, suggesting that the effect of the treatment became more severe the longer it lasted. In total, 1358 DEGs

were shared by the three time points, which can be considered to be common drought-responsive genes (Figure 2.b; Supplementary Table 2). With so many DEGs, drought causes major changes to the transcriptome and is therefore be considered to be a severe stress.

To observe if rehydration upon drought reverts plants to the same state as seen under control conditions, plants exposed to rehydration after drought treatment were compared to the control (not drought-stressed), which still revealed 3432 DEGs (Supplementary Table 3). Comparing DEGs under rehydration (T4) to DEGs under drought at T3, we found that 1677 drought responsive genes were affected by the sequential rehydration treatment (Figure 2.c; Supplementary Table 3.e). This means that a one-day rehydration period is insufficient to fully overcome the effects of drought stress on Arabidopsis.

Enriched GO terms of genes involved in the drought and subsequent rehydration response

The 1358 common drought-responsive DEGs (Figure 2) were examined for overrepresented gene ontology (GO) terms. The top 20 enriched GO terms indicates three main gene expression responses to drought: the defence response, which includes GO terms such as response to stimulus, response to abiotic stimulus, response to chemical stimulus, response to stress, response to temperature stimulus; the photosynthesis response, including GO terms photosynthesis and light reaction; and the metabolic processes response, including GO terms such as secondary metabolic process, oxoacid metabolic process, carboxylic acid metabolic process, etc. (Supplementary Table 3.a). To determine the role of DEGs involved in a biochemical pathway, an 'enriched pathway analysis' was performed, which identified 32 pathways that were associated with the DEGs, including the biosynthesis of phenylpropanoids, biosynthesis of plant hormones, and photosynthesis (Supplementary Table 3.b). In addition, the targets of several transcription factors that are related to stress responses are enriched, such as transcription factors HY5 (light response), AtbHLH15 (light and hormone response), AP2, MYB88, and MYB124 (abiotic stress response), WRKY53 (drought response), and MYC2 (drought and biotic

stress response) (Osterlund et al., 2000);(Xie et al., 2010; Mizoi et al., 2012) (Supplementary table 3.c).

When analysing the large set of DEGs identified when comparing the gene expression after rehydration with gene expression in unstressed, control, plants (Supplementary Table 4.a) the GO terms that are associated with defence response are still enriched, as they were upon drought treatment (Supplementary Table 4.b). DEGs involved in the biosynthesis of alkaloid-derived pathways and biosynthesis of phenylpropanoids were also overrepresented (Supplementary Table 4.c), as were targets of transcription factors AtbHLH15 (involved in light and hormone response), AP2, MYB88, and MYB124 (involved in abiotic stress response), WRKY53 (involved in drought response) AGL15 (involved in flowering), and PYE (involved in the response to iron deficiency) *(Adamczyk et al., 2007; Long et al., 2010)* (Supplementary Table 4.d) and several DEGs known to be drought-responsive, such as *RESPONSIVE TO DESICCATION 29a (RD29a)*, *RESPONSIVE TO DESICCATION 29a (RD29b)*, and *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2a (DREB2a)* (Shinozaki and Yamaguchi-Shinozaki, 2007). In conclusion, one day of rehydration did not dramatically revert the drought-induced gene expression profile back to normal.

Comparing DEGs under rehydration to DEGs under drought at T3, a set of 1677 DEGs were shared by the two time points. Enriched GO terms that are associated with stress responses such as response to stimulus, response to chemical stimulus, response to stress, response to endogenous stimulus, and response to abiotic stimulus are highly enriched (Supplementary Table 4.e). DEGs involved in the metabolic pathways, biosynthesis of plant hormones, and biosynthesis of alkaloids are overrepresented (Supplementary Table 4.f). We did not find overrepresented transcription factor targets in this gene set (Supplementary Table 4.g).

Differential expressed genes by combinatorial and sequential rehydration

Plants exposed to the two combinatorial stresses were compared with plants subjected to the single drought treatment, at comparable time points, to determine if there is an effect of a biotic pre-treatment on the response to drought (Figure 1). For the combination of *Botrytis* followed by drought, 101 DEGs were identified (Figure 3.a), most of them representing down-regulated genes, and the large majority of them (80) were differentially expressed at T2 (Supplementary Table 5.a). Only one DEG appeared in two time points (T2 and T3), the *AMINO ACID PERMERASE* 6 (*AAP6*) gene (At5g49630), encoding a high-affinity Asp/Trp uptake transporter to transport amino acids in phloem sap (Hunt et al., 2010).

A total of 734 DEGs were identified when comparing the combined *Pieris* and subsequent drought treatment to the drought treatment (Figure 3.b, Supplementary Table 6). The number of DEGs decreased over time from 503 (T1) to 20 (T3). Most DEGs were down-regulated at T1, while most DEGs were up-regulated at T2 and T3. In total 165 DEGs were common between T1 and T2, but only 10 DEGs between T2 and T3, and 7 between T3 and T1. Of the 20 DEGs found at T3, no less than six were common to all three time-points. Genes including two known drought-responsive genes CALCIUM-*DEPENDENT PROTEIN KINASE 28* (*CPK28*) (At5g66210) and *EARLY RESPONSE TO DEHYDRATION 6* (*ERD6*) (At1g08930) were expressed at all three time points (Kiyosue et al., 1998; Zou et al., 2015). When comparing the combinatorial treatments, 49 of all 786 DEGs were found in both treatments (Figure 4.a).

To investigate if the biotic pre-treatments still had any effect on gene expression after rehydration to recover from the drought treatment, DEGs were identified when comparing the gene expression upon the three rehydration treatments (after drought alone and after *Botrytis/Pieris* pre-treatment and drought). The *Botrytis* pre-treatment affected the expression of only 24 genes (Supplementary Table 7), while the *Pieris* pre-treatment affected the expression of 1779 genes (Supplementary Table 8). Only 7 DEGs, including gene *CHALCONE SYNTHASE (CHS)* (At5g13930) which is required for purple

anthocyanins accumulation in leaves and stems (Li and Strid, 2005), were found to be common for both combinatorial treatments upon rehydration (Figure 4.b, Supplementary Table 9.b). This illustrates that herbivory by *Pieris rapae* has a much higher impact on the transcriptome during sequential drought and rehydration, than the infection with *Botrytis cinerea*.
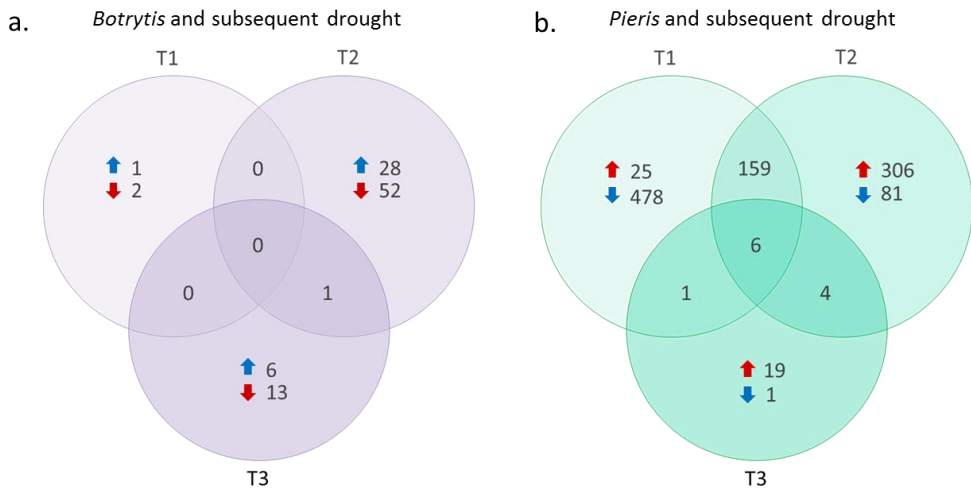
Figure 3. Venn diagram showing the numbers of DEGs of Col-0 at points T1-T3 (see figure 1) when comparing plants treated with (a) *Botrytis cinerea* and drought (purple;) or (b) *Pieris rapae* and drought (green) with the drought alone treatment. The numbers of up- or down-regulated genes are indicated with blue and red arrows.
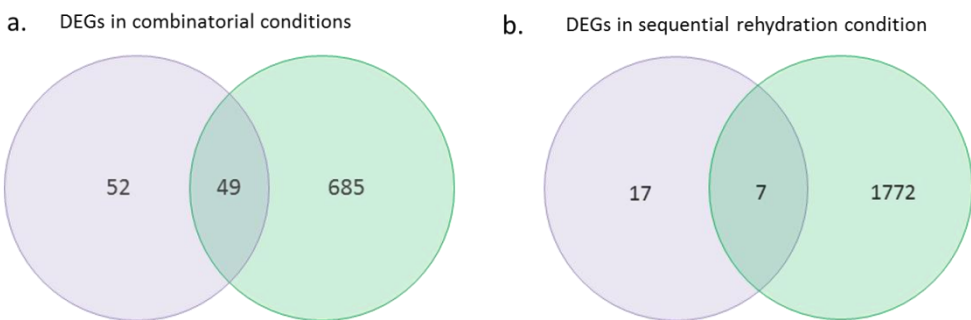
Figure 4. Venn diagram showing the numbers of DEGs of Col-0 when (a) comparing plants pre-treated with (*Botrytis cinerea* (purple) or *Pieris rapae* (green) followed by drought to control plants only treated with drought; or (b) when comparing plants pre-treated with *Botrytis cinerea* (purple) or *Pieris rapae* (green) followed by drought and subsequent rehydration, to control plants treated only with drought and rehydration. The numbers of up- or down-regulated genes are indicated with blue and red arrows .

Enriched GO terms in combinatorial stress response

Among the 101 DEGs identified under the stress combination of *Botrytis* on subsequent drought treatment. Genes including drought responsive gene *RD28* (At2g37180) (Taji et al., 1999), cytokinin-related gene *CYTOKININ OXIDASE 5* (*CKX5*) (*At1g75450*) (Werner et al., 2003), auxin-related gene *ARABIDOPSIS THALIANA METHYL ESTERASE 18* (*ATMES18*) (At5g58310) (Yang et al., 2008) (Supplementary Table 5.b). Among the 734 DEGs responsive to the *Pieris* pre-treatment before subsequent drought, defence response associated GO terms such as response to chitin, defence response, innate immune response, response to stress, response to biotic stimulus, and regulation of defence responses were highly enriched (Supplementary Table 6.f). Known stress-response genes include mitogen-active protein kinase (MAPK)-related genes such as *MPK3* (*At3g45640*)*, MKK4* (*At1g51660*)*, MKK5* (*At3g21220*), and MAP kinase substrate 1 (*MKS1*, *At3g18690*). The DEGs involved in metabolic pathways such as plant-pathogen interaction pathway was especially enriched in this gene set (Supplementary Table 6.g), as well as targets of transcription factors such as ATBZIP60 (unfolded protein response) and GL1 (development, anthocyanin biosynthesis) were only enriched in this gene set (Humbert et al., 2012)(Supplementary Table 6.h).

Common genes affected by both biotic pre-treatments in combination with drought

A total of 49 DEGs were shared by both combinatorial treatments (Supplementary Table 9). Among the 49 DEGs, 17 DEGs were down-regulated and 14 DEGs were up-regulated under these treatments. Remarkably, for 42 genes the biotic pre-treatments had an opposite effect on the up- or downregulation of their expression, either at one or more time points. Among these are known stress response genes, such as *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 5* (*ERF5*) (At5g47230), *ERF6* (At4g17490), *ERF104* (At5g61600) and Delta1-Pyrroline-5-Carboxylate Synthase 1 (P5CS1) (AT2G39800). In general, these genes showed an initial down-regulation and subsequent up-regulation upon pre-treatment with *Botrytis*, and an initial up-regulation and subsequent down-regulation upon the *Pieris* pre-treatment. This

reveals specific temporal regulation during drought that is influenced by the biotic pre-treatment factor.

CCR2 and MDAR3 play a role in response to the consecutive combination of Pieris and subsequent drought

Genes that showed a high fold-change in expression when comparing the *Pieris* plus drought treatment with the drought treatment alone, were selected for further analysis of their relevance for the response. Phenotypic analysis was performed on T-DNA insertion mutants of three up-regulated candidate genes (*WALL ASSOCIATED KINASE (WAK)-LIKE 10*; *WAKL10*; At1g79680) (expression fold change (FC)>7), *CINNAMOYL COA REDUCTASE; CCR2*; At1g80820) (FC>12), *MONODEHYDROASCORBATE REDUCTASE 3*; *MDAR3;* At3g09940) (FC>30), and one down-regulated gene, At3g10720, encoding an invertase/pectin methylesterase inhibitor (FC<0.2) (Supplementary Table 6). The *wakl10* mutant plants exhibited reduced rosette dry weight when compared with their Col-0 wild-type under control conditions (Figure 5.a). No mutants exhibited significant differences in response to *Pieris* treatment when compared to the wild type for leaf eaten area (Figure 5.b). Under drought stress, *mdar3* mutants showed increased rosette dry weight when compared with the wild type (Figure 5.c). A decreased rosette dry weight was observed for the *ccr2* and *mdar3* mutants when pre-treated with *Pieris* followed by drought, when compared to the wild type (Figure 5.d; Supplementary Figure 2). This means that only for the mutant of At3g10720 we did not observe any effect on the phenotype under any of the tested conditions. All other genes are somehow involved in the growth of plants, with particularly *CCR2*, encoding a cinnamoyl CoA reductase involved in lignin biosynthesis, and *MDAR3*, encoding a monodehydroascorbate reductase, to be important to maintain wild-type growth under the combined *Pieris*-drought combination.
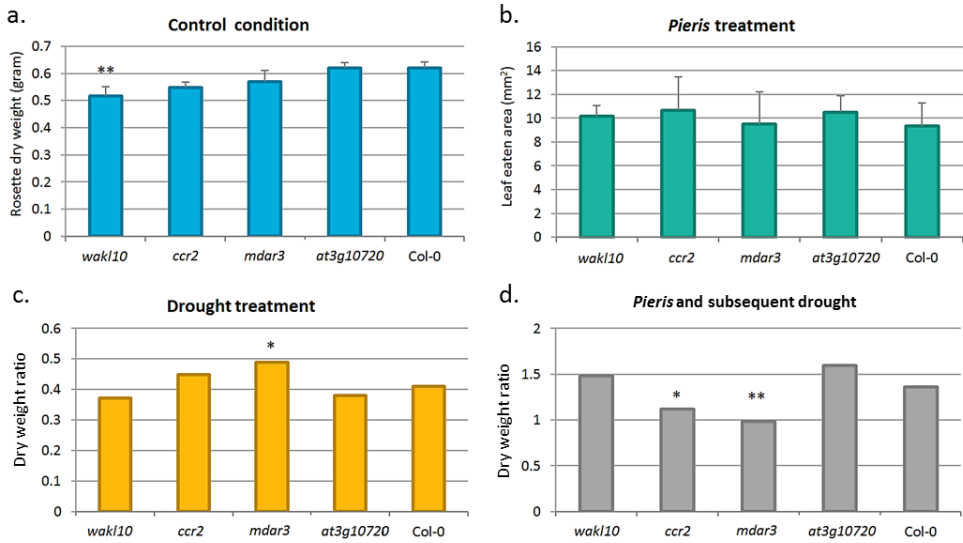
Figure 5. Phenotypes of T-DNA insertion mutants of DEGs *WAKL10* (*At1g79680*), *CCR2* (*At1g80820*), *MDAR3* (*At3g09940*) and unnamed gene *At3g10720*, identified when comparing *Pieris rapae* pre-treated with non-pre-treated plants subjected to drought (Sup. Table .....).
a. Rosette dry weight of Col-0 wild type and mutant plants grown in control conditions.
b. Leaf areas eaten by *Pieris* of these genotypes.
c. Rosette dry weight ratios of these genotypes after exposure to drought, compared to control plants.
d. Rosette dry weight ratios of these genotypes after pre-treatment with *Pieris* and subsequent exposure to drought, compared to plants exposed to drought only.
One-way ANOVA was used for Figures 5.a and b. Two-way ANOVA was used for Figures 5.c and d. Error bars were calculated from at least six plants. * indicates mutants with a significant difference when compared to Col-0 wild type (* $p<0.05$, **$p<0.01$).

Alternative RNA splicing affecting gene expression upon exposure to stress-inducing conditions

Alternative splicing (AS) is known to be sometimes an important factor in controlling the gene expression response to environmental perturbations (Staiger and Brown, 2013). To determine if there is any differential alternative RNA splicing, plants exposed to drought were compared to control plants at each of the three time points. For 19 genes a total of 23 AS variants were found (Table 1; Supplementary Table 10.a). When including the rehydration treatment in the analysis, and comparing rehydrated plants after drought exposure to plants continuously grown under control conditions, 17 genes with a total of 20 AS variants were found (Table 1; Supplementary Table 10.b).

When examining both plants exposed to biotic pre-treatments plus drought to plants only exposed to drought, five genes with as many AS variants were found for the *Botrytis* pre-treatment and 16 genes corresponding to 16 AS variants for the *Pieris* pre-treatment. There was no overlap between these two sets. When including the rehydration treatment, only the *Botrytis* pre-treatment was found to yield AS variants, for 5 genes (Table 1; Supplementary Table 10. c and d).

Table 1. Number of alternative splicing variants induced by different stresses

| Treatment Type	Drought	BD*	PD*	Rehydration after drought	Rehydration after BD	Rehydratoin after PD
A3	10	1	5	4	4	0
A5	3	3	5	2	0	0
RI	6	1	3	7	1	1
MXE	1	0	2	1	0	0
SE	3	0	1	0	0	0
Total	23	5	16	14	5	1

A3 = Alternative splicing at 3' of intron; A5 = Alternative splicing at 5' of intron, RI = Intron retention, MXE = mutually exclusive exon, SE = exon skipping. BD = combination of *Botrytis* and subsequent drought; PD = combination *Pieris* and subsequent drought.

### *PIN7 plays a role in response to Pieris, drought, and the consecutive stress combination treatments*

One gene, for which AS variants were found, that caught our attention is *PIN-FORMED 7* (*PIN7*) (At1g23080), as it is known to be involved in auxin transport contributing to root architecture (Lewis et al., 2011). We found two splicing variants for this gene, *PIN7-1* and *PIN7-2*, differing at the 5' splice donor site of intron 1 leading to addition/removal of 4 amino acids (Figure 6.a). The splicing affects a region of the protein that is located in the cytoplasm. The expression of these two AS variants of *PIN7* was determined using specific splice-site-flanking primers, which showed that the expression level of the *PIN7-1* variant is significantly higher than of *PIN7-2*, under the single drought and the *Pieris* plus subsequent drought treatments (Figure 6.b). *PIN7-1* was marginally, though significantly, higher expressed when plants were pre-treated with *Pieris* than when plants were only drought-treated. In order to verify if this gene has any phenotypic importance for the response to *Pieris* and/or drought, a T-DNA insertion *pin7* mutant was obtained and grown under the different conditions. This mutant showed some remarkable phenotypic differences when compared to the wild type. When grown under control, non-stressing, conditions the *pin7* mutant rosettes comprised significantly more dry biomass than Col-0 (Figure 7.a), of which much less was consumed by *Pieris* (Figure 7.b). However, when plants were exposed to drought, the *pin7* mutants performed much worse than Col-0 in terms of rosette dry biomass ratios when comparing drought-treated to well-watered plants (Figure 7.c). Pre-treatment with *Pieris* alleviated this strong inhibition by drought somewhat, though the *pin7* mutants stayed notably smaller than Col-0 plants (Figure 7.d). This suggests that *PIN7* is important for Col-0 tolerance to drought although it enhances herbivory by *Pieris*.
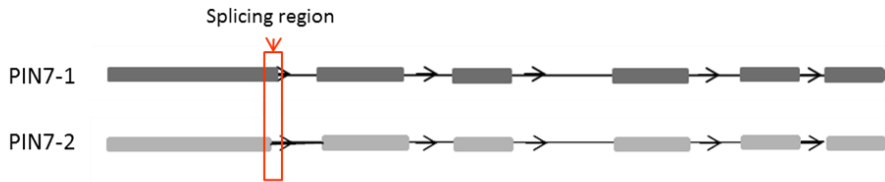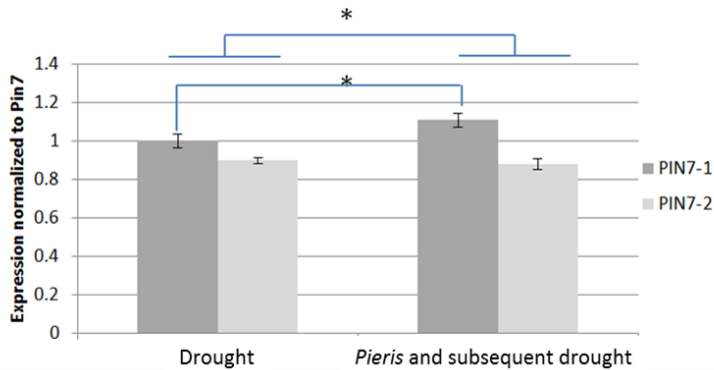
Figure 6 . Alternative splicing (AS) variants of *PIN7* are differentially expressed upon *Pieris* and drought exposure.

a. Two splicing variants of *PIN7*, indicating the alternatively spliced region with a red box. Exons are indicated as grey boxes and introns with lines, arrows indicate direction of transcription. AS variant *PIN7-1* is indicated in dark grey and *PIN7-2* in light grey.

b. Relative expression of the two *PIN7* splicing variants when comparing drought treated with well-watered control plants, and comparing the *Pieris* and subsequent drought with the well-watered control plants.

A Student's t-test was used to compare expression of each splicing variant in the different conditions. Two-way ANOVA was used to test the differences of the two AS variants between different conditions. * indicate significant differences ($p<0.05$). Standard error bars were calculated from at least six plants.
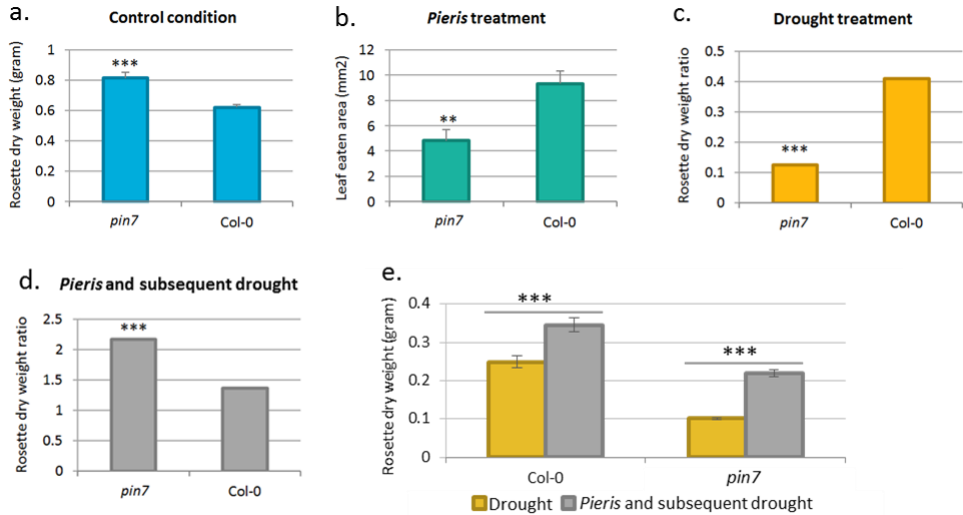
Figure 7. The *PIN7* gene is important for Arabidopsis tolerance to drought and herbivory.
a. Rosette dry weight of *pin7* mutant and Col-0 wild-type plants when grown in control conditions.
b. The area of rosette leaf consumed by *Pieris* of *pin7* mutant and Col-0 wild-type plants.
c. Rosette dry weight ratios of *pin7* mutant and Col-0 wild-type plants when comparing drought-treated plants to well-watered plants.
d. Rosette dry weight ratios of *pin7* mutant and Col-0 wild-type plants when comparing *Pieris* pre-treated and drought exposed plants to plants only exposed to drought.
e. Rosette dry weight of plants in drought and the combinatorial conditions.
A student's t-test was used for a and b. Two-way ANOVA was used for c and d. Error bars were calculated from at least six plants. *indicate significant differences, **$p<0.01$, ***$p<0.0001$.

Discussion

Transcriptomic responses to drought and subsequent rehydration

In response to myriad biotic and abiotic stress triggers, plants mobilize signaling molecules thereby boosting the accumulation of so-called second messengers, such as reactive oxygen species (ROS), calcium ions (Ca^{2+}), phospholipids, mitogen-activated protein kinases (MAPKs), Sucrose NonFermenting-1 (SNF1)-related protein kinases (SnRKs), and phytohormones (Takahashi et al., 2011; Tuteja and Sarvajeet, 2012; Smékalová et al., 2014). Some genes related to secondary messengers and plant hormones were found among the 1358 common drought-responsive DEGs, such as the up-regulation of *SnRKs family* genes such as *SnRK2.6 and SnRK2.7*, plant hormones-related genes such as *AUXIN RESPONSE FACTOR 2, AUXIN-INDUCED PROTEIN 13 (IAA13)*, *JAMONATE-ZIM-DOMAIN PROTEIN 9* (*JZA9*)*, and ABSCISIC ACID RESPONSIVE ELEMENT-BINDIGN FACTOR 2/3 (ABF2/3)*; and down-regulation of *CALCIUM-DEPENDENT PROTEIN KINASE 31* (*CPK31*), and plant hormone-related genes such as ET-stabilized transcription factors *ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1)*, auxin biosynthesis gene YUCCA9 (Supplementary Table 2). . Many *LIGHT HARVESTING CHLOROPHYLL A/B BINDING (LHCB)* protein genes were down-regulated under drought and the sequential rehydration. The *LHCB* family genes encode proteins that are part of the light harvesting antenna that receives and transfers light energy to the reaction centre of photosystem II (PSII) (Jansson, 1994). The *LHCB* genes are required for ABA-mediated stomatal aperture and the *lhcb* knock out mutants were found incapable to control stomatal aperture under ABA treatment by (Xu et al., 2012). The down-regulation of the *LHCB* family genes under drought thus reflects the effect of drought-induced stomatal closure, inhibiting gas exchange. Thus, limiting CO_2 assimilation and impairs photosynthesis.

A set of 1677 DEGs were expressed at T3 under drought as well as under the sequential rehydration treatment (T4). 30% of the DEGs were overlapped with the common drought-responsive DEGs. GO terms 'response to chemical stimulus' and the plant hormone biosynthesis pathway are overrepresented in

this data set, both indicating hormone-related signalling to be involved. Plant hormone-related genes exhibited different expression at T4 in comparison to T3. Ethylene-related genes such as *ERF6* and *ERF8* were down-regulated in both conditions. *JASMONATE-ZIM-DOMAIN-PROTEIN (JAZ) 3/7/8/9* and *INDOLE-3-ACETIC ACID INDUCIBLE (IAA) 13/18* were up-regulated under in both conditions, while *IAA29* and *OCTAEDECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) were down-regulated under drought, but up-regulated under the sequential rehydration condition. JAZ family genes function in signal transduction in JA signalling (Chini et al., 2007), and the same holds for the pathogen defence responsive gene *ORA59*, linking it to the ET signalling pathway (Pre et al., 2008). The auxin biosynthesis *YUCCA 2/8/9* genes and the auxin efflux carrier *PIN-FORMED 4* (*PIN4)* gene were also down-regulated under drought and up-regulated under sequential rehydration, indicating an inhibition of auxin biosynthesis and transport in plants by drought. ABA-related genes such as DREB2a, *RESPONSIVE TO DEHYDRATION 22* (*RD22*), *RESPONSIVE TO DESICCATION 29a (RD29a)*, *RESPONSIVE TO DESICCATION 29b (RD29b)* were up-regulated under drought at T3 (day7) but down-regulated in sequential rehydration condition. This is showing that the well-watered state is indeed sensed by plants and that for mostly down-stream drought responsive genes are still expressed upon rehydration.

Temporal regulation of genes influenced by biotic pre-treatment

A set of 49 identified DEGs were shared by the two combinatorial treatments, of which 31 genes exhibited opposite directions of expression response when comparing the two pre-treatments at T2 (Supplementary Table 9). Another eleven out of 49 DEGs responded faster to the *Pieris* pre-treatment than to the *Botrytis* pre-treatment, being found at T1 upon *Pieris* pre-treatment, but at T2 after inoculation by *Botrytis*. The 11 genes exhibited the same expression at early time point but an opposite transcriptional output at later time points. One of the gene, *ERF6*, was exhibited redundant function as *ERF5* in response to *Botrytis* infection (Moffat et al., 2012). However, the scenario is different in the sequential drought condition. The *ERF5* and *ERF6* were down-regulated by *Botrytis* pre-treatment at T2 during sequential drought, but gene *ERF6* exhibited enhanced down-regulation at T3 and this was not found for *ERF5*. *ERF5* and

ERF6 were up-regulated under *Botrytis* treatment and were down-regulated drought (Coolen et al., 2016)(Chapter 5). The down-regulation of the two genes in the sequential drought condition indicates an negative interaction effects between *Botrytis* and drought on *ERF5* and *ERF6*, and the effect on *ERF6* is stronger than on *ERF5*. To *Pieris* pre-treatment response, up-regulation of the two genes in the *Pieris* and subsequent drought condition was observed at T2, but only *ERF6* exhibited down-regulation at T1. This is showing a negative influence of the *Pieris* pre-treatment on *ERF6* but not on *ERF5*.

In total 18 out of the 49 DEGs exhibited faster response to the *Pieris* pre-treatment than to the *Botrytis* pre-treatment, showing a higher impact of the *Pieris rapae* on transcriptome during the sequential drought. This could be due to the severity of the *Pieris* herbivory was much stronger than 24 h *Botrytis* treatment. Windram et al (2012) used the same concentration of *Botrytis* spores as in this study, and showed that the first symptom of *Botrytis* infection on leaf can be observed at 20 h after inoculation (HAI) and the expansion of lesion was at 36 HAI.

Combinatorial stress responses cannot be predicted by comparing single stress responses

Previous studies have suggested tailored responses of plants to simultaneous combinations of biotic and abiotic stresses (Prasch and Sonnewald, 2013; Ramegowda and Senthil-Kumar, 2015), which is the combinatorial stress responses cannot be predicted by comparing single stress responses. Our experimental conditions and protocols are the same as described by Coolen et al;, (2016) (Chapter 5). Thus, our data set for combinatorial stress responses were compared with the results obtained under single stresses as by Coolen et al;, (2016) (Chapter 5). When comparing DEGs from the stress combination of *Botrytis* and subsequent drought with single drought and *Botrytis* treatments (Coolen et al., 2016)(Chapter 5), only 4% of DEGs identified as *Botrytis* and subsequent drought responsive genes were not induced by either single *Botrytis* or drought treatments (Supplementary Table 11). When comparing DEGs from the stress combination of *Pieris* and subsequent drought with single drought and *Pieris* treatments, only 3% of DEGs identified as *Pieris* + drought responsive

genes were not induced by either *Pieris* or drought. This is showing that most combinatorial stress responsive genes can be identified by comparing results from single stresses. Genes such as *ERF5* and *ERF104* are the two combinatorial stress responsive genes and can be identified by comparing results from single stresses. *ERF5* was up-regulated under both *Botrytis* and *Pieris* treatments, and down-regulated in drought condition. In combinatorial stress conditions, the gene was down-regulated under *Botrytis* and subsequent drought stress and up-regulated under *Pieris* and subsequent drought stress. *ERF104* is up-regulated under *Botrytis*, *Pieris* and drought conditions, but was down-regulated under the *Botrytis* and subsequent drought stress and was up-regulated under the *Pieris* and subsequent drought stress. This is showing that the effects of the interaction between biotic and subsequent drought responses cannot be predicted from single stress responses.

PIN7-regulated auxin is essential for plants responding to Pieris attack and drought stress

A number of alternative splicing (AS) variants were identified from each combinatorial treatment. No observed genes exhibited AS variants under both combinatorial treatments, indicating that the effects of the two biotic pre-treatments on alternative RNA splicing in sequential drought conditions were different. In addition, DEGs were compared from the combinatorial treatment with genes carrying AS variants and as expected no overlap was found. This indicates differential regulation of combinatorial stress in gene expression and alternative RNA splicing. The alternative splicing of a gene causes different isoforms and the isoform expression levels can be different depending on the condition, but this does necessarily need to have an effect on the total mRNA transcripts levels. Although a small overlap between DEGs and DEGs with AS variants were observed under salt stress (Ding et al., 2014), the over-represented function of the DEGs and the DEGs with AS variants fell into two different categories, showing a separate regulation of the two set of genes in response to stress.

The relative expression of two AS variants of auxin efflux carrier *PIN7* were measured and showed enhanced expression of AS variant *PIN7-1* under the

stress combination of *Pieris* and subsequent drought, when compared to the single drought treatment. This shows that the *PIN7-1* could play a role in response to the combinatorial stress. The two isoforms of *PIN7* could have distinct biological functions, as their protein sequence is slightly different. Analysis of AS variants of the *ZINC-INDUCED FACILITATOR1* (*ZIFL1*) gene showed that two of its isoforms (*ZIFL1.1* and *ZIFL1.3*) exhibited distinct localization and played different roles in root tonoplast cells to modulate root polar auxin transport (*ZIFL1.1*) and in plasma membrane of stomatal guard cells for drought tolerance (*ZIFL1.3*) (Eckardt, 2013). The same AS variants of PIN7 we describe for *A. thaliana* were deposited in GenBank for the Brassicaceae *Camelina sativa*, though these variants had not yet been reported for *A. thaliana*, according to TAIR (www.arabidopsis.org). The biological function and the targets of the two *PIN7* variants in response to stresses remains to be determined.

Role of PIN7, MDAR3, and CCR2 in response to stresses

The biological function of the combinatorial stress responsive genes (*PIN7*, *MDAR3*, and *CCR2*) were validated using T-DNA mutants. The *PIN7* gene encodes an auxin efflux carrier expressed in the vascular tissues and columella in the mature lateral root and primary root, leaf primordia, and hypocotyl (Scarpella et al., 2006; Guyomarc'h et al., 2012; Sassi et al., 2012; Lavenus et al.). The knockout mutant *pin7* exhibited decreased number of lateral root in comparison to the wild type (Lewis et al., 2011), which could be the reason for the enhanced susceptibility to drought stress. *Pieris* uses glucosinolate as feeding and oviposition stimulus (de Vos et al., 2008). Thus, one of our hypothesis for the decreased leaf consumption by *Pieris* is due to less indole-3-carbinol (I3C) accumulation in *pin7* leafs. However, an antagonistic interaction has been suggested between the glucosinolate breakdown products I3C and auxin (Katz et al., 2015). Another hypothesis for the enhanced tolerance to *Pieris* in *pin7* could be due to the negative regulation between auxin and JA biosynthesis (Cecchetti et al., 2013). The *pin7* mutant could contain higher JA accumulation than the wild type and that regulates *Pieris* defence genes expression. The *MDAR3* gene is a member of the monodehydroascorbate reductase gene family and plays an important role in recapturing ascorbic acid (AsA) and regulates redox homeostasis in plant cell (Ramel et al., 2009; Brini et

al., 2011). The *MDAR3* expression is regulated by *ERF6* and the expression of MADAR3 can be inhibited by ABA and heat treatments (Sewelam et al., 2013), as well as drought stress (this study). The AsA level is decreased under drought because AsA has impair on ABA- and H_2O_2-mediated stomatal closing (Pastori and Foyer, 2002). Inhibit MDAR3 expression reduces AsA level in plants cell and results increased tolerance to drought stress by closing stomata. The *CCR2* gene encodes one of cinnamoyl CoA reductase (CCR) isoforms and is involved in lignification and phenolic biosynthesis (Lauvergeat et al., 2001). The lignification is inovlved in response to multiple biotic and abiotic stresses. For instance, The phenolic acids can affect *Pieris* oviposition and feeding stimulants (van Loon, 1990), the lignification and *CCR* proteins were increased drought and salt stress in *Leucaena leucocephala*(Srivastava et al., 2015). A study in maize showed that the transcript level of *CCR2* is increased under drought stress and the increased expression of *CCR2* results decreased cell wall expansion, increased deposition of lignin, and reduced root elongation (Fan et al., 2006). The *ccr2* mutant could promote root elongation and that enhances the sensitivity of *ccr2* mutant to drought stress.

Conclusion

In this paper, we have shown the gene expression and alternative splicing of genes that are associated with drought and two combinatorial pre-treatment stress responses in Arabidopsis. An increased number of DEGs across time points under drought stress were identified, showing an increased severity of drought experienced by plants. We found that one-day rehydration period is insufficient to fully overcome the effects of drought stress on Arabidopsis. More DEGs under the *Pieris* and subsequent drought stress were identified than under the *Botrytis* on subsequent drought stress, showing that the *Pieris* herbivory is more severe than *Botrytis* infection. A small set of DEGs were shared by the two combinatorial stresses and most of the genes showed opposite transcriptional output in the two combinatorial conditions, indicating different regulations of the two combinatorial stress in gene expression. We validated some combinatorial stress responsive genes using loss-of-function mutants and observed antagonistic interaction between *Pieris* and drought. Some unique combinatorial stress responsive genes were discovered, but their biological function in stress conditions still remains unresolved. We confirmed that the expression level of alternative splicing variant *PIN7-1* changes under drought and the combination of *Pieris* and subsequent drought, but further research will be needed to establish if and how this affects the phenotype.

Acknowledgement

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Supporting Information

Additional supporting information may be download via link

https://www.dropbox.com/sh/hb95t5dwvxbsc1u/AAB2Z7Q1DPSwCCU-oaTBBi2Pa?dl=0

CHAPTER 7

Phenotypic and genetic responses of Arabidopsis thaliana natural accessions to consecutive Pieris rapae herbivory and drought stress

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Abstract

This study focuses on investigating the responses of natural accessions of *Arabidopsis thaliana* to the consecutive exposure to a biotic agent (herbivory by *Pieris rapae*) and an abiotic factor (drought) as well as to each of the single treatments. Three parameters: rosette fresh weight (FW), dry weight (DW), and water content (WC) were used to evaluate the effect of exposure to *Pieris* and/or subsequent drought stress in 16 *Arabidopsis* accessions. We found WC is a suitable parameter for the *Pieris* and drought stress interaction assessment and showed it to be independent of leaf biomass accumulation under drought conditions. To gauge the effect of *Pieris* pre-treatment on sequential drought response *in planta*, differential expression levels of nine drought-responsive genes, relevant to the cross-talk between Abscisic acid (ABA) and jasmonic acid (JA) signaling pathways, were measured. While no correlation with the observed phenotypic variation was found, the expression of all investigated gene candidates (except *RD29b)* proved affected in response to drought preceded by exposure to the herbivore activity. Results presented herein allowed us to propose a model of gene regulatory networks effectively delineating the effects of the interaction between *Pieris* and drought stress triggers on drought-responsive gene expression.

Keywords

Introduction

Biotic and abiotic stress factors, such as pathogen attack, insect herbivory, drought, extreme temperatures, UV radiation, salinity, etc. (Pareek et al., 2010; Robert-Seilaniantz et al., 2010) often exert negative effects on plant growth and development causing immense losses in agricultural production (Hirt, 2004). Drought is one of the most devastating abiotic stress elicitors in plants, resulting in accumulation of abscisic acid (ABA), a phytohormone triggering stomatal closure to reduce water loss via transpiration (Rai and Takabe, 2006; Xiong and Ishitani, 2006). As a consequence, water uptake and assimilation are reduced, leading to lower nutrient uptake and diminished biomass (Verslues et al., 2006).

The ABA-dependent signaling pathway has been reported crucial in mediation of plant stress-responsive gene expression under drought condition, while also being involved in osmotic and pathogen stress response mechanisms (Rai and Takabe, 2006; Xiong and Ishitani, 2006). A comprehensive overview of ABA-dependent and ABA-independent signaling trails *in planta* as triggered by drought stress was proposed by Shinozaki and Yamaguchi-Shinozaki (2007). Zeaxanthin epoxidase, catalyzing the first committed step in the ABA biosynthetic pathway, is encoded by *ABA DEFICIENT 1* (*ABA1*) (Xiong and Zhu, 2003), whose expression is enhanced in response to drought as well as osmotic stress, high salinity, and ABA treatment (Xiong et al., 2002; Barrero et al., 2006) and down-regulated by JA (Anderson et al., 2004). Furthermore, multiple transcription factors (TFs), such as RESPONSIVE TO DESICCATION 26 (RD26), MYB2, MYC2, RD22, and ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2 (ABF2), RESPONSIVE TO DESICCATION 29B (RD29b) participate in the ABA-dependent pathway and are involved in the cross-talk between ABA and jasmonic acid (JA) signaling (Shinozaki and Yamaguchi-Shinozaki, 2007). Their genes are expressed in response to multiple biotic and abiotic triggers, including drought, high salinity, osmotic stress, *Fusarium oxysporum* infection, and herbivory by *Pieris rapae* (Abe et al., 2003*;* Anderson et al., 2004; Fujita et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Vos et al., 2013)*.* Among the aforementioned TFs, MYC2, a positive regulator of *Pieris* defense as well as drought tolerance (Vos et al., 2013), is of special significance. Transgenic

overexpression of *MYB2* and *MYC2* resulted in plants exhibiting enhanced sensitivity to ABA treatment, with the *MYB2* transformants proving less responsive to the phytohormone in comparison with their *MYC2* overexpressing counterparts (Abe et al., 2003). Further, the promoter of *RD22* contains two cis-elements that can bind respectively with MYB2 and MYC2 (Abe et al., 2003), while gene expression of RD29b is regulated by ABF2, as well as ANAC019 in response to dehydration treatment (Jensen et al., 2010; Uno et al., 2000; Yamaguchi-Shinozaki and Shinozaki, 1994). Moreover, the DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2 (DREB2a), playing a role in the ABA-independent pathway, was reported as sensitive to drought (Shinozaki and Yamaguchi-Shinozaki, 2007). Although the upstream sequence of the initial transcriptional site of *DREB2a* contains an ABA-responsive AREB element, and its expression can be activated by ABF2 (AREB1)*,* AREB2*,* and AREB3,only low expression levels of the gene were observed after exposure to the plant hormone (Kim et al., 2011), while enhanced expression of *DREB2a* was detected after 24 hours of rehydration treatment (Yamaguchi-Shinozaki and Shinozaki, 1994). What is more, plants under drought stress were found to accumulate high levels of proline (Delauney and Verma, 1993). *DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (*P5CS1*) is one of the proline biosynthetic genes (Strizhov et al., 1997) and is required for the build-up of the amino acid levels under osmotic, high salinity, and drought stress conditions (Szekely et al., 2008; Verslues and Sharma, 2010). The gene can be regulated via both the ABA-dependent and the ABA-independent pathway (Verslues and Sharma, 2010). The enumerated drought-responsive genes are representative of the cross-talk between the ABA and JA cascades, as well the cross-take between ABA-dependent and ABA-independent signal transduction mechanism that triggered by the abiotic stress factor in question.

Various interactions between biotic and abiotic stress response mechanisms in plants have been reported and summarized by Suzuki et al. (2014). The interplay between biotic and drought stress feedback involves complex cross-talk via both metabolic and signaling pathways (Atkinson and Urwin, 2012; Prasch and Sonnewald, 2013; Rasmussen et al., 2013). For instance, the biotic herbivory of *Pieris rapae* larvae induces signaling through the jasmonic acid and

ethylene (JA-ET) cascade (De Keersmaecker et al., 2005), which can interacts negatively with the drought-induced ABA signaling (Pieterse et al., 2012). The latter observation suggests that *Pieris* pre-treatment might lead to increased drought susceptibility. Thus, understanding how plants adapt to the wide range of stress factors and the genetics underlying their response will undoubtedly contribute to the improvement of plant tolerance to extreme conditions via optimized breeding.

This study was designed to gain novel insights into the mechanism of plant response to combinatorial stress, namely insect attack and subsequent drought, through observation of the variation of *Arabidopsis thaliana* natural accessions subjected to the investigated stress factor combination. *Arabidopsis* plants were exposed to *Pieris rapae* larvae, to elicit response to herbivory, and then treated with polyethylene glycol (PEG) which induces osmotic stress simulating drought. Control plants were subjected to single stress treatments (*Pieris* herbivory only and single PEG treatment without any pre-treatment). Responses to the applied stress-inducing conditions were investigated using phenotypic analysis in combination with expression profiling of the aforementioned nine drought-responsive genes.

Materials and methods

Plant material and growth conditions

Sixteen *Arabidopsis thaliana* accessions that showed maximum genetic divergence from the HapMap population were selected based on single nucleotide polymorphism (SNP) marker information (Li et al., 2010). These included: Br-0 (CS76101), Bur-0 (CS76105), Col-0 (CS76113), Est-1 (CS76127), Fei-0 (CS76129), Got-7 (CS76136), Ler-1 (CS76164), Lov-5 (CS76175), NFA-8 (CS76199), RRS-7 (CS28713), RRS-10 (CS22689), Sha (CS76227), Tamm-2 (CS76244), Ts-1 (CS76268), Tsu-0 (CS28780), and Van-0 (CS76297).

Prior to experimental procedure commencement, seeds of the test *A. thaliana* accessions were stratified at 4 °C in the dark for five days. Thereafter, one seed per accession was sown on one Grodan® Rockwool cube of 40×40×40 mm. The plants were watered three times per week (on Mondays, Wednesdays, and Fridays) with a nutrient solution developed for *Arabidopsis* (pH, 7; EC, 1.4 mS/cm). The solution consisted of 1.7 mM NH_4^+, 4.5 mM K^+, 0.4 mM Na^+, 2.3 mM $Ca2^+$, 1.5 mM $Mg2^+$, 4.4 mM NO_3^-, 0.2 mM Cl^-, 3.5 mM SO_4^{2-}, 0.6 mM HCO_3^-, 1.12 mM PO_4^{3-}, 0.23 mM SiO_3^{2-}, 21 µM Fe^{2+} chelated using 3 % diethylenetriaminepentaacetic acid (DTPA), 3.4 µM Mn^{2+}, 4.7 µM Zn^{2+}, 14 µM BO_3^{3-}, 6.9 µM Cu^{2+}, and <0.1 µM MoO_4^{4-}. The plants were grown in a climate-controlled growth chamber set to short day conditions, i.e., 10 h day/14 h night at 21 °C day/19 °C night and 70 % relative humidity. Irradiation was set to 200 µmol m^{-2} s^{-1}.

## *Preparation of Pieris rapae larvae*

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

for 1 h, first-instar (L1) larvae were placed on *Arabidopsis* leaves using a fine paint brush as described by Van Wees et al. (2013).

Stress treatments

Control plants did not receive any stress treatment (Figure 1). For single *Pieris* treatment, 17-day-old plants were exposed to one *Pieris* first-instar larva (one larva per one plant)for one day. Thereafter, the caterpillars were removed and the plants were allowed growth under the aforementioned control conditions for six days ("*Pieris*"). For single drought treatment, 19-day-old plants were irrigated with the nutrient solution (described above) that contained 7.7 % w/v polyethylene glycol (PEG) 8000 for six days ("drought"). For combinatorial stress, 17-day-old plants were exposed to one *Pieris* first-instar larva for one day. The caterpillars were then removed and the plants continued to grow under the control conditions for one day to be subsequently subjected to irrigation with the Hyponex solution containing 7.7 % w/v PEG 8000 for six days ("*Pieris* and subsequent drought").
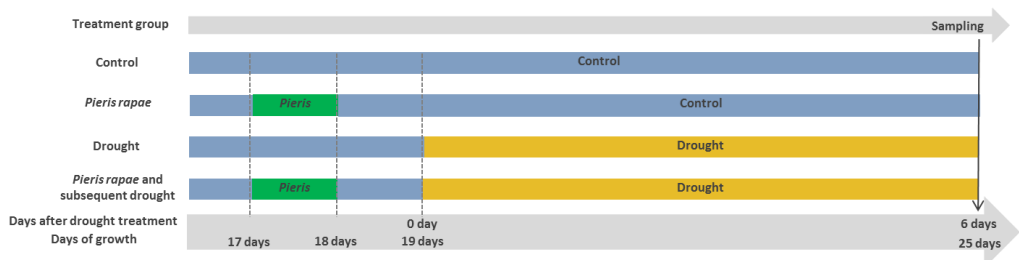
Figure 1. Experimental design of the applied plant treatment. *A. thaliana* plants were subjected to *Pieris rapae* herbivory, drought, or combinatorial stress (*Pieris* herbivory and drought) as well as allowed growth under control conditions. The various treatment conditions are indicated in different colors: control, blue; drought, yellow; *Pieris*, green; rehydration after drought, gray. The vertical arrow indicates the sampling time point. Vertical dashed lines indicate periods before *Pieris* pre-treatment, after *Pieris* treatment, and before exposure to drought stress conditions.

Real-time reverse transcription PCR (qRT–PCR)

RNA was extracted from *A. thaliana* rosettes following the protocol proposed by Onate-Sanchez and Vicente-Carbajosa (2008). cDNA was then synthesized from 800 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad) at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. cDNA thus obtained was diluted (1:10) and used as a template in quantitative PCR (qPCR). According to the transcriptome data collected from several studies concerning plant responses to drought, osmotic, and *Botrytis* infection stress triggers, relying on the Genevestigator (http://refgenes.org/rg) web tool application, *RETICULON-LIKE PROTEIN B11* (*RTNLB11*) proved the gene characterized by the most stable expression level across the experiments and was, therefore, selected as a reference gene. The qPCR was performed using the iQ SYBR® Green Supermix (Bio-Rad) on the CFX96 real-time PCR system (Bio-Rad) set to 95 °C for 4 min, followed by 40 cycles of 95 °C for 10 sec and 55 °C for 30 sec. Primer sequences of all investigated genes are provided in Supplementary Table 1.

Statistical analysis

Broad-sense heritability, H^2 was calculated using equation (1):

$$H^2 = \frac{Var(G)}{Var(G) + Var(E)} \qquad (1),$$

where *G* stands for genotype, *E* for environment and *Var* for variance.

Pearson's correlation coefficient (r) analysis, one-way, two-way, and three-way analysis of variance (ANOVA, to test the Accession×Gene×Treatment interaction), as well as canonical variate analysis (CVA) were performed using the GenStat software (16th Edition). The three-way ANOVA used to assess the Accession×*Pieris*×Drought interaction was performed as described by Sokal RR (1981). The logarithm (log) transformed quantification cycle (C_q) values of drought-responsive genes were used for the three-way ANOVA tests. Student's t-test was used to compare the investigated accessions subjected to two alternative treatments. Statistical tests on the measured parameters: rosette fresh weight (FW), rosette dry weight (DW), and rosette water content (WC)

ratio were performed upon four plants per accession. The water content (WC) ratio was calculated using equation (2):

$$WC= \frac{FW-DW}{FW}$$

(2).

Statistical tests on the log transformed C_q values were performed upon three plants per accession.

Results

Effect of Pieris rapae herbivory and subsequent drought treatment on 16 natural accessions of Arabidopsis

Sixteen *A. thaliana* accessions, exhibiting maximum genetic divergence based on SNP marker information (Li et al., 2010), were selected and examined for the effect of *Pieris rapae* herbivory (*Pieris*) and/or drought stress on plant performance (Figure 1). Single *Pieris* treatment significantly reduced the rosette fresh weight (FW) and rosette dry weight (DW) in the accessions Bur-0, Col-0, Got-7, NFA-8, RRS-7, and Tamm2 (Figure 2 a and b). Strikingly, the representatives of Est-1 accession showed significantly higher FW and DW values upon *Pieris* treatment than under control conditions. Sole drought treatment significantly reduced both FW and DW quotients in Bur-0, Col-0, Fei-0, NFA-8, RRS-10, RRS-7, Tamm-2, and Van-0. Decrease in DW was also detected in Got-7, Sha, and Ts-1, indicating that most accessions experienced serious growth retardation upon drought treatment. This finding prompted us to examine the water content (WC) ratio, which was significantly enhanced in Got-7, Sha, Tamm-2, and Ts-1 and decreased in Tsu-0, pointing to the latter as the most drought-susceptible accession (Figure 2 c). *Pieris* pre-treatment followed by that simulating drought resulted in lowered FW values in Bur-0, Col-0, Est-1, Got-7, RRS-7, and Tamm2 and diminished DW rates in Est-1 and RRS-7, in comparison to those characteristic of plants subjected to drought conditions without pre-treatment. Significantly reduced WC values were observed in Bur-0, Col-0, Got-7, Lov-5, Sha, Tamm-2, and Ts-1, showing that the most drought-resistant accessions (evaluated as such based on their WC ratio parameters) lost their relative drought tolerance after *Pieris* pre-treatment. Broad-sense heritabilities of the investigated traits were high, mostly above 0.5, up to 0.65, with the exception of the shift in WC ratio upon *Pieris* treatment, which showed moderate heritability (Table 1). Results obtained thus far offered a favorable prospect for genetic analysis of the test traits in the 16 accessions.
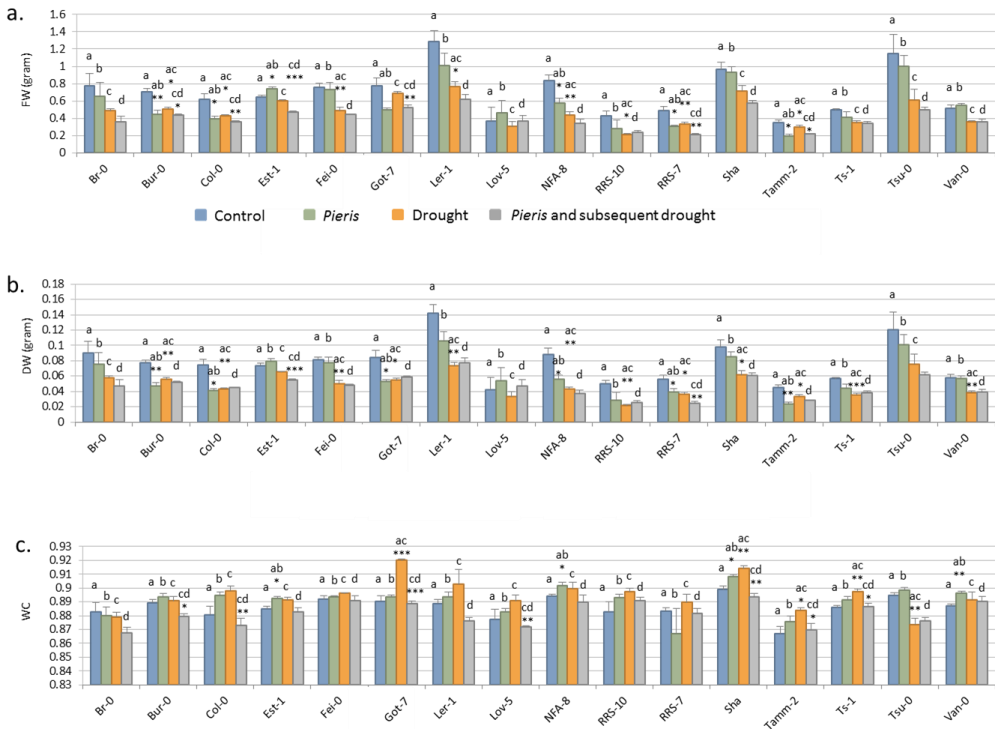
Figure 2. Phenotypic analysis of rosettes of 16 *A. thaliana* accessions in response to *Pieris rapae* herbivory, drought, and drought induced after *Pieris* pre-treatment. **a.**, Rosette fresh weight (FW); **b.**, rosette dry weight (DW); **c.**, water content (WC), calculated as the (FW-DW)/FW ratio. Phenotypes observed under control conditions are indicated in blue, those resulting from exposure to *Pieris* alone – in green, drought alone – in yellow, and *Pieris* pre-treatment followed by drought – in grey. The standard error of the mean (SE) was calculated based on four replicates. Two-way ANOVA was used to compare *Pieris*- and drought-induced responses to the control and to compare combinatorial stress feedback to that caused by drought. *p*-values: * <0.05, ** <0.01, *** <0.001

Table 1. Broad-sense heritability, H^2 of rosette fresh weight (FW), dry weight (DW), and water content (WC= FW-DW/FW) of 16 *A. thaliana* accessions upon exposure to *Pieris rapae* herbivory, drought, and combinatorial stress conditions (n= 4).

Treatment	FW	DW	WC
Control	0.63	0.60	0.59
Pieris	0.58	0.55	0.37
Drought	0.65	0.60	0.64
***Pieris* and drought**	0.63	0.59	0.53

Correlations among traits

Pearson's correlation coefficients between FW, DW, and WC were calculated for each set of experimental conditions. Positive correlations between FW and DW were found in all investigated settings. While the correlation between FW and WC was significant under all applied conditions except the combinatorial treatment, the latter correlation was largely lost when considering DW and WC upon sole drought induction (Table 2). This observation pointed to WC as an informative trait in the study of sensitivity to drought.

Table 2. Pearson's correlation coefficients upon comparison of rosette fresh weight (FW), dry weight (DW), and water content (WC) of 16 *A. thaliana* accessions under each set of experimental conditions. Significant correlations are shaded in gray.

Treatment group		Phenotypic traits	
		DW	WC
Control	FW	0.99	0.61
	WC	0.47	
Pieris	FW	0.98	0.49
	WC	0.32	
Drought	FW	0.94	0.32
	WC	-0.01	
***Pieris* and subsequent drought**	FW	0.97	0.17
	WC	-0.08	

To observe the variation of phenotypic responses of the investigated accessions under stress conditions, three-way ANOVA was performed to test the interactions between traits, treatments, and accessions. Significant main effects of accessions, traits, and treatments were observed ($p < 0.001$; Table 3) and significant interactions between accessions, traits, and treatments were found. Upon exposure to divergent treatments, the plants showed a large variation in phenotypic responses.

Table 3. Three-way ANOVA to test the interaction between accessions, traits, and treatments. All main effects and interactions were significant.

Source of variation	d.f	s.s	m.s	v.r	*p*-value
Accessions (Acc)	15	7.98	0.53	69.91	3.06E-132
Traits	2	211.82	105.91	13914.40	0.00E+00
Treatments	3	2.32	0.77	101.71	1.96E-55
Acc×Traits	30	3.96	0.13	17.35	3.96E-67
Acc×Treatments	45	1.18	0.03	3.45	1.75E-12
Traits×Treatments	6	1.21	0.20	26.46	1.04E-28
Acc×Traits×Treatments	90	0.67	0.01	0.98	5.34E-01
Residual	576	4.38	0.01		
Total	767	233.53			

d.f= degree of freedom; s.s= sum of square; m.s= mean square; v.r= variance

To test the interactions between treatments and accessions, three-way ANOVAs were performed for the three traits separately (Table 4). The analyses showed significant main effects of *Pieris* and drought treatments (*p* <0.001) in all cases, as well as interaction effects between accessions and the two single stress triggers (*Pieris* and drought) for FW and DW (*p* <0.001) and between accessions and drought-simulating treatment regarding WC (*p* <0.001). The only significant interaction effect between accessions, *Pieris* and drought was observed for WC (*p* <0.05).

Taken together, the obtained results point to a large variation in the phenotypic response of plants exposed to *Pieris* herbivory, drought, or the combination of stress triggers (exposure to *Pieris* followed by drought simulation). Calculation of the WC ratio allows identification of natural variation in the drought response, including the influence of *Pieris* pre-treatment.

Table 4. Three-way ANOVA evaluating the interaction between treatments and accessions regarding rosette fresh weight (FW), dry weight (DW), and water content (WC). Main effects were significant for each trait. Significant interaction between Pieris herbivory, drought, and accessions was found in case of the WC parameter.

Source of variation	d.f	FW				DW				WC			
		s.s	m.s	F	p-value	s.s	m.s	F	p-value	s.s	m.s	F	p-value
Accessions (Acc)	15	6.34	0.42	36.76	2.78E-48	5.25	0.35	32.59	1.05E-44	3.42E-03	2.28E-04	12.33	4.12E-21
Pieris	1	0.44	0.44	38.64	3.11E-09	0.29	0.29	27.33	4.45E-07	2.33E-04	2.33E-04	12.58	4.89E-04
Drought	1	1.2	1.2	104.39	7.59E-20	1.26	1.26	117.58	1.12E-21	1.25E-03	1.25E-03	67.36	3.23E-14
Acc×*Pieris*	15	0.43	0.03	2.48	2.32E-03	0.37	0.02	2.29	0.01	4.51E-04	3.01E-05	1.63	0.07
Acc×Drought	15	0.5	0.03	2.91	3.61E-04	0.31	0.02	1.89	0.03	2.14E-03	1.43E-04	7.71	2.28E-13
***Pieris*×Drought**	1	0.02	0.02	1.76	0.19	0.16	0.16	14.59	0	1.21E-03	1.21E-03	65.68	6.07E-14
Acc×*Pieris*×Drought	15	0.18	0.01	1.03	0.43	0.24	0.02	1.52	0.1	5.07E-04	3.38E-05	1.83	0.03
within	192	2.21	0.01			2.06	0.01			3.55E-03	1.85E-05		

d.f= degree of freedom; s.s= sum of square; m.s= mean square; F= F probability

Table 5. List of the nine tested drought-responsive genes and their corresponding stress sensitivity.

| Gene ID	Gene name	Full name	Response to	Literature
AT5G67030	ABA1	ABA DEFICIENT 1	ABA biosynthesis, osmotic stress, drought, high salinity, ABA	Xiong et al., 2002; Barrero et al., 2006
AT1G32640	MYC2	MYC DOMAIN PROTEIN 2	Drought, *Pieris rapae*, wounding, oxidative stress, pathogens, ABA, JA	Abe et al., 2003; Dombrecht et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007; Kazan and Manners, 2013; Vos et al., 2013
AT2G47190	MYB2	MYB DOMAIN PROTEIN 2	Drought, high salinity, ABA	Abe et al., 2003; Yoo et al., 2005; Shinozaki and Yamaguchi-Shinozaki, 2007
AT5G25610	RD22	RESPONSIVE TO DESICCATION 22	Drought, ABA	Yamaguchi-Shinozaki and Shinozaki, 1993; Abe et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007;
AT4G27410	RD26	RESPONSIVE TO DESICCATION 26	Drought, high salinity, ABA, JA	Fujita et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007
AT1G45249	ABF2	ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2	Drought, high salinity, ABA	Kim et al., 2004; Lee et al., 2010
AT5G52300	RD29b	RESPONSIVE TO DESICCATION 29B	Drought, osmotic stress, ABA	Uno et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2007;
AT5G05410	DREB2a	DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2	Drought	Qin et al., 2008b
AT2G39800	P5CS1	DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1	Proline biosynthesis, drought, osmotic stress	Strizhov et al., 1997; Szekely et al., 2008; Verslues and Sharma, 2010

Gene expression variation under Pieris, drought, and the combinatorial treatments

To explore whether the genetic background and the observed phenotypic differences influenced the transcriptional output of the investigated plant population upon *Pieris*, drought, and the combinatorial stress treatments, expression levels of nine known drought-responsive genes (Table 5) were compared to those characteristic of plants maintained under control conditions. The expression quotients of interest were determined via analysis of the genetic material of rosette leaves of 14 *Arabidopsis* accessions, as the relevant organs of the other two accessions were lost during harvesting. All primers used for the quantitative reverse transcription PCR (qRT-PCR) analysis were designed to amplify the same coding sequence region from the mRNA pools of the investigated accessions and were pre-tested for efficiency.

Pearson's correlation coefficients between the three investigated traits and the nine drought-responsive gene expression levels were calculated and significant correlations were found (Supplementary Table 4), while the obtained transcription quotients could not be correlated with the treatment effects on growth of the tested natural accessions of *Arabidopsis*.

When examining gene expression responses in general, two groups of accessions could be distinguished; one, for which ^{2}log quantification cycle (C_q) values (^{2}logC_q) decreased upon drought treatment but increased upon application of the combination of *Pieris* and sequential drought triggers and another, showing hardly any response to drought but a decrease in ^{2}logC_q values upon exposure to the combinatorial stress treatment (Figure 3). Further, divergent effects of the *Pieris* pre-treatment in response to sequential drought were found in the investigated accessions.
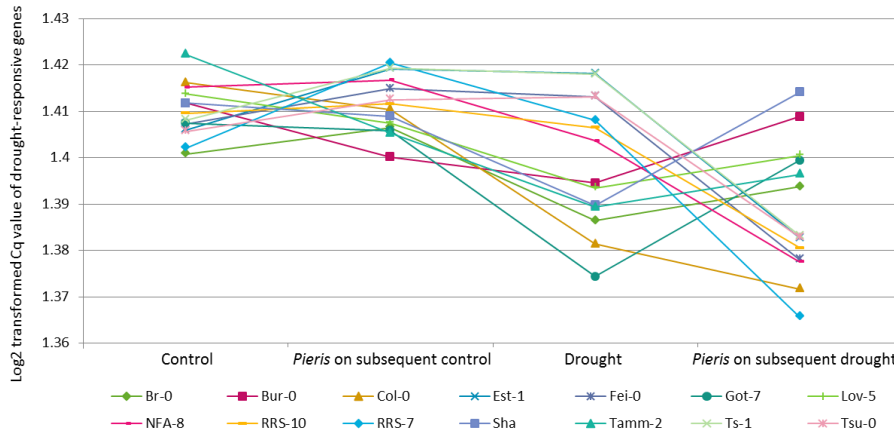
Figure 3. Variation in mean average C_q values (on a log2 scale) representing expression of the nine investigated drought-responsive genes in rosettes of 14 *A. thaliana* accessions in response to drought, *Pieris* herbivory, and *Pieris* pre-treatment followed by drought simulation.

Pearson's correlation coefficients between gene expression levels were calculated using relevant C_q values. For most genes, a positive correlation was observed (Table 6), while a negative one was noted between *ABA1* and *DREB2a* quotients, confirming their independence. No significant correlation was found between *MYB2* transcript abundance values and those of the remaining investigated genes, showing that *MYB2* is not related to other drought-responsive genes in the three tested experimental settings.

Table 6. Pearson's correlation coefficients of the investigated drought-responsive genes calculated on the basis of the relevant C_q values. Gray shading indicates significant correlation ($p < 0.05$).

|	ABA1	ABF2	DREB2a	MYB2	MYC2	P5CS1	RD22	RD26	RD29b
ABA1	1								
ABF2	0.17	1							
DREB2a	-0.31	0.26	1						
MYB2	0.11	-0.03	0.02	1					
MYC2	-0.02	0.47	0.4	-0.06	1				
P5CS1	0.36	0.62	-0.04	-0.09	0.61	1			
RD22	-0.11	0.51	0.57	0.09	0.76	0.48	1		
RD26	0.08	0.45	0.42	-0.08	0.72	0.62	0.61	1	
RD29b	0.20	0.70	0.18	0.02	0.32	0.54	0.31	0.4	1
Genes	ABA1	ABF2	DREB2a	MYB2	MYC2	P5CS1	RD22	RD26	RD29b

To assess the effect of the applied treatments on plant gene expression, canonical variate analysis (CVA) was performed upon the relevant C_q values. CVA is a multivariate analysis method that has a similar function to the principle component analysis (PCA) applied to evaluate the possible correlation between variables (Heymann and Noble, 1989). The difference is that CVA can be used to discriminate between alternative treatment groups as it accounts for differences in group variation in course of their separation (Heymann and Noble, 1989). Thus, the CVA axes maximize the variance between groups relative to that within them (Zelditch et al., 2012). The resulting biplot (Figure 4) represents 92.9% of total variance and shows that the variation among accessions was lower than the variation among treatments, based on the separation of plants subjected to control conditions, drought, and the combination of *Pieris* and subsequent drought simulation, but not on separation of accessions. As a result, strong effects of *Arabidopsis* accessions on gene expression come to the fore, while those of single stress treatments cannot be distinguished in the presented plot. When considering individual components, the CVA-1 component explained 77.6 % of the total variance, especially for *DREB2a*, *RD22*, *P5CS1*, and *RD29b* while the CVA-2 explained 15.3% of the total variance, most notably in case of *ABF2*, *RD22*, *MYC2*, *P5CS1*, and *DREB2a* (Supplementary Table 2).
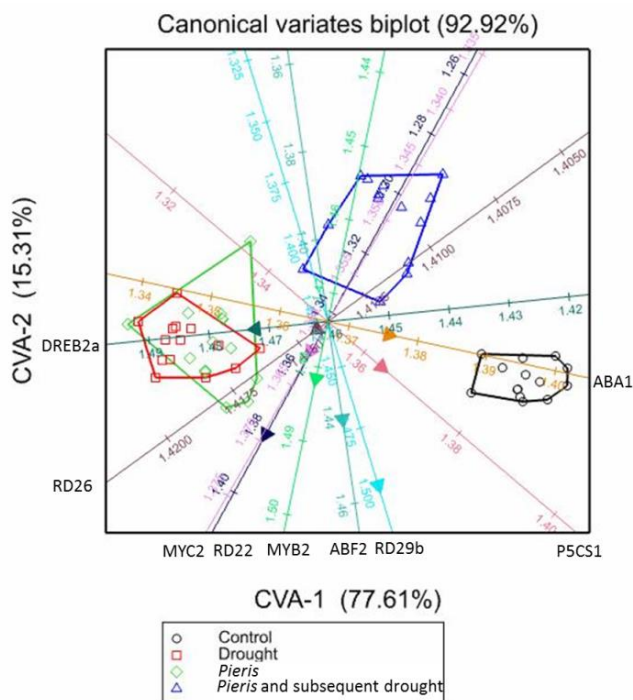
Figure 4. Canonical variates analysis (CVA) explained 92.92% of total variance in drought-responsive gene expression upon application of alternative stress treatments. The CVA-1 explained 77.61% of total variance and the CVA-2, 15.31%. The biplot displays a clear separation between the control group, the one subjected to drought, and that exposed to combinatorial stress. Each biplot axis represents one gene, while the numbers on the axes, the corresponding C_q values on the log2 scale. The angles between the axes illustrate correlation between the variables and arrows, the direction, with lines in opposite direction indicating negative correlation (e.g., DREB2a and ABA1). Black color represents control conditions; green, *Pieris* treatment; red, drought simulation; blue, combinatorial stress induction. Each dot within each treatment group represents one accession.

As initially expected, drought stress (as compared to control conditions) induced the expression of ABA-dependent signaling genes *ABA1*, *RD26*, *MYC2*, *RD22*, *RD29b*, *ABF2*, and *P5CS1*, although the levels of induction were quite variable between accessions (Figure 5). Strikingly, the ABA-dependent signaling gene *MYB2* was either up- or down-regulated depending on the accession. For instance, its expression fold change under drought conditions (relative to control) was two times lower in Fei-0 (log2FC= -1) but up to four times higher in Ts-1 (log2FC= 2). Also *DREB2a*, an ABA-independent signaling gene showed variable response levels upon comparison of accessions.

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Unexpectedly, analysis of the single *Pieris* treatment outcomes also revealed differential expression of drought-responsive genes depending on their genetic background (Supplementary Figure 1). *RD29b* and *P5CS1* were up-regulated, whereas the expression levels of *RD26*, *RD22*, and *DREB2a* decreased. This observation suggested that transcriptional activity of the aforementioned genes, thought to be largely drought-responsive, was also susceptible to biotic stress triggers (such as *Pieris* herbivory), thus leading us to hypothesize that, depending on the genetic background, a combination of stress factors can have a synergetic or an antagonistic effect on the expression levels of drought-responsive genes. To address this hypothesis, gene expression levels were analyzed by comparing the drought vs. control expression difference with the combinatorial stress vs. control expression difference (Figure 5). *Pieris* pre-treatment reduced the expression of *ABA1* and *P5CS1* in response to sequential drought, when compared to the outcomes of the single drought treatment, while *MYB2*, *RD22*, and *ABF2* were up-regulated. For *RD26*, *MYC2*, and *DREB2a*, the direction of the response varied considerably depending on the accession, indicating a large effect of natural genetic variation on their transcriptional activity. This supposition was confirmed by a three-way ANOVA, accounting for C_q values of drought-responsive genes, that showed a significant main effect of drought stress for all genes ($p < 0.001$, Supplementary Table 3) and a significant main effect of *Pieris* on the expression levels of *RD26, MYB2 DREB2a, RD29b,* and *ABF2* ($p < 0.05$). Moreover, significant interactions were found between genes and accessions, meaning that a combination of biotic and abiotic stress elicits a specific response in each accession.

a. ABA1
b. RD26
c. MYC2
d. MYB2
e. RD22
f. DREB2a
g. RD29b
h. ABF2
i. P5CS1
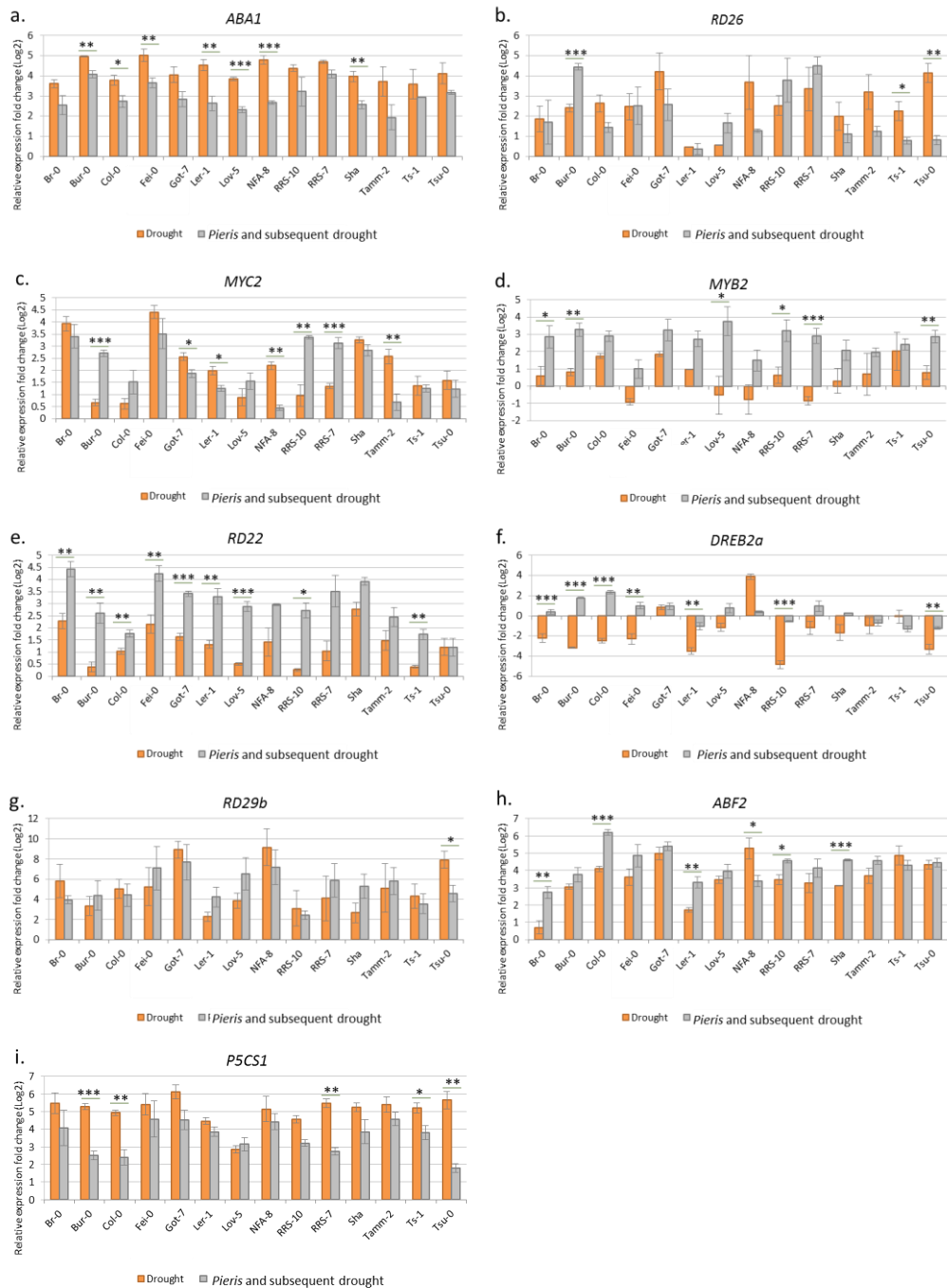
Figure 5. Relative expression levels of drought-responsive genes: **a.**, *ABA1*; **b.**, *RD26*; **c.**, *MYC2*; **d.**, *MYB2*; **e.**, *RD22*; **f.**, *DREB2a*; **g.**, *RD29b*; **h.**, *AFB2*; **i.**, *P5CS1* in rosettes of the investigated *A. thaliana* accessions upon comparison of drought vs. control and combinatorial stress vs. control treatments. Log2 scale at 1, 2, 3, 4, 5, 6, 7, and 8, corresponds to 2, 4, 6, 8, 16, 32, 64, 128, and 256 times actual fold change, respectively. The standard error of the mean (SE) was calculated based on three plants per accession. Two-way ANOVA was used to compare the responses to the combinatorial stress to those caused by drought simulation. Significant differences (*p*-values): * <0.05, ** <0.01, *** <0.001.

Discussion

This work uncovers the differences in responses of natural accessions of *A. thaliana* to single and combinatorial stress treatments involving biotic stress imposed by herbivory of *P. rapae* larvae and abiotic stress due to drought.

WC is a suitable parameters to present drought response

Our research showed that the water content ratio (WC, (FW-DW)/FW), but not the fresh weight (FW) or the dry weight (DW) parameters of *Arabidosis* rosettes alone, is a trait relevant to identification of genotypes that are either tolerant, susceptible, or characterized by mechanisms aiming at evasion of detriments of drought. Low WC ratio values coincide with inhibited plant growth, as the parameter is positively correlated with solutes and metabolic activity in plants (Bac-Molenaar et al., 2016; Loudet et al., 2003).

Different responses to drought stress in Arabidopsis

Drought tolerance is characterized by maintaining similar DW and WC quotients under drought stress to those corresponding to control conditions, which was observed in Br-0, Est-1, and Lov-5. Further, Tsu-0 exhibited drought tolerance, which is consistent with earlier findings on the relative water content in this accession under drought conditions (Bouchabke et al., 2008). In contrast, accessions Bur-0, Col-0, Fei-0, Ler-1, NFA-8, RRS-10, RRS-7, and Van-0 proved drought-sensitive, displaying reduced DW values but no differences in WC.

Under drought stress, plants can avoid dehydration by retaining water, reducing photosynthesis, and enhancing root development (Verslues et al., 2006). Drought avoidance is characterized by diminished DW outputs along with increased WC. Such phenotypes were found for the accessions Got-7, Sha, Tamm-2, and Ts-1. Several potential downstream targets of the two flowering-time regulators, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), were identified by QTL mapping of WC under two contrasting nitrate regimes (Loudet et al., 2003). FRI can activate the expression of *FLC*, which inhibits flowering by repressing transcription of downstream genes in the floral pathway (Michaels and Amasino, 1999). It is, moreover, possible that FLC is involved in WC regulation through quenching of the downstream flowering integrator

SUPPRESSOR OF OVEREXPRESSION OF CO 1 (*SOC1*) to control stomatal opening (Kimura et al., 2015; Loudet et al., 2003). These considerations suggest that Got-7, Sha, Tamm-2, and Ts-1 (early flowering accessions) mobilize drought-avoidance mechanisms by accelerating their metabolic processes to finish their life cycle with limited biomass accumulation.

Another way to circumvent sensitivity to drought is for plants to stay small, as their high water use efficiency (WUE) renders smaller specimens more tolerant to the stress factor (Blum, 2005). In the present study, five accessions characterized by low biomass were investigated, Lov-5, RRS-10, RRS-7, Tamm-2, and Ts-1; of these, however, only Lov-5 proved drought-tolerant. Our observation of different responses of *Arabidopsis* accessions to drought stress indicates a variation in plant adaptation to extreme environments.

Role of drought-responsive genes in plant stress mechanisms triggered by consecutive Pieris and drought treatments

In our study, expression of all investigated genes (except *RD29b*) was affected by the interaction between plant response mechanisms triggered by *Pieris* herbivory and those caused by the subsequent drought stress, with both the ABA-dependent and the ABA-independent signaling pathways involved. The cross-talk between the aforementioned stress response cascades was described by Nakashima et al. (2014).

ABA1 is involved in ABA biosynthesis and is down-regulated by JA (Anderson et al., 2004; Xiong and Zhu, 2003). Enhanced *ABA1* expression under drought simulating conditions observed in this study points to increased accumulation of the phytohormone in response to the stress factor (Figure 5 a). In contrast, reduced transcriptional activity of *ABA1* detected under combinatorial stress conditions indicates a negative interaction between the *Pieris*-induced JA signaling pathway and the drought-activated ABA-dependent response mechanism. The aforementioned results proved our experimental design sufficient for the study of the interaction between *Pieris*- and drought-triggered stress responses *in planta*.

MYB2 and MYC2 are transcription factors acting as positive regulators of *Pieris* and drought stress responses, thus playing an important role in the cross-talk between ABA and JA signaling trails (Abe et al., 2003; Anderson et al., 2004; Harb et al., 2010). Herein, *Pieris* pre-treatment enhanced *MYB2* expression in plants subsequently exposed to drought. This finding is in accord with previously reported results showing that *MYB2* can be respectively up- and down-regulated by two upstream transcription factors, WRKY18 and WRKY40 (Shang et al., 2010), positively responding to JA (Xu et al., 2006). Further, opposite effects of *Pieris* pre-treatment on MYC2 gene expression were observed between the investigated accessions. Both MYB2 and MYC2 bind to the promoter region of *RD22* activating its transcription (Abe et al., 2003). In our study, exposure to *Pieris* larvae resulted in *RD22* up-regulation in most accessions upon subsequent drought simulation, when compared to the single drought treatment. This finding exemplifies a synergetic effect of *Pieris* pre-treatment and drought simulation, as *RD22* was repressed in plants exposed to the herbivore alone.

Enhanced expression of *DREB2a* under a 24-hour dehydration treatment (relative to control) was reported previously (Yamaguchi-Shinozaki and Shinozaki, 1994). Herein, however, the gene was down-regulated in most accessions after six days of drought simulation. As the increased severity of drought stress is positively associated with the concentration of ABA in plants (Kim et al., 2012), the observed reduction in *DREB2a* transcriptional activity could be due to its sensitivity to the phytohormone levels. To confirm this supposition, measurements of ABA concentration in plants on consecutive days of drought treatment are necessary. Moreover, a positive influence of *Pieris* herbivory on *DREB2a* expression under subsequent drought conditions was observed, when compared to the single drought treatment. The effect could be due to the interaction between salicylic acid (SA), JA, and ABA activity in Arabidopsis plants. According to Yoshioka and Shinozaki (2009), the involvement of *DREB2a* in the cross-talk between plant hormones is relevant to the interaction of SA and ABA rather than that between ABA and JA. Thus, it is possible that the effect of *Pieris* pre-treatment on DREB2a gene expression is not JA- but SA-dependent.

Expression of *RD29b* is regulated by several transcription factors, encoded by drought-responsive genes, such as ABF2 (Uno et al., 2000), DREB2a (Qin et al., 2008a), and ANAC019 (that can be further modulated by MYB2 and MYC2 (Bu et al., 2008; Hickman et al., 2013; Jensen et al., 2010). The former TF can interact with ABA-responsive elements and plays a positive role in drought feedback (Abe et al., 2003). In this study, we expected *Pieris* pre-treatment to lead to up-regulation of *ANAC019* and, thus, exert an effect on *RD29b* expression under sequential drought stress conditions. However, no significant differences in *RD29b* transcriptional activity under combinatorial stress (relative to single drought treatment) were observed between the investigated accessions (except Tsu-0). This could be due to the complex regulation of *RD29b* expression in response to drought. For instance, besides ANAC019, ABF2, and DREB2a, positive regulators of *RD29b* whose gene expression increases under drought conditions, transcription of the gene in question can be further increased by ETHYLENE INSENSITIVE 2 (EIN2) and SA- AND ABA- DOWNREGULATED ZINC FINGER GENE (SAZ) whose levels decrease under drought treatment (Jiang et al., 2007; Wang et al., 2007).

Furthermore, enhanced expression of *P5CS1*, pointing to increased proline biosynthesis, was observed in all investigated accessions upon drought simulating treatment. The promoter region of *P5CS1* contains a drought-responsive element (DRE) which can be bound by the JA-responsive protein ETHYLENE RESPONSE FACTOR 1 (ERF1) (Anderson et al., 2004), with the latter negatively regulated by MYC2, VSP2, and ABA treatment (Anderson et al., 2004; Dombrecht et al., 2007). The factor can boost *P5CS1* expression, as was found in *35S:ERF1* plants (Cheng et al., 2013). Thus, the subsequently observed reduced expression of *P5CS1* under combinatorial stress conditions could be due to the antagonistic interaction between the down-regulation of *ERF1* and increased proline accumulation characteristic of drought exposure.

Based on the one described by Shinozaki and Yamaguchi-Shinozaki (2007), we hereby propose a model of gene regulatory networks relevant to the consecutive *Pieris* and drought stress responses in plants (Figure 6). Our study focused on measurement and assessment of the effect of *Pieris* herbivory on expression levels of known drought-responsive genes upon sequential drought

exposure; we, however, payed less attention to *Pieris*-responsive genes. Some of the latter, such as *ERF1*, *ANAC019*, and *VSP2*, can be regulated by both JA and ABA. Thus, it would be of further interest to determine the effect of *Pieris* pre-treatment on transcriptional activity of the three genes in response to sequential drought simulation. No effect of the interaction between *Pieris* and drought stress triggers was observed in case of *RD29b* only. Our hypothesis as to the cause of this outcome pertains to the aforementioned complexity of its upstream regulation. For instance, since EIN2 and SAZ were identified as involved in down-regulation of *RD29b* in response to drought stress, additional evaluation of expression levels of their encoding genes is necessary. Lastly, to comprehensively delineate the gene regulatory network triggered in response to the combinational stress *in planta*, full transcriptome analysis is called for.
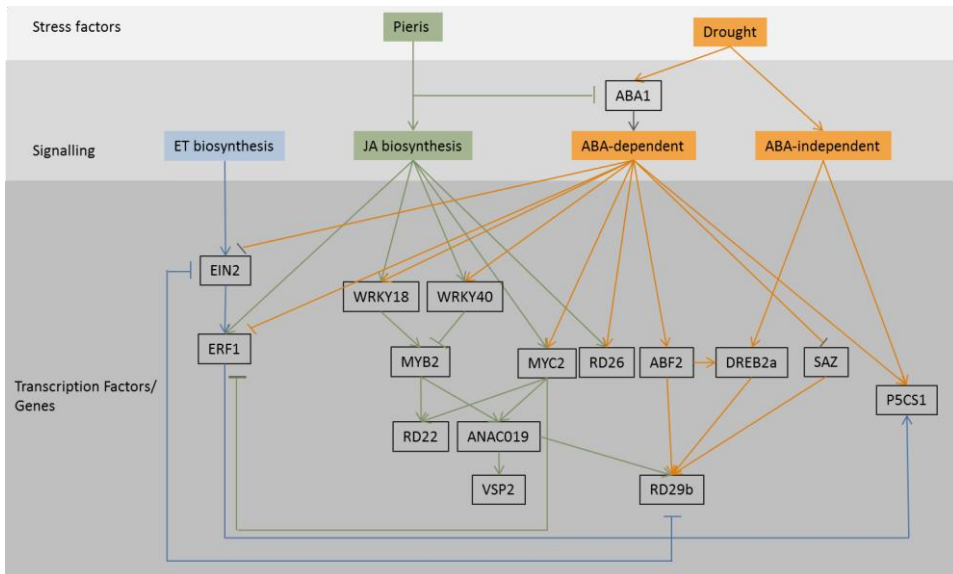
Figure 6. Model of the gene regulatory networks relevant to the consecutive *Pieris* and drought stress responses in plants, involving *ABA1, MYB2, MYC2, RD22, RD26, ABF2, DREB2a, RD29B, P5CS1, WRKY18, WRKY 40, ANAC019, VSP2, EIN2,* and *ERF1*. Signaling pathways triggered by drought are indicated in orange, while those implicated in the response mechanism to *Pieris rapae* herbivory are marked in green. The figure was updated based on Shinozaki and Yamaguchi-Shinozaki (2007).

No divergent expression profiles of the investigated drought-responsive genes among *Arabidopsis* accessions

Our findings on differential gene expression are in line with previous results of a study comparing seven *Arabidopsis* accessions in response to SA treatment that identified a number of genes with multiple alleles characterized by diversified transcriptional activity (Kliebenstein et al., 2006). Similarly, contrasting expression of abiotic stress-responsive genes (including *RD22*) was observed in Col-0, when compared with C24 (Miller et al., 2015). Moreover, diverse expression levels in response to drought and cold stress were detected upon comparative analysis of ten *Arabidopsis* accessions (including Bur-0, Col-0, and Ts-1; Lasky et al., 2014). Further, while similar expression of *REDUCED CHLOROPLAST COVERAGE 3* (*REC3*) was reported for the three aforementioned accessions subjected to drought (relative to control), *ETHYLENE RESPONSE FACTOR022* (*ERF022*) showed contrasting transcriptional levels in Ts-1 as compared to Bur-0 and Col-0 (Lasky et al., 2014). We, however, did not observe divergent expression profiles of the investigated drought-responsive genes in Ts-1 relative to Bur-0 and/or Col-0, except in case of *RD26*.

No correlation between phenotypic responses and variation in gene expression

In the present study, no correlation between the three investigated traits and the nine drought-responsive genes was found (Supplementary Table 1). In addition, the relevant expression level quotients could not be correlated with the treatment effects on growth in *Arabidopsis* natural accessions. Hence, the obtained results suggest that the representatives of the investigated accessions employ not one, but different strategies to overcome drought stress, or at least not one involving the genes whose expression was evaluated. Considering the substantial variation in both phenotypes and gene expression levels among accessions upon comparison of their responses to single and combinatorial stress treatments, their whole-transcriptome analysis would be of great interest. Since we selected accessions that are genetically divergent, the observed large variation among them was not unexpected.

Thus, the limited transcriptomic information at our disposal prevented us from effective determination of the relationship between phenotype variation and genetic responses among accessions. For instance, correlation analysis of the expression data of more than 400 *Pseudomonas syringae pv tomato* DC3000 (Pst) type III effector protein AvrRpt2-responsive genes showed that 49% of variation of the phenotypic responses to Pst infection could be explained by genetic differences among accessions (Van Poecke et al., 2007). Therefore, full transcriptome analysis of the accessions of interest would be useful in providing adequate genetic information corresponding to stress conditions (relative to control). Another hypothesis aiming at explanation of the poor correlation is that the simple Pearson's test is not sufficient to determine the relationship between complicated phenotype stress responses and relevant genotype data. Application of powerful analytical methods, such as algorithm locally linear embedding graph generator (LEGG) (Van Poecke et al., 2007) and cluster analysis, could help to effectively tackle these obstacles.

Conclusion

In this paper, we have evaluated phenotypic responses and gene expression in *Arabidopsis* upon exposure to *Pieris* herbivory, drought, and combinatorial stress conditions. The observed responses of the investigated accessions to drought simulation were divergent, indicating a variation in plant adaptation to extreme environments. We identified the phenotypic trait of water content (WC) as a parameter allowing effective estimation of the effects of the interaction between *Pieris* and drought stress responses. No significant correlation between phenotypic responses and gene expression was found, suggesting that 1) whole transcriptomes of the investigated accessions ascertaining sufficient genetic information and 2) application of superior correlation analysis methods, such as LEGG, will be needed to delineate the relevant interactions. Different effects of *Pieris* pre-treatment on drought-responsive gene expression levels upon sequential drought simulation were observed, with the exception of *RD29b*. Based on the obtained results, we proposed an improved model of gene regulatory networks triggered in response to consecutive *Pieris* and drought stress factors. However, to afford a comprehensive overview of the network in question, appropriate full transcriptome analyses are necessary.

Acknowledgement

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CHAPTER 8

General discussion

Pingping Huang

This thesis deals with the responses of *Arabidopsis thaliana* (Arabidopsis) plants to sequential combinations of biotic and drought stress triggers, with the identification of relevant regulatory genes as its main objective. *Pieris rapae* and *Botrytis cinerea* were employed as biotic stress activators. Following the biological treatment(s), the investigated plants were subjected to abiotic stress conditions (drought). Both *Pieris* and *Botrytis* trigger expression of defense-responsive genes from the jasmonate (JA) signalling pathway; however, the respective defense mechanisms mostly follow alternative courses. The *Pieris* response is mediated mainly through the MYC pathway branch, whereas *Botrytis* infection, activates the ethylene response factor (ERF) branch (Dombrecht et al., 2007). The two sub-networks can interact with each other antagonistically. Hence, some JA-ET-responsive genes were expected to be shared in both investigated biotic stress mechanisms but others might show opposite effects. In addition, the MYC and ERF branches engage, respectively, in positive and negative interactions with the drought-induced abscisic acid (ABA) signalling pathway (Dombrecht et al., 2007). Therefore, the hypothesis that the JA-ET-responsive genes and therefore also *Pieris* and *Botrytis* pre-treatments exert contrasting influence on the ABA-mediated drought stress response was addressed. Transcriptiome analysis identifed 31 genes, which exhibited opposite directions of expression response when comparing the two combinatorial stresses, and indeed found differential regulation of several plant hormone-related genes such as *ERF4*, *ERF5, ERF6* and *ERF104* (Chapter 6).

GWA-mapping and RNA-seq

Two genetic approaches were used to study the effects of sequential stress combinations in plants in comparison with single factor responses and to identify genes relevant especially to the combinatorial stress mechanisms.

RNA sequencing (RNA-seq) using the Next Generation Sequencing approach is a straightforward technique affording identification of genes showing differential transcription levels under various conditions. Next to determining levels of transcription, RNA-seq will reveal alternative splicing (AS) variants influenced by diverse environments. This can be interesting, as for instance analysis of AS variants of the *ZINC-INDUCED FACILITATOR1* (*ZIFL1*) gene

showed that two of its isoforms (*ZIFL1.1* and *ZIFL1.3*) exhibited distinct localization and played different roles, either to modulate root polar auxin transport by localizing to the root cell tonoplasts (*ZIFL1.1*), or to affect drought tolerance when expressed in stomatal guard cells and localised to the plasma membrane (*ZIFL1.3*) (Remy et al., 2013). In the response to combinatorial stresses, AS variants of the auxin efflux carrier *PIN7* were found. The AS variant *PIN7-1* exhibited enhanced expression compared to *PIN7-2* under the stress combination *Pieris* and subsequent drought in comparison to the single drought treatment, suggesting that this particular isoform is more relevant in the response to the combinatorial stress than the other isoform (Chapter 6).

Genome-wide association (GWA) analysis identifies genes for which there is genetic variation that leads to differences in the genotype's response to treatments while RNA–seq, using a single genotype, identifies genes that respond in expression to the treatments. While gene expression differences are very interesting to identify genes that play a role in environmental responses, it will not reveal anything on the genetic variation for environmental response, that can be illustrative for breeders to focus on when trying to improve crop robustness and tolerance to environmental fluctuations. However, some overlap was found between the genes identified in both approaches. For instance, in case of the effect of *Pieris* and subsequent drought stress response, one identified quantitative trait locus (QTL) overlapped with differential expression of two genes, *PRX34* and *AT3G49350* (Chapters 3 & 6), while for *Botrytis* infection, a similar overlap was seen for gene *AT3G02910* (Chapters 3 & 6).

The GWA-mapping approach affords discerning connections between DNA sequence polymorphisms and their phenotypic consequences. Genetic variation can either exert its impact through single-nucleotide polymorphisms (SNPs) associated with gene transcription differences or those affecting protein functions. For instance, Arabidopsis natural accessions carrying a single SNP at the *HIGH-AFFINITY K+ TRANSPORTER 1;1* (*AtHKT1;1*) gene showed an aberrant expression of the gene leading to a difference in leaf Na^+ accumulation (Baxter et al., 2010); and a single SNP substitution in *CRY2* led to an amino acid substitution affecting CRY2 protein stability under short photoperiods, thus leading to early flowering in *Arabidopsis thaliana* (El-Assal et al., 2001).

GWA-mapping has been used in many studies and proved successful in detecting SNPs causal to their corresponding phenotypic traits (Atwell et al., 2010; Bac-Molenaar et al., 2016; Meijon et al., 2014). The challenges of the technique were described by Weigel (2012) and include 1) false positives: the identified SNPs may not be the causal ones or the association may incorrectly identify SNPs as associated, 2) population structure: association may be found due to close relationships between genotypes, rather than shared SNPs based on short-range linkage disequilibrium, and 3) allelic heterogeneity: different SNPs at the same locus lead to similar phenotypes, but they will not be detected through GWA analysis (Barboza et al., 2013).

Using a Bonferroni-corrected threshold ($-\log(p)=6.5$) to reduce false positives is advised by Atwell et al (2010). However, the Bonferroni corrected threshold is extremely conservative and often not convenient for growth related traits such as FW, DW, WC, and PLA because these traits are genetically influenced by a multitude of small-effect QTLs. An arbitrary threshold of $-\log(p)=4$ as described by van Rooijen et al. (2015) was used to assign associated SNPs in Chapter 3 and Chapter 4, and a number of SNPs exhibited genetic variance in response to stresses were discovered, with associated genes for which mutants showed a relevant aberrant phenotype. Although the number of false positive associated SNPs will be larger than when the Bonferroni threshold is used, there will be sufficient SNPs found that are indeed associated with the phenotype. Especially for complex polygenic traits this seems to be a practically viable approach for GWAs analysis.

In many GWAs studies, statistical approaches were applied to correct population structure (Yu et al., 2006; Atwell et al., 2010; Brachi et al., 2010). However, correcting population structure can increase the frequency of false negatives and led to miss the causative genetic markers (Brachi et al., 2010). GWA analysis in Chapter 2, 3, and 4, used the 350 accessions, which eliminated population structure based on testing results of 149 SNPs for genetic diversity (Li et al., 2010; Platt et al., 2010). In combination with mutant test, I confirmed the biological function of some SNPs associated candidate genes under different conditions.

Several studies combined association mapping with linkage mapping, which helped substantially in identifying causal genetic variation eg. in case of flowering time (Brachi et al., 2010), resistance to *Hyaloperonospora arabidopsidis ex parasitica (Hpa)* (Nemri et al., 2010), endoreduplication (Sterken et al., 2012), or shoot regeneration (Motte et al., 2014). Thus, to confirm the loci associated with combinatorial stress response and to reveal false negatives, follow-up studies of QTL analysis using biparental populations might be useful. Although in my studies, the allelic effects of some genes on combinatorial stress responses could be highlighted, the physiological function of many allelic variants in the response to stress conditions remains to be discovered. Future experiments, such as gene expression studies aiming at determining the effects of SNP variation or different allelic groups on gene expression or complementation tests to examine the gain or loss of function effects of the SNPs or alternative types of alleles under diverse stress conditions, should be performed.

Using different trait values was also recommend for GWA analysis. For instance, residuals that represent the variations of the accessions (genotype) in response to stresses (environment) (Filiault and Maloof, 2012) are suitable for univariate GWA analysis (Chapter 3 & 4). Principal component (PC) values are suitable for multi-trait multi-environment analysis (Chapter 2).

GWA analysis identifies small-effects QTLs

Moderate to high heritability was found for the measured traits in the single and combinatorial stress conditions, indicating the variation among accessions in their responses to stresses due to their genetic variation (Chapter 3 & 4). Normally, moderate to high heritability offers good prospects to identify QTLs affecting the trait. In my study, each identified individual QTL explained a small part of the genetic variance (on average 4-6%). This is not a surprising because the traits we measured (FW, DW, WC and PLA) are very general growth traits, which are likely to be genetically affected by allelic variation at many loci. Few loci with large genetic variance were not able to be identified by GWAs analysis approach (Brachi et al; 2010). The large number of small-effect QTLs could make it very difficult to identify these QTLs (Kooke et al., 2016). Thus, the

causal variants could be due either to a small number of genes, with relatively large effects; or due to many genes, with relatively small effects. Analysis of a simple trait often results in less QTLs, but with larger effect sizes, than for a complex trait (Louthan and Kay, 2011).

Different models in GWA-mapping

In the present study, the interactions of *Arabidopsis* accessions with an array of enviromental conditions were tested. Various phenotypic parameters were taken into account, and different models of GWA-mapping were applied to determine the effects of SNP variants on multiple stress responses. Models such as multi-trait QTL mixed models (MTMMs) (Chapter 2), Multi-environment (ME) analysis, , and univriate analysis (Chapter 3 & 4). Residuals resulting from the regression of the response to the combinaitonal stress on the response to single stress were used as trait values (Chapter 3 & 4).

A recent study, appying QTL analysis to investigate yield-related traits in pepper, showed a partial overlap of the identified significant QTLs when comparing MTME, ME, multi-trait (MT), and univariate single environment (SE) mapping approaches (Alimi et al., 2013). In my study, the comparison of ME GWA-mapping with univariate analysis resulted in identification of eight shared SNPs with QTL by environment interaction (Q×E) effects of subsequent drought for those *Pieris* pre-challenged plants and four common SNPs with Q×E effects in subsequent drought stress response for those infected with *Botrytis* (Chapter 3). This is not a surprise because the univriate analysis used residuals which also represent Q×E effects. The 12 SNPs that were identified by both approaches indicates these could be true causative QTLs. The partly overlap between the univariate and ME approahces was oversation found by El-Soda et al. (2015) as well, where a positive correlation between the −log10 values of the 214k SNPs resulted from two approaches were reported.

Korte et al. (2012) suggest that ME mapping using MTMM is more powerful than the univariate approach as it takes the variation within and between trait(s) into consideration, while the latter affords identification of SNPs associated with main effects only, without accounting for the Q×E interactions. However, as my

study used residuals that represent genotype x environment (G×E) variation for the univariate analysis, it does take at least some of the Q×E into consideration. Several studies reported limited efficacy of the ME GWA-mapping in detection of Q×E effects for strong QTLs (Bac-Molenaar et al., 2015; Korte et al., 2012; Morrison and Linder, 2014). In these studies, more significant SNPs were identified using the univariate method than when the ME mapping was applied. The same was the case in my studies. This does not mean the ME method is the less efficient one, as many of the SNPs identified in the univariate analysis could be false positives (Chapter 3). Thorough follow-up research would be needed to evaluate these results properly, but this is very time-consuming and labor intensive.

Different interactions between biotic and abiotic stress

Abiotic stresses can significantly affect plant responses to biotic strains and vice versa (Atkinson and Urwin, 2012; Appel et al., 2014; Rejeb et al., 2014). The interaction between biotic and abiotic stress responses is often mediated by different plant hormones and the outcome can be synergistic, antagonistic, or neutral (Audenaert et al., 2002; Achuo et al., 2006; Suzuki et al., 2014; Ramegowda and Senthil-Kumar, 2015; Prasch and Sonnewald, 2015). In Chapter 3, 4, 6 and 7, different phenotypic and transcriptomic responses to the single and combinatorial stress were observed. For instance, we found that the biotic pre-treatment promotes the susceptibility of plants to drought stress. For instance, by reducing rosette dry weight (DW) during sequential drought, but increasing the water content (WC) (Chapter 7). Three types of interaction between biotic and abiotic stress were observed. For example, neutral effects were found of the interaction between *Pieris* and osmotic stress on *bzip25* mutants, antagonistic effects were found between *Pieris* and osmotic stress on the *prx34* mutant and between *Botrytis* and osmotic stress in the *rmg1* mutant (Chapter 3), while a synergistic effect was observed between drought and *Botrytis* in the *pal4* mutant (Chapter 4).

Combinatorial stress responses cannot be predicted by comparing single stress responses

Ramegowda and Senthil-Kumar (2015) suggest there has to be a unique response to combinatorial stresses as the responses of plants to blends of triggering factors cannot be predicted based on mechanisms responding to their individual components. For instance, 61% of the changes in gene expression in response to the combinatorial stress could not be predicted from the single stress responses (Rasmussen et al., 2013). Transcriptome analysis of simultaneous effects of drought, heat, and virus infections in *Arabidopsis* identified only 11 genes involved in all single, double, and triple stress treatments (Prasch and Sonnewald, 2013). This is not much, considering Arabidopsis has around 30,000 genes, with often at least half of them expressed at any time point in any organ.

Genes involved in multiple stress responses may not be able to identified under single stress conditions. For instance, the expression of the resistance gene RPS6 was only regulated in case of simultaneous heat, drought, and virus treatment, but not under any of the double or individual stresses (Prasch and Sonnewald, 2013). In my study, when comparing the *Botrytis* and subsequent drought with single drought and *Botrytis* treatments, 4% of DEGs identified as *Botrytis* and subsequent drought responsive genes were not induced by either single *Botrytis* or drought treatments; comparing DEGs from the stress combination of *Pieris* and subsequent drought with single drought and *Pieris* treatments, 3% of DEGs identified as *Pieris* and subsequent drought responsive genes were not induced by either *Pieris* or drought (Chapter 6).

The aim to study combinatorial stress responses is to understand how plants react in a close to natural environment, as a combinatorial stress is more likely to occur in a natural environment than a single stress. Transcriptome analysis identified several combinatorial stress responsive genes that cannot be induced by neither by the biotic nor drought, showing that these combinatorial stress responsive genes could be the potential candidate genes for plant breeding use.

Experimental design

The combinatorial treatment in the GWA-mapping experiments was slightly different from the one used for the RNA-seq analysis, with a 24 h recovery period included in between the first stress condition and the sequential drought in case of the GWA-mapping (Chapter 3, Supplementary Figure 1). As the interaction between *Pieris* and drought and between *Botrytis* and drought on plants could be observed for the expression of *bZIP25 and PRX34* (for *Pieris* & drought*)* and for *Di19 and WRR4* (for *Botrytis* & drought) under combinatorial treatment in comparison to single drought treatment (Chapter 3), I consider the 24 h recovery period still acceptable for observing the interaction between the first challenge and the sequential drought stress response.

The investigated plants differed in recovery speed from the *Botrytis* and *Pieris* treatments. For instance, the RNA-seq analysis reported a much lower number of DEGs in the *Botrytis*-challenged plants following subsequent drought treatment as compared to those subjected to initial *Pieris* herbivory and subsequent drought. This observation could stem from the fast recovery of plants from the exposure to *Botrytis*, with few residual infection-induced signals at the time of the application of drought. In order to get a stronger interaction between *Botrytis* and drought, it will be better to apply a quick induction of drought in plants. Such could be achieved by applying polyethylene glycol (PEG), which will emphasize the osmotic component of drought, or to ensure that moisture evaporates faster from soil eg. by increasing air circulation.

The impact of the first stress treatment on plant response to sequential ones can be strong but depends on the nature of the initial challenge (Chapter 5). While prior herbivory strongly affected *Botrytis* resistance (Coolen, 2016), we identified six times more DEGs under the *Pieris* and subsequent drought than under *Botrytis* and subsequent drought, showing a stronger effect of prior *Pieris* exposure on gene expression than prior *Botrytis* infection (Chapter 6). However, this also could mean that *Pieris* herbivory is more severe than *Botrytis* infection. For instance, Windram et al (2012)) used the same concentration of *Botrytis* spores as in this study, and showed that the first symptom of *Botrytis* infection on leaf can be observed at 20 h after inoculation (HAI) and the expansion of

lesions at 36 HAI. In order to have a stronger effect of *Botrytis* infection, a higher spore concentration (e.g. using spore concentration at $1X10^6$ per ml), increase the number of droplets per leaf, or to inoculate *Botrytis* spores on plants in the late afternoon (e.g. inoculate at 18 hour) (Ingle et al., 2015) could be used.

Conclusions and recommendations

The research described in this thesis provides insights into plant response mechanisms triggered by sequential combinations of biotic and drought stress factors, facilitating our understanding of the intricate network of defense processes *in planta*. A number of candidate genes to combinatorial stress responses were identified by GWA analysis and RNA-seq. The physiological function of some candidate genes in different stress conditions were characterized using T-DNA insertion mutants and gene expression study. However, the physiological function of many allelic variants in stress conditions remain to be discovered. The present study provides an important step towards in-depth understanding of plant responses to the investigated stress factors and highlights the importance of an array of genes, crucial to the underlying defense processes, as targets for breeding by allele mining aimed at ultimate improvement of crop tolerance to relevant stress factor combinations.

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Summary in English

Biotic and abiotic stresses are often occurring either simultaneous or consecutively in the natural environment and the impact of the two types of stresses on plants are often negative. Abiotic stresses can significantly affect plant responses to biotic stresses and vice versa. In the research described in this thesis, *Arabidopsis thaliana* was used as a model to study plant responses to different sequential combinations of biotic factors (infection with *Botrytis cinerea* or herbivory by *Pieris rapae*) and drought. Genome-wide association (GWA) mapping and RNA sequencing (RNA-seq) approaches were used to identify combinatorial stress responsive genes. The main objective of the research described in this thesis is to identify genes that contribute to tolerance to the aforementioned sequential stress combinations.

In Chapter 2, I describe multi-trait QTL mixed models (MTMMs) GWA-mapping with traits from 11 single environmental factors and several of their consecutive combinations. Thirty significant SNPs that associated with genes responding to multiple stresses were identified and the average QTL-effect-sizes were found to be stronger for combinatorial stresses than single stresses. This is showing that plants appear to deploy broad-spectrum defensive mechanisms influencing multiple traits in response to combined stresses. Association analyses were performed to identify QTLs contributing to contrasting and similar responses to biotic versus abiotic stresses and belowground versus aboveground stresses. Candidate genes such as *RMG1* and *WRKY38* were identified in response to multiple environments and found to exhibit contrasting responses to biotic and abiotic stress. The *rmg1* mutant was more tolerant to *Pieris* herbivory and more susceptible to osmotic and salt stresses compared to wild-type plants.

In Chapter 3, polyethylene glycol (PEG) was used to mimic drought. Univariate and ME GWA-mapping were performed with phenotypic traits such as project rosette leaf area (PLA), rosette dry weight (DW) or fresh weight (FW), and water content (WC) for *Pieris* + PEG and *Botrytis* + PEG responses. A number of SNPs were identified by both methods, of which eight SNPs were

identified for the consecutive *Pieris* and PEG responses. The SNPs were associated to genes with IDs At2g36550/At2g36560, and AT3G45910; four common SNPs were found for the response to *Botrytis* and PEG, the SNPs associated with genes with IDs At1g48670, At1g56280, At2g13690 and a larger region comprising At5g48120 (*DI19*) – At5g48180. Allelic effects of genes At2g36560 and *DI19* in response to combinatorial stress were found. Several SNPs were found to be in linkage disequilibrium with known stress-responsive genes. New roles were found for some of these known stress-responsive genes, e.g. for *PRX34* and *bZIP25* in their responses to the combined *Pieris* and PEG treatment, and *RMG1* to *Botrytis* and PEG. We observed antagonistic interaction between the two biotic stress and drought, and a negative effect of the two biotic pre-treatments on subsequent PEG response.

Chapter 4 focused on the consecutive drought and *Botrytis* infection responses. Univariate GWA-mapping was performed on the residuals of DW and identified the effect of a number of significant SNPs that were associated with the DW plasticity under stresses. The biological functions of six candidate genes were validated under single drought or *Botrytis* stress and the combinatorial stress using T-DNA insertion mutants and the transcriptome data of the most extreme eight accessions. An allelic effect was observed on *MYB46* gene expression under combinatorial stress compared to the single *Botrytis* treatment. The role of *MYB46, PAL4*, and *HSFA1b* in response to the interaction of drought and *Botrytis* infection was highlighted. Antagonistic interaction between drought and *Botrytis* infection were observed, as well as a negative effect of drought pre-treatment on subsequent response to *Botrytis* infection.

Chapter 5 reports on whole-transcriptome profile analysis of Arabidopsis Col-0 exposed to three single stress triggers (*Pieris*, *Botrytis*, drought) and six consecutive combinations. A set of 394 genes were differentially expressed during all three stress conditions, though often in opposite directions. A significant effect of the first-stress-signatures could be identified in the sequential stress profiles, and the effect was often related to responses to phytohormones, strengthening the notion that hormones are general modulators of interactions between different types of stress.

Chapter 6 focuses on the analysis of the transcriptome data on consecutive biotic (*Pieris* and *Botrytis*) and drought stress. Herbivory by *Pieris* has a much higher impact on the transcriptome during sequential drought and rehydration, than the infection with *Botrytis*. A set of 42 common genes were found to be responsive to both *Pieris* + drought and *Botrytis* + drought stresses and opposite effects of the two biotic pre-treatments were observed on ethylene-related genes *ERF5, ERF6, ERF104* and drought responsive gene *P5CS1*. Several genes were only differentially expressed under combinatorial stress, showing the unique responses to the consecutive biotic and drought stress. Genes with alternative splicing (AS) variants were identified in different stress conditions, but none of them were found among the differentially expressed genes, showing a separate regulation of gene expression and AS in response to stresses. Phenotypic differences found for the *mdar3* and *pin7* mutants when compared to wild type suggest that the *MDAR3* and *PIN7* genes play an important role in the response to the combinatorial stress.

Chapter 7 describes the investigations of the responses of Arabidopsis accessions to the consecutive exposure to *Pieris* and drought stress. WC is a suitable parameter for evaluation of these interactions. To observe the effect of *Pieris* pre-treatment on drought responsive genes in response to drought stress, the expression levels of nine drought-responsive genes were measured under different treatments but this showed no correlation with the observed phenotypic variation. In the end, a mode of gene regulatory network is proposed that is sufficient to explain the effect of *Pieris* pre-treatment on the nine drought responsive genes in response to drought stress.

The study presented here provides an important step towards an in-depth understanding of plant responses to the investigated stress factors and highlights the importance of an array of genes, crucial to the underlying defense processes, as targets for breeding by allele mining, ultimately aimed at improvement of crop tolerance to frequent combinations of stress factors.

中文摘要

在自然环境下，生物胁迫和非生物胁迫经常同时发生或先后发生，而且它们常常会对植物生长带来不利影响。非生物胁迫可以显著影响植物对生物胁迫的响应，其显著程度受到由于胁迫时间、种类、和严重程度的影响；反之亦然。本论文旨在研究生物胁迫（菜青虫，或灰霉病）与非生物胁迫（干旱）先后处理时对拟南芥生长的影响，所用的主要技术方法为用全基因组关联分析（GWAS）来寻找相关抗性基因位点，以及用 RNA 测序（RNA sequencing）方法来寻找相关抗性基因。研究主要目标是是挖掘双重胁迫下的植物抗性基因。

第二章中，我们用了用多性状数量性状位点（QTL）分析模型（MTMMs）分析了 11 个单胁迫环境和六个双胁迫环境，找出了响应最显著的 30 个单核苷酸多态性（SNPs）。我们发现在双重胁迫下的 QTL 效应值大于单一胁迫情况下的值。结果表明，在双重胁迫的情况下，植物启动了广谱抵御机制，导致很多表型性状发生了改变。关联分析鉴定出一些 QTL 位点，他们在生物/非生物胁迫，或地上/地下胁迫条件下时有相似的或者截然相反的反应。对于生物胁迫和非生物胁迫，双重胁迫抗性基因例如 *RMG1* 和 *WRKY38* 则展现出了既然不同的相应。

在第三章中，聚乙二醇 （PEG）-8000 则被用作模拟干旱胁迫环境。表型性状例如莲座叶面积（PLA） ，莲座干重（DW），鲜重（FW）和水含量（WC）被用于与单变量和多环境 GWAs 两种方法分析用。八个相应菜青虫和 PEG 的双重胁迫基因和四个相应灰霉病和 PEG 的双重胁迫基因所描述的两种 GWAs 分析方法所鉴定出来。我们观察到了基因 At2g36560 and *DI19* 的等位基因效应对于双重胁迫的影响。一些备选基因的生理作用已经得到 T-DNA 突变体证明。*prx34* 突变体证明了该基因对菜青虫啃食或 PEG 处理下的植物响应起正向作用，但是对双重胁迫起负面效应；d*i19* 突变体证明了改基因对灰霉病菌和 PEG 胁迫单独处理条件下的植物响应有正面作用，但是对双重胁迫没有效应；*arf4* 突变体证明了该基因对于灰霉菌和 PEG 单独处理条件下的植物没有效应，但是对双重胁迫有正面效应。

第四章侧重于连续的干旱和灰霉菌胁迫反应。我们使用了莲座干重（DW）残差和单变量 GWAs 分析方法，找到了一系列在干旱和灰霉菌先后处理情况下情况下影响表型的重要 SNPs. 我们观察到了 *MYB46* 基因对于干旱和灰霉菌的的等位基因效应。通过测试备选基因 的 T-DNA 突变体，*MYB46*，*PAL4* 和 *HSFA1b* 对应连续的干旱和灰霉病感染有相应。我们 发现干旱和灰霉菌的交感作用是相反的，并且干旱预处理对随后的灰霉菌感染有负面影响。

第五章报告了拟南芥对于连续的生物胁迫和非生物胁迫反应的的 RNA 测序的结果。 拟 南芥分别被菜青虫，灰霉菌，和干旱三种单个胁迫和六个双胁迫所处理过。一共有 394 个基 因对这三种单个胁迫都有反应。预处理胁迫对于随后的胁迫有着明显的影响，而且这种影响 是和植物荷尔蒙有关联的。

第六章侧重于分析针对于菜青虫/灰霉菌和随后的干旱胁迫的 RNA 测序结果。 菜青虫对 于随后干旱胁迫的影响力大于灰霉菌对于随之的干旱胁迫。一共有 42 个基因响应于菜青虫和 干旱双胁迫以及灰霉菌和干旱双胁迫。乙烯相关的基因 *ERF5, ERF6, ERF104* 和干旱胁迫响 应基因 *P5CS1* 展现出了相反的菜青虫和灰霉菌对于随之的干旱胁迫效应。随后我们鉴定出了 在胁迫环境下展现出了选择性剪切（AS）的基因，并且发现这些基因并在胁迫环境下并没有 产生基因表达量的变化。着说明了选择性剪切和基因表达的变化是对于胁迫环境的两种独 立的相应。突变体例如 *mdar3* 和 *pin7* 证明了这两个基因对于菜青虫和干旱双胁迫环境响应 起到了重要作用。

第七章我们调查了拟南芥对于连续的菜青虫和干旱胁迫的反应。表型数据水含量（WC） 是一个非常适合用来评价这两种胁迫的相互作用的参数。我们分别测试了九个干旱抗性基因 在菜青虫和干旱的单独胁迫情况下和双胁迫情况下的的基因表达量，并没有发现在胁迫环境 下的基因表达量的差异和拟南芥的表型有着显著联系。最后，基于这九个干旱基因在胁迫环 境下的不同表达，我们提出了一个应答菜青虫和干旱双胁迫的基因调控网络的模型。

本文研究提供了对于理解植物对所描述的胁迫环境的响应迈出了重要一步，突出了双重胁迫的后选抗性基因并描述了潜在的生物防御过程，最终目的在于改善作物对于生物和非生物胁迫双重胁迫的耐受性。

Acknowledgements

The accomplishment of this research involved the support of many people, in the academic formation and social life, in big and small ways. For this, I would like to express my sincere gratitude.

First of all, I would like to thank my promoter **Prof. Dr. Maarten Koornneef**, co-promotor **Dr. Mark Aarts**, and **Prof. Dr. Bas Zwaan** who interviewed me and gave me the opportunity to work on this project. Dear Maarten, thank you very much for your quick response in reviewing all my thesis chapters with detailed comments. I appreciate your patience in answering my questions and I'm grateful for your encouragement to pursue my Ph.D. study. Dear Mark, I admire your intelligence and I respect your belief in fundamental science. I liked the freedom you gave me within the research and I appreciate your advice of attending important scientific events. I hope you can find a balance between work and stress load and I look forward to see more high quality articles from you in future. Dear Bas, I appreciate your guidance and your advice on my project very much when Mark wasn't available. You were always there when I needed help. Especially I want to thank you for getting *Pieris rapae* larvae for me from outside the Netherlands.

My Ph.D. project is part of the big project "Mining natural genetic variation of combinatorial stress responses of Arabidopsis to identify new tolerance pathways for biotic and abiotic stress" (STW grant, project number: STW10988) and belongs to the program "Learning from Nature" (website http://www.stw.nl/nl/programmas/learning-nature-protect-crops-lfn). Different research groups from different universities were involved in the program, including ambitious Ph.D. students, post-docs, and professionals. **Dr. Nelson Davila** and **Silvia Coolen**, you are not only my collaborators within the combinatorial stress responses project but also are real friends in life. I wish all the best to you and let's keep in touch. Together with other "co-team" members **Dr. Manus Thoen**, **Dr. Karen Kloth**, **Dr. Willem Kruijer**, and **Dr. Joost van Heerwaarden**, we accomplished our MTMM manuscript. Furthermore I would

scientist as well as a beloved friend. I admire your indomitable spirit and your open-mindedness. I learned a lot from you during your stay. I wish you all the best with your work and your family. I want to thank **Dr. Christian Bachem** and **Dr. Bjorn Kloosterman**, I wouldn't have thought about applying for a Ph.D. position without your inspiration and your encouragement.

Furthermore I want to thank my beloved friends **Yanchun Zheng**, **Sakura Mao**, and **Lianlan Xu**. Thank you for the trust, understanding, and support in the past 16 years. Thank you for traveling from China to the Netherlands to prepare my wedding when I was totally frustrated at work. I love you always. To my dear friends who I met in the Netherlands, thank you for the joy and thank you for being there when I needed you, especially to **Xianwen Ji**, **Cheng Liu**, **Tom van Stein**, **Hanna van Stein**, **Yenthe van Stein, Xiaoqian Shi**, **Tim Kunne**, **Izan Ramlee**, **Ameem Yahaya**, **Aryan Yahaya**, **Freddy Yeo**, **Marian Oortwijn, Bart van Tuijl**, **Weicong Qi**, **Xi Cheng**, and **Jihua Cheng.**

To my husband **Michel Arts**, it is my destiny to meet you and to marry you. You played the role of bringing more balance in my life. I can't name any specific reasons to answer the question why I love you so much, I just know that I can't picture my life without you. Veel dank aan mijn schoonouders **Ben Arts** and **Wil Arts-de Graaf**, dank jullie voor jullie zorg en ik ben blij met jullie als schoonouders.

Finally, I would like to express my gratitude to my parents **He Huang** and **Lijun Zhao**. Especially to my father, I wouldn't have made it so far without his support. 我为有您这样的父亲而骄傲。

About the author

Pingping Huang was born on 25th April, 1984 in Chuzhou, P.R.China. She studied a major of Biology during high school at Shenzhen University Normal College Middle School and continued with a bachelor program of Biotechnology at the China Agriculture University (CAU) in P.R.China. During her study at CAU, she worked as a project assistant at the university student council. Subsequently, she continued her study at the Hogeschool van Arnhem en Nijmegen and the Hogeschool Van Hall Larenstein, and obtained the Bachelor degree in International Horticulture and Marketing. In the year 2008, she continued her master study in Plant Science with a specialization in Plant Breeding and Genetic Resources at the Wageningen University (WUR) and there she obtained her MSc degree. During her MSc study, she worked on her master thesis "Identifying and functional characterization of point mutations in sucrose synthase 2 in tomato" with Dr. Christian Bachem and Dr. Antoine Gady at the Laboratory of Plant Breeding (WUR). Additionally she worked on her master minor thesis of "fine mapping of Beta-carotene QTLs in tomato fruit" with Dr. Jim Giovannoni at the Boyce Thompson Institute in the U.S.A. After she completed her master study, shortly she was a guest worker as a technician at the Laboratory of Plant Breeding (WUR). Meanwhile, she applied for and accepted the position of Ph.D. in a 4-year project on "Mining natural genetic variation of combinatorial stress responses of Arabidopsis to identify new tolerance pathways for biotic and abiotic stress" that was funded by the Dutch Technology Foundation (STW) and subsequently became a Ph.D. student at the Laboratory of Genetics. This thesis presents the outcome of her Ph.D. research work.

List of publications

Published or Accepted

1. Manus P.M. Thoen*, Nelson H. Davila Olivas*, Karen J. Kloth*, Silvia Coolen*, **Ping-Ping Huang*,** Mark G.M. Aarts, Johanna A. Bac-Molenaar, Jaap Bakker, Harro J. Bouwmeester, Colette Broekgaarden, Johan Bucher, Jacqueline Busscher-Lange, Xi Cheng, Emilie F. Fradin, Maarten A. Jongsma, Magdalena M. Julkowska, Joost J.B. Keurentjes, Wilco Ligterink, Corné M.J. Pieterse, Carolien Ruyter-Spira, Geert Smant, Christa Testerink, Björn Usadel, Joop. J.A. van Loon, Johan A. van Pelt, Casper C. van Schaik, Saskia C.M. van Wees, Richard G.F. Visser, Roeland Voorrips, Ben Vosman, Dick Vreugdenhil, Sonja Warmerdam, Gerrie L. Wiegers, Joost van Heerwaarden, Willem Kruijer, Fred A. van Eeuwijk & Marcel Dicke. (2016) *Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping*. New Phytologist (Accepted)

2. Silvia Coolen*, Silvia Proietti*, Richard Hickman*, Nelson H. Davila Olivas*, **Ping-Ping Huang*,** Marcel C. Van Verk, Johan A. Van Pelt, Alexander H.J. Wittenberg, Martin De Vos, Marcel Prins, Joop J.A. Van Loon, Mark G.M. Aarts, Marcel Dicke, Corné M.J. Pieterse and Saskia C.M. Van Wees. (2016) *Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses*. the Plant Journal. 86(3):249-67. doi:10.1111/tpj.13167

3. Nelson H. Davila Olivas, Silvia Coolen, **Pingping Huang**, Edouard Severing , Marcel C.van Verk, Richard Hickman, Alexander H. J. Wittenberg, Martin de Vos, Marcel Prinse ,Joop J.A. van Loon, Mark G. M. Aarts , Saskia C.M. van Wees, Corné M.J. Pieterseb, Marcel Dicke. (2016) *Effect of prior drought and pathogen stress on Arabidopsis transcriptome changes to caterpillar herbivory*. New Phytologist. 210: 1344–1356. doi:10.1111/nph.13847

4. Gady A, Vriezen W, Van de Wal M, **Huang P**, Bovy A, Visser R, Bachem C (2011) *Induced point mutations in the phytoene synthase 1 gene cause differences in carotenoid content during tomato fruit ripening*. Molecular Breeding: 1-12.

(*shared first authorship)

To be submitted

1. **Pingping Huang**, Mohamed El-Soda, Katarzyna W. Wolinska, Nelson H. Davila Olivas, Joop J.A. Van Loon, Marcel Dicke, Kaige Zhao,, Jan van Kan, Mark G. M. Aarts. *Genome wide association analysis reveals candidate genes controlling a negative effect of biotic stresses on consecutive osmotic stress response.*

2. **Pingping Huang**, Mohamed El-Soda, Katarzyna W. Wolinska, Silvia Coolen, Hans van Pelt, Corné Pieterse, Jan van Kan, Mark G. M. Aarts. *Genome-wide association mapping of the Arabidopsis thaliana growth response to the sequential combination of drought and Botrytis cinerea infection.*

3. **Pingping Huang**, Edouard Severing, Nelson H. Davila Olivas, Silvia Coolen, Marcel C. van Verk, Richard Hickman, Alexander H. J. Wit-tenberg, Martin de Vos, Marcel Prins, Saskia C.M. van Wees, Corné M.J. Pieterse, Joop J.A. van Loon, Marcel Dicke and Mark G. M. Aarts. *Opposite interaction effects on the ethylene signalling pathway of two biotic stresses (Botrytis cinerea and Pieris rapae) on subsequent drought stress responses in Arabidopsis thaliana.*

4. **Pingping Huang**, Michael Mintgen, Mark G.M. Aarts. *Phenotypic and genetic responses of Arabidopsis thaliana natural accessions to consecutive Pieris rapae herbivory and drought stress.*

Education certificate

Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School **EXPERIMENTAL PLANT SCIENCES**
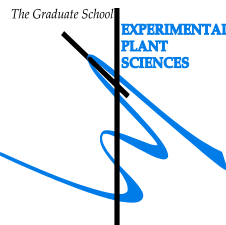
Issued to: **Pingping Huang**

Date: **7 October 2016**

Group: **Laboratory of Genetics**

University: **Wageningen University & Research centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Mining Natural Genetic Variation of Combinatorial Stress Responses of Arabidopsis to Identify New Tolerance Pathways for Biotic and Drought Stress	Oct 11, 2011
► **Writing or rewriting a project proposal**	
Title: Mining Natural Genetic Variation of Combinatorial Stress Responses of Arabidopsis to Identify New Tolerance Pathways for Biotic and Drought Stress	Jun 2011
► **Writing a review or book chapter**	
► **MSc courses**	
Genetic Analysis, Tools and Concepts (GATC) -GEN 30306	Nov 2011
► **Laboratory use of isotopes**	

Subtotal Start-up Phase   *13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
EPS PhD student day, Wageningen University & Research	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 30, 2012
EPS PhD student Days " Get2Together", Soest, NL	Jan 29-30, 2015
► **EPS Theme Symposia**	
Annual EPS Theme 2 Symposium 'Interaction between Plants and Biotic Agents', Wageningen University & Research	Feb 10, 2012
Annual EPS Theme 3 Symposium 'Metabolism and Adaptation', University of Amsterdam	Mar 24, 2013
► **Lunteren days and other National Platforms**	
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	Apr 04-05, 2011
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	Apr 02-03, 2012
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	Apr 22-23, 2013
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	Apr 14-15, 2014
► **Seminars (series), workshops and symposia**	

	Plant science seminars	2011-2015
	6th Plant-Insect interactions workshop, University of Amsterdam	Nov 23, 2011
	Symposium 'Omics Advances for Academia & Industry'	Dec 11, 2014
►	**Seminar plus**	
►	**International symposia and congresses**	
	Gordon conference 'Salt and Water Stress', USA	Jun 24-29, 2012
	Gordon conference 'Salt and Water Stress', USA	Aug 03-08, 2014
	European Joint Retreat for PhD students in Plant Sciences, Gif sur Yvette, France	Jul 05-08, 2011
	European Joint Retreat for PhD students in Plant Sciences, Norwich, UK	Aug 15-17, 2012
►	**Presentations**	
	Poster: Annual Meeting "Experimental Plant Sciences", Lunteren	Apr 04-05, 2011
	Poster: PhD Retreat, France	Jul 05-08, 2011
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Sep 29, 2011
	Poster: Annual Meeting "Experimental Plant Sciences", Lunteren	Apr 02-03, 2012
	Poster: Gordon conference 'Salt and Water Stress'	Jun 24-29, 2012
	Poster: PhD retreat UK	Aug 15-17, 2012
	Poster: Discovering combinatorial stress tolerant genes - STW meeting	Sep 18, 2012
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Sep 29, 2012
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Jan 29, 2013
	Poster Annual Meeting "Experimental Plant Sciences", Lunteren	Apr 22-23, 2013
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Jun 18, 2013
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Sep 19, 2013
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Mar 28, 2014
	Poster: Annual Meeting "Experimental Plant Sciences", Lunteren	Apr 14-15, 2014
	Poster: Gordon conference salt and water stress	Aug 03-08, 2014
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Sep 26, 2014
	Talk: Discovery combinatorial stress tolerant genes - STW meeting	Mar 10, 2015
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Sep 29, 2014
►	**Excursions**	

Subtotal Scientific Exposure	*26.4 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
PhD summer school 'Environmental Signalling'	Aug 22-24, 2011
Introduction to R for Statistical Analysis	Oct 24-25, 2011
PhD Summerchool on Environmental Signaling	Aug 22-24, 2011
Mixed model based QTL mapping in GenStat	May 14-16, 2012
PhD summer school 'Natural Variation of Plants'	Aug 21- 24, 2012
Bioinformatics 'A User's Approach'	Aug 26-30, 2013
Current Trends in Phylogenetics	Oct 14-18, 2013
Transcription Factors and Transcriptional Regulations	Dec 17-19, 2013
► **Journal club**	
Literature discussion at Genetics	2011-2015
► **Individual research training**	

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Subtotal In-Depth Studies   *9.7 credits**

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4) Personal development	*date*
► **Skill training courses**	
Competence Assessment	May 24, 2011
Project and Time Management	Oct-Dec 2012
English for Professional Purposes	Jan-Apr 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	

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Subtotal Personal Development   *3.1 credits**

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TOTAL NUMBER OF CREDIT POINTS*	**52.7**

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.