You are kindly invited to attend the defence of my PhD thesis on **Genetics and regulation of combined abiotic and biotic stress tolerance in tomato** by Christos Kissoudis.

Friday, February 26th 2016
At 16.00 in the Aula of Wageningen University, General Foulkesweg 1a, Wageningen

You are kindly invited to the reception after the defence at the same address.

Christos Kissoudis
christos.kissoudis@wur.nl

PARANYMPHS
Cynara Romero
cynara.romero@wur.nl

Mas Muniroh
mas.muniroh@wur.nl
Genetics and regulation of combined abiotic and biotic stress tolerance in tomato

Christos Kissoudis
Thesis committee

Promotor
Prof. Dr Richard G.F. Visser
Professor of Plant Breeding
Wageningen University

Co-promotors
Dr C. Gerard van der Linden
Research Group Leader, Wageningen UR Plant Breeding
Wageningen University & Research Centre

Dr Yuling Bai
Associate professor, Wageningen UR Plant Breeding
Wageningen University & Research Centre

Other members
Prof. Dr Harro J. Bouwmeester, Wageningen University
Prof. Dr Marcel Dicke, Wageningen University
Dr Christa S. Testerink, University of Amsterdam
Dr Adriaan Verhage, Rijk Zwaan, De Lier

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences
Genetics and regulation of combined abiotic and biotic stress tolerance in tomato

Christos Kissoudis

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 26 February 2016
at 4 p.m. in the Aula.
Christos Kissoudis
Genetics and regulation of combined abiotic and biotic stress tolerance in tomato, 212 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summaries in English and Dutch

ISBN 978-94-6257-656-8
Contents

Chapter 1 ................................................................................................................. 7
General Introduction

Chapter 2 ................................................................................................................. 17
Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk

Chapter 3 ................................................................................................................. 49
Combined biotic and abiotic stress resistance in tomato

Chapter 4 ................................................................................................................. 71
Responses to combined abiotic and biotic stress in tomato are governed by stress intensity and mechanism of resistance

Chapter 5 ................................................................................................................. 95
Hormone signalling and regulation of tomato response to combined biotic and abiotic stress

Chapter 6 ................................................................................................................. 121
Roles and contribution of tomato WRKY genes to salt stress and powdery mildew resistance

Chapter 7 ................................................................................................................. 149
General discussion

References .............................................................................................................. 167

Summary ............................................................................................................... 199

Samenvatting ...................................................................................................... 203

Acknowledgements ............................................................................................ 207

Education Certificate ......................................................................................... 210
Chapter 1

General introduction
Producing more with less

We are living on a planet with finite resources and since it is the only place that can sustain our welfare and civilization both for the present and the foreseeable future, we must ensure that our population can grow sustainably and in harmony with the environment. Since the industrial revolution, due to recurrent advances in (food) technology, hygiene and medicine the human population has boomed at an accelerated pace, increasing almost 4-fold since 1900 and expected to exceed 9 billion in 2050. This growth was sustained by the significant advances in technology with the mechanization and intensification of agriculture which, combined with leaps in genetics and plant breeding science, resulted in the green revolution (between the 1930s and 60s) . This doubled yields for the major staple crops and ensured that enough food could be produced to support the growing human population (Pingali 2012).

Currently however the same challenge not only has re-appeared, as population growth is continuing to rise, but is increasingly harder to address due to the risk of depleting the finite/non-renewable natural resources of the earth. The consumption of natural resources such as fossil fuels, on which industrialization is majorly based, has additionally led to an inevitable increase in atmospheric CO\textsubscript{2}, which is expected to continue to rise, even if appropriate measures are taken (Pan et al. 2014). The effects of the elevated CO\textsubscript{2} concentrations on our climate can already be detected, with record high temperatures being observed and expected to further rise to different projected magnitudes depending on the measures taken as well as changes in precipitation patterns (Trenberth 2011). In addition, agricultural intensification, while advantageous, has questionable sustainability, itself contributing to CO\textsubscript{2} rise (Gitz and Ciais 2004) and further evidenced by the rapid depletion of water resources (agriculture is the biggest fresh water consumer among all human activities), land deterioration from continuous cultivation including increased salinization in irrigated lands and coastal areas and agrochemical and fertilizer pollution (Savci 2012). Moreover the genetic erosion of crops with only a few cultivars per crop covering the majority of cultivated land makes them increasingly vulnerable to disease pandemics and the changing environmental conditions (Keneni et al. 2012) with increasing efforts being undertaken to incorporate genetic variation from wild species to increase crop stress resilience (Warschefsky et al. 2014).

Thus, under the current conditions the goal is to increase global agricultural productivity while limiting inputs to maintain sustainability. Key to this goal, except increasing the yield potential of crops and optimizing cultivation methods, is to be
able to maintain crop yield and performance under less than optimal conditions such as abiotic stress conditions (drought, salinity, nutrient insufficiency etc.) and pathogen and pest attacks. Maximizing performance under such limiting conditions is also of great importance in bridging the yield inequalities that are observed in different parts of the world, which are largely due to different resource inputs (Mueller et al. 2012).

**Stress combinations**

Field conditions by themselves are stress environments and that is the reason why field crops hardly ever realize their true yield potential as for instance evidenced by huge yield gaps when comparing experimental plots with farm data (Lobell et al. 2009). This presumes that crop plants are subjected to a variety of stresses during their lifecycle. These stresses, though in many occasions probably mild, may have a significant impact on productivity. Stress factors include not only abiotic stress factor like drought and temperature but also various pathogens and pests, such as insects and nematodes. For these biotic stress factors intervention with agrochemicals is often necessary when there is no genetically-based resistance to ensure high yields. As in many occasions either chemical protection is not complete or genetic resistance is partial or not durable, crops are experiencing biotic stress that can be concurring with abiotic stress.

Climate change significantly influences both abiotic stress incidence and pathogen ecophysiology. Most projections indicate an increased frequency of adverse environmental conditions affecting agriculture (Trnka et al. 2014). On the other hand there is already evidence for spread of pathogens towards the earth poles due to increased winter temperature, while longer seasons due to warmer weather allow more generations during a single season (Bebber 2015; Garrett et al. 2006). This generates higher probabilities of intra-species evolution of more virulent strains. Climate change can also change the responses of the hosts. This may result in decreased or enhanced resistance, although for stress conditions aggravated by climate change such as drought and heat stress most studies indicate a dampening effect on resistance (Bostock et al. 2014; Cheng et al. 2013a).

Generalizations however cannot be easily made due to the multitude of overlapping layers involved in abiotic and biotic stress response (Fig.1) evident by the numerous reports of pathosystem-specific responses. ABA signalling for example can contribute to increased resistance or susceptibility, depending on the host-pathogen combination and stress conditions (Adie et al. 2007; Hok et al. 2014; Mang et al. 2012; Ulferts et al.
Moreover evidence so far indicates that the responses under combined stress are unique (Rasmussen et al. 2013). Thus to maximize the chance to identify key regulatory components involved in adaptation and tolerance specific combinations of stresses should be treated as distinct stress conditions.

Figure 1. Overlapping components of plants responses to abiotic and biotic stress.

The need to expand research to crops: tomato as a stepping stone between Arabidopsis and crops

With the current and upcoming challenges in covering population nutritional demands the increasing importance of research translation is accentuated, as basic research has focused since the dawn of the genomics era on the non-crop model dicot species Arabidopsis thaliana (Koornneef and Meinke 2010). Tomato (Solanum lycopersicum) can be an optimal “bridge” between the model plant and other crops as it combines attributes of both a model plant with genetic and genomic resources such as mapping populations and mutants (Emmanuel and Levy 2002; Park et al. 2014;
Pascual et al. 2015) with a larger pool of readily crossable wild relatives that can contribute novel alleles for agronomic trait improvement (Koenig et al. 2013). Tomato is also economically the most important vegetable crop in the world (Lin et al. 2014). The tomato plant exhibits features such as fleshy fruit, a sympodial shoot, and compound leaves, all being important agronomic traits that other model plants (e.g., rice and Arabidopsis) do not possess (Kimura and Sinha 2008). Tomato is closely related to many cultivated species such as potato, eggplant, pepper and tobacco, enabling accelerated (or even instant) research translation to all these crops. Moreover, it is cultivated both in the greenhouse and in open field conditions in temperate Mediterranean climates, and the latter is thought to be most affected by climate change (Giorgi and Lionello 2008).

Genomic resources are increasing with the sequence of cultivated and wild relatives boosting tomato breeding, with a huge potential of relieving the bottleneck that was experienced when tomato was originally domesticated in its region of origin and when it was transported later on to Europe (Aflitos et al. 2014; Lin et al. 2014). Wild relatives are especially important, as approximately 20-fold higher SNP rates are discovered in these species in comparison with the variation among the cultivated varieties (Aflitos et al. 2014). Having the same number of chromosomes and ploidy level as the cultivated varieties (2n=2x=24), high genomic synteny (Stack et al. 2009) and being in most occasions readily intercrossable has made the wild relatives an important source of alleles increasing the economic value of tomato and its robustness against diseases and pests (Aflitos et al. 2014; Bleeker et al. 2012). Linkage drag of introgressed regions, in many occasions due to recombination inhibition by the presence of chromosomal rearrangements such as inversions (Verlaan et al. 2011), results in the carry-over of undesirable wild alleles constraining wild species utilization. The increased adoption of marker and genomics-assisted breeding and the construction of introgression line (IL) libraries (Chitwood et al. 2013; Eshed and Zamir 1995) in combination with single base resolution of nucleotide variation and association with desired phenotypes (Lin et al. 2014) offers unlimited power in further improving tomatoes overcoming the existing barriers.

**Salt stress adaptation and tolerance**

High soil salinity is considered a major threat for agricultural productivity in semi-arid or coastal areas due to its increased occurrence in irrigated lands, which account for a major part of world food production (Flowers 2004). Thus, salinity is also an important abiotic stress limiting (tomato) crop cultivation. Salt stress persistence
throughout the plants’ lifetime likely leads to co-occurrence with additional stress factors, either abiotic (e.g. heat, drought) or biotic (fungi, insects etc.) justifying further research on the impact of stress combinations that include salinity.

Salinity stress is characterized by an osmotic and an ionic component. The initially perceived osmotic stress results in growth inhibition due to turgor reduction, and reduction in photosynthesis due to stomatal closure. Ionic stress builds up gradually and the intracellular accumulation of Na\(^+\) can eventually lead to direct toxic effects due to enzyme inhibition or indirect effects due to reduced K\(^+\) influx (Munns and Tester 2008). The plant’s tolerance to salinity stress is characterized by the adaptation potential to osmotic stress and the ability to cope with ionic stress. Ionic stress can be either avoided by limiting Na\(^+\) uptake from the roots or restricting its transport to the shoot, or tolerated by efficiently compartmentalizing the increased Na\(^+\) concentrations in the aerial parts in places where it cannot directly interact with the cellular functions and exert its toxicity, like the vacuole (Adem et al. 2014; Plett and Møller 2010). Additional scavenging of excess reactive oxygen species (ROS) due to photosynthesis inhibition and membrane damage is also of great importance in achieving tissue tolerance (Adem et al. 2014; Miller et al. 2010).

Various approaches have been employed that aim at increased salt tolerance in tomato but efforts were less successful than expected, possibly due to the polygenic nature of salt tolerance (Cuartero et al., 2006). QTL discovery was undertaken using segregating populations originating most frequently from crosses between salt sensitive tomato cultivars and salt tolerant wild tomato species such as \textit{Solanum pimpinellifolium} and \textit{Solanum pennellii} (Asins et al. 1993; Frary et al. 2010; Villalta et al. 2007). The results confirmed the complex genetic architecture of salinity tolerance, with tolerance traits having medium to low heritability and individual QTLs explaining a fraction of the total variation (Monforte et al. 1996; Villalta et al. 2007).

Salinity tolerance determinants in tomato have also been studied at the biochemical and molecular level. Elevated antioxidant enzymes activities are critical for the efficient scavenging of ROS in the salt tolerant wild species \textit{Solanum pennellii} (Frary et al. 2010; Mittova et al. 2003). The significance of Na\(^+\) concentration in the leaves for tomato salinity tolerance however is obscure. Correlation analyses in populations segregating for salinity tolerance have demonstrated a reduced association of Na\(^+\) accumulation and yield parameters (Asins et al. 2010; Villalta et al. 2007). On the other hand, transgenic approaches manipulating Na\(^+\) exclusion and compartmentation provide support for their relative importance in achieving salt tolerance (Huertas et al. 2012; Zhang and Blumwald 2001). K\(^+\) homeostasis is also
important, as shown by the overexpression in tomato plants of K+/H+ antiporters, which resulted in a greater capacity to retain intracellular K+ and in enhanced salinity stress tolerance (Leidi et al. 2010; Rodríguez-Rosales et al. 2008). Recently, the importance of homeostasis of plant hormones such as ABA, auxin, cytokinin, ethylene and jasmonates during salinity stress has been revealed, which were shown to be directly controlling plant growth and senescence under stress conditions (Albacete et al. 2008; Ghanem et al. 2008; Ghanem et al. 2012). Since many of these hormones participate in both abiotic and biotic stress responses, they may be involved in crosstalk between these responses and possibly be determinants of plant phenotypic responses under combined stress conditions.

**Powdery mildew resistance in tomato**

Powdery mildew caused by the biotrophic Ascomycete *Oidium neolycopersici* is a significant threat for tomato cultivation both in the field and in the greenhouse (Panthee and Chen 2010). Breeding efforts are focused on identifying resistance genes in the wild tomato germplasm and introducing them in commercial cultivars through marker-assisted selection (Seifi et al. 2014a). Several loci conferring resistance to *O. neolycopersici* have been identified. *Ol-1* and *Ol-4* are dominant resistance genes that originate from *S. habrochaites* and *S. peruvianum*, respectively (Bai et al. 2004; vander Beek et al. 1994) and are located on the long arm of chromosome 6. The *Ol-1* mediated resistance is characterized by multiple-cell slow hypersensitivity response (HR), while the *Ol-4* gene is homologous to the *Mi-1* gene encoding a CC-NBS-LRR protein and confers resistance through fast single-cell HR (Li et al. 2007; Seifi et al. 2014). The *ol-2* gene, discovered in an accession of *S. lycopersicum var. cerasiforme* (Ricciardi et al. 2007), is located near the centromere region of chromosome 4, and encodes a loss-of-function allele of a gene homologous to the *Mlo* gene of barley (Bai et al. 2008b). *ol-2* confers race non-specific resistance through increased papillae formation and callose deposition at the site of the attempted penetration (Li et al. 2007).

**Stress crosstalk and response to stress combinations in tomato**

There are only few studies focused on tomato responses under abiotic and biotic stress combination. Increased soil salt concentration resulted in enhanced susceptibility to soil borne diseases and *Phytophthora* spp. (DiLeo et al. 2010; Triky-Dotan et al. 2005). On the other hand, drought stress resulted in reduction of susceptibility to powdery mildew and *Botrytis cinerea* concomitant with ABA concentration increase (Achuo et
Evidence for abiotic and biotic stress resistance crosstalk in tomato has been mostly found with mutants defective in the ABA signaling pathway. The ABA deficient mutant *sitiens*, which exhibits reduced tolerance to salinity stress (Mäkelä et al. 2003), was more resistant to *Botrytis cinerea*, involving elevated SA- but not JA-responsive gene expression (Audenaert et al. 2002). The enhanced resistance observed is a result of timely production of ROS that in turn induce cell wall modifications that restrict pathogen penetration (Asselbergh et al. 2007). The *sitiens* mutant was also reported to be more resistant to the biotrophic bacterium *Pseudomonas syringae* (Thaler and Bostock 2004) and the biotrophic fungus *O. neolycopersici* (Achuo et al. 2006). More recently it has been demonstrated that the ABA-inducible MYB transcription factor AIM1 regulates responses to both salt stress and *B. cinerea* infection (AbuQamar et al. 2009). Down regulation of ABA-inducible AIM1 results in increased sensitivity to salinity stress, elevated accumulation of Na+ and susceptibility to the pathogen, suggesting an involvement of ABA-regulated ion fluxes in the defense responses against *B. cinerea*.

Biochemical and molecular studies of basal disease resistance regulation in tomato have revealed certain differences compared to the observations in the model plant, *Arabidopsis thaliana*. Salicylic acid and jasmonic acid/ethylene mediated defense responses are effective against biotrophs and necrotrophs, respectively, and act antagonistically with each other in Arabidopsis. In tomato however, salicylic acid-mediated defense gene expression appears to be ineffective for resistance against the biotroph *O. neolycopersici*, but enhances resistance against the necrotroph *Botrytis cinerea* (Achuo et al. 2004). Furthermore, jasmonate-deficient (*defl*) mutant tomato plants exhibited among others increased susceptibility to biotrophic bacteria and the oomycete *Phytophthora infestans* (Thaler et al. 2004), indicating hormonal interactions and their functions on defense pathways in tomato might significantly deviate when compared with *Arabidopsis*.

**Objectives and scopes of this thesis**

The research described in this thesis was initiated to increase our understanding of the biological processes underlying adaptation and resistance to combined salt stress and powdery mildew but also to highlight ways to efficiently breed for tolerance to stress combinations in crops. Our strategy involved the employment of different approaches to identify regulatory components of combined salt stress and powdery mildew (PM) resistance in tomato by a forward genetic approach utilizing an IL
population segregating for both traits, as well as targeted approaches that investigate the response of well-defined tomato Ol-genes (Ol-1, ol-2 and Ol-4) to PM under different salt stress regimes. In addition, the effect of major stress regulating hormones was examined in tomato lines that combined resistance genes with manipulations of hormonal pathways, with the aim to reveal critical contributions of hormone signalling to combined stress adaptation. Finally transcription factors of the WRKY family as putative cross regulatory components of both abiotic and biotic stress tolerance were investigated to further assess their potential in breeding for combined stress tolerance. This included assessing any complexities arising from pleiotropic effects.

In Chapter 2, mechanisms underlying cross regulation of abiotic and biotic stress adaptation and tolerance, ranging from morpho-physiological to biochemical and genetic or epigenetic aspects, are extensively reviewed and discussed. Emphasis was additionally given to specific disease resistance mechanisms such as R-gene resistance and pre-invasive defense responses such as callose and papillae. Many of the potential overlapping mechanisms are covered and targets for genetic improvement of crops to combined stress are provided and discussed.

In Chapter 3, a S. habrochaites LYC4 IL population was evaluated under salt stress and PM individually and under combination of these stresses. The IL population segregated for both salt stress tolerance and PM resistance and various new genetic loci contributing to tomato salt stress tolerance, partial PM resistance and/or both were discovered. Salt stress had an additive negative effect increasing susceptibility to PM and reducing phenotypic variation for disease resistance. The results provide genomic targets for allele mining for salt stress tolerance and disease resistance in S. habrochaites LYC4, which has been recently sequenced, as well as insights on the genetic architecture of combined stress tolerance.

In Chapter 4, we examined the impact of different salinity stress severities representing mild and severe stress and of different monogenic PM resistance mechanisms on the outcome of combined salt stress and PM challenge. The resistance mechanisms included an R-gene mediated hypersensitivity response and pre-invasive defense mediated by the susceptibility gene mlo. We observed a significant interaction of PM resistance with salt stress severity, which was dependent on the resistance mechanism. R-gene resistance was stable across all treatments examined and could be the most readily available source for achieving tolerance to abiotic and biotic stress combinations.

In Chapter 5, we evaluated the effects of three major hormonal pathways, ABA, JA
and ET, on the PM resistance conferred by the above mentioned resistance genes (Ol-1, ol-2 and Ol-4) under combined stresses. A significant negative effect of ethylene overproduction was observed on PM resistance mediated by Ol-1 and ol-2, which was aggravated under combined stress. On the other hand, ABA deficiency alleviated the increased PM susceptibility and senescence observed under combined stress for Ol-1, while it resulted in a minor increase in PM susceptibility for ol-2 under control conditions. JA deficiency effects were minor and tended to decrease increase PM resistance conferred by Ol-1 and ol-2. Ol-4 was exceptionally robust to all hormonal perturbations.

In Chapter 6, we identified and cloned tomato WRKY transcription factors that were likely to be involved in stress response based on their homology to Arabidopsis thaliana WRKY genes with known involvement in the stress response. Overexpression and silencing transgenic lines were examined for their tolerance to salt stress, to powdery mildew infection as well as to these stress factors combined. The results confirmed broad functions for the tomato WRKY genes with several contributing to increased salt tolerance and/or PM resistance. Exceptional phenotypes were observed in SIWRKY23 transgenic overexpression and silencing lines, with overexpression increasing salt tolerance but resulting in PM hyper-susceptibility and silencing resulting in necrotic symptoms and PM resistance. This highlights a potentially significant role in the cross-regulation of abiotic and biotic stress signalling.

In Chapter 7, we summarize all findings and discuss these in both molecular and physiological context, and we examine their relevance for improved breeding efficiency aimed at increased tolerance to powdery mildew and salt stress combinations. We discuss the potential of these results to be extrapolated to other crops, pathosystems and abiotic stresses and provide recommendations on targets for future research to enhance our understanding of combinatorial stress adaptation.
Chapter 2

Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk

Christos Kissoudis¹,², Clemens van de Wiel¹, Richard G.F. Visser¹, C. Gerard van der Linden¹

¹Laboratory of Plant Breeding, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

²Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

Published in Frontiers in Plant Science (2014)
Abstract

Plants growing in their natural habitats are often challenged simultaneously by multiple stress factors, both abiotic and biotic. Research has so far been limited to responses to individual stresses, and understanding of adaptation to combinatorial stress is limited, but indicative of non-additive interactions. Omics data analysis and functional characterization of individual genes has revealed a convergence of signalling pathways for abiotic and biotic stress adaptation. Taking into account that most data originate from imposition of individual stress factors, this review summarizes these findings in a physiological context, following the pathogenesis timeline and highlighting potential differential interactions occurring between abiotic and biotic stress signalling across the different cellular compartments and at the whole plant level. Potential effects of abiotic stress on resistance components such as extracellular receptor proteins, R-genes and systemic acquired resistance will be elaborated, as well as crosstalk at the levels of hormone, ROS and redox signalling. Breeding targets and strategies are proposed focusing on either manipulation and deployment of individual common regulators such as transcription factors or pyramiding of non- (negatively) interacting components such as R-genes with abiotic stress resistance genes. We propose that dissection of broad spectrum stress tolerance conferred by priming chemicals may provide an insight on stress cross regulation and additional candidate genes for improving crop performance under combined stress. Validation of the proposed strategies in lab and field experiments is a first step towards the goal of achieving tolerance to combinatorial stress in crops.

Keywords: salinity, drought, disease resistance, R- genes, crosstalk, hormones, transcription factors, post-translational modifications
Introduction

Plants are sessile and cannot escape stressful conditions originating from the physical environment (abiotic stress) and from interactions with insects and microorganisms such as fungi and bacteria (biotic stress). The on-going change in climate conditions due to mostly anthropogenic causes such as the increase in CO₂ emissions (Peters et al. 2011) exaggerates agricultural land deterioration due to temperature rise. This results in increased evapotranspiration, intensifying drought episodes (Zhao and Running 2010) and increasing soil salinization, augmenting the 7% of the total and 30% of the irrigated agricultural land already affected by salinity (Munns and Tester 2008). Available data and projections on the effect of climate change on pathogen spread are not conclusive, although the evidence points to increased reproductive potential and geographic expansion that will lead to interactions with both more hosts and different pathogen strains, increasing the chances for the rise of more virulent strains (Garrett et al. 2006). Therefore, the chances of plants encountering abiotic and/or biotic stress in the future are likely to be higher, with more frequent stress interactions.

Plants have developed a multitude of defense responses that allow them to adapt, survive and reproduce under stress conditions (Pieterse et al. 2009). With the advancement of ~omics technologies and on-going functional characterizations of individual genes, it has become apparent that environmental adaptation is under tight regulation, which is critical for plant survival (López et al. 2008). Many components of this regulatory network are involved in responses to different stresses but may function antagonistically or some responses are prioritized over others, compromising plant resistance to multiple stresses simultaneously (Glazebrook 2005; Yasuda et al. 2008).

Major components of the regulatory networks underlying environmental stress adaptation, pathogen recognition and defense include reactive oxygen species signalling (ROS) (Miller et al. 2008), plant hormones (Bari and Jones 2009; Peleg and Blumwald 2011), changes in redox status (Munne-Bosch et al. 2013) and inorganic ion fluxes, such as Ca²⁺(Martí et al. 2013). Based on ~omics data analyses these components appear to be at least partly shared between both abiotic and biotic stress signalling, indicating crosstalk and convergence of mechanisms in these pathways and the existence of a general stress response (Walley et al. 2007).

The nature of pathogen perception dictates that physical barriers such as the cuticle, stomata and cell walls are also critical for timely pathogen recognition and interception (Asselbergh et al. 2007). As data generated by ~omics analyses derive
from a mixture of different cell types and tissues, these spatially important interactions may be missed and these datasets may lead to erroneous conclusions about components shared and their significance in abiotic and biotic stress crosstalk. Moreover, as combinatorial stress potentially results in novel interactions between signalling components, extrapolation of results from studies with single stress conditions should be done with care.

Here we will elaborate on the mechanisms involved in adaptation and tolerance to combinatorial abiotic and biotic stress, with a focus on dehydration/salt stress and fungal and bacterial pathogens interaction. This review will particularly emphasise interactions that potentially arise during the pathogenesis timeline and were as yet given little attention. We will discuss molecular components with potentially critical roles in abiotic and biotic stress tolerance crosstalk, and propose breeding approaches towards effective crop improvement against combinatorial stress.

**Evidence of crosstalk**

**Evidence at the phenotypic and physiological level**

Studies on the commonly occurring combination of drought and heat stress have revealed that physiological and molecular responses of plants exposed to both stresses are markedly different from their response to the individual stresses (Rizhsky et al. 2004). Similarly, there are numerous reports about abiotic stress (mostly drought and salinity) affecting pathogen resistance, which is indicative of interaction between abiotic and biotic stress. There are reports of disease resistance attenuation by high humidity and high temperature (Wang et al. 2005; Wang et al. 2009). In most cases abiotic stress predisposes plants to subsequent pathogen infection (Sanogo 2004; Triky-Dotan et al. 2005; You et al. 2011), although positive effects on resistance to foliar pathogens have also been reported (Achuo et al. 2006; Wiese et al. 2004).

There is evidence that different levels of abiotic stress have a significantly different impact on disease susceptibility (Desprez-Loustau et al. 2006; Soliman and Kostandi 1998). Salinity stress, in particular, exerts its damaging effect through both osmotic effects and ion toxicity resulting from ion accumulation (mainly Na\(^+\) and Cl\(^-\)). As NaCl is an antifungal agent (Blomberg and Adler 1993) it could potentially exert a direct toxic effect on fungal growth after accumulation inside the plants (Fig.1). In line with this argument are the many examples of reduction of fungal pathogenicity by metal accumulation (Fones et al. 2010; Poschenrieder et al. 2006), and a similar trend is observed for NaCl accumulation (Soliman and Kostandi 1998). Therefore salt stress-
pathogen interactions may be highly influenced by stress intensity, which affects the degree of accumulation of salt in the plant.

Figure 1. A scheme for the effects of abiotic and biotic stress at the plant level. A combination of abiotic stress with pathogen infection potentially derails hormone and systemic ROS homeostasis. Pathogen infection has been shown to impair stomata closure under non-stress conditions, with the dynamics of this interaction under abiotic stress being unknown. Senescence is a common component of both abiotic and biotic stress that can potentially be amplified under combinatorial stress. Systemic ROS signals generated after pathogen encounter may alter water relation and salt uptake through their effects in root hydraulic conductance and ion transport. Abiotic stress through ABA signaling negatively affects signals that trigger systemic acquired resistance, enhancing pathogen spread from the initial site of infection. Ion accumulation ($\text{Na}^+$, $\text{Cl}^-$) under salt stress can have a direct toxic effect on pathogen growth.
The different tolerance strategies of the host against ion toxicity (ion exclusion at the roots and/or ion compartmentalisation in the above ground organs inside the vacuoles) can impact on the outcome of plant-pathogen interactions under salt stress. Therefore, it appears that the outcome of the interaction in most occasions is plant, genotype, pathogen and stress intensity dependent. Moreover abiotic stress, except for potentially dampening or strengthening signalling responses for pathogen defense deployment, could create more or less favourable conditions for pathogen growth by additionally influencing the physiological status of the plant such as water and ion content. This could create more or less favourable conditions for pathogen growth.

Vice versa, plant responses to abiotic stress can be affected by prior interactions with pathogenic fungi. Pathogen infection has been shown to reduce photosynthesis and water use efficiency (WUE) and induce abnormal stomata opening patterns, and all of these are critical for plant tolerance to abiotic stress (Bilgin et al. 2010; Grimmer et al. 2012). Salicylic acid (SA) signalling induced after infection with biotrophic fungi, can attenuate abscisic acid (ABA) signalling that is orchestrating plant adaptive responses to abiotic stress (Kim et al. 2011c). Infection by a root pathogen was shown to increase shoot Na⁺ and Cl⁻ content under saline conditions in Phaseolus vulgaris (You et al. 2011) (Fig.1). Finally genetically heightened resistance to pathogens is often accompanied by a fitness cost that may generally affect the plant performance under both abiotic stress and stress-free conditions (Huang et al. 2010; Todesco et al. 2010).

A direct interaction of pathogen virulence factors with stress tolerance components of the plant host was demonstrated for the P. syringae type III effector HopAM1 that targets HSP70 (Jelenska et al. 2010) involved in heat tolerance and stomata closure under stress (Clement et al. 2011). Overexpression of HopAM1 in Arabidopsis thaliana results in increased sensitivity to ABA and salt stress, providing proof of direct manipulation of abiotic stress signalling components (Goel et al. 2008).

Interaction of plants with microorganisms can also be beneficial to abiotic stress tolerance. For instance, infection of plants with RNA viruses improved tolerance to drought (Xu et al. 2008). Infection with the vascular pathogen Verticillium spp. increased Arabidopsis thaliana drought tolerance due to de novo xylem formation, which enhances water flow (Reusche et al. 2012). Symbiosis with fungal endophytes (Marquez et al. 2007) as well as association of plant roots with non-pathogenic rhizobacteria and mycorrhizal fungi increases plant vigour under stress conditions through, among others, interactions with hormonal pathways and the sustainment of water and source-sink relations (Dodd and Perez-Alfocea 2012). Remarkably, rhizobacteria colonization is also shown to enhance plant resistance to fungal
pathogens and insects, via systemic signalling that triggers immunity (induced systemic resistance, ISR) (Berendsen et al. 2012).

Further evidence for abiotic and biotic stress resistance crosstalk comes from studies of the effects of exogenous application of chemicals that sensitize plant defense responses, a phenomenon called priming (Goellner and Conrath 2008). For example, application in *Arabidopsis thaliana* of β-aminobutyric acid (β-ABA), a non-protein amino acid, results in enhanced resistance to a wide range of stresses including heat, drought and salinity stress, as well as enhanced resistance to biotrophic as well as necrotrophic fungi (Ton et al. 2005). Exogenous application of SA renders many crop plants more tolerant to an extensive array of abiotic stresses (Horváth et al. 2007), and similar observations have also been reported after treatment with jasmonates (Walia et al. 2007).

**Evidence for crosstalk from whole genome expression analyses**

Evidence for regulatory crosstalk between abiotic and biotic stress response at the molecular level comes mostly from observations of expression patterns of genes under independent imposition of the single stress conditions. In *Arabidopsis thaliana* a significant number of genes up-regulated by salinity stress are also induced in response to biotic stresses (Ma et al. 2006). Whole genome expression meta-analysis experiments under different abiotic and biotic stress treatments revealed a significant number of genes that are commonly regulated under abiotic and biotic stress conditions (Ma and Bohnert 2007; Shaik and Ramakrishna 2013; 2014). Functional categories enriched in the 197 commonly regulated genes identified by (Ma and Bohnert 2007) include response to ABA, SA, jasmonic acid (JA) and ethylene (ET), major stress hormones controlling adaptation to abiotic and biotic stress. Several members of signalling pathways involving mitogen activated protein kinase (MAPK), Ca²⁺, reactive oxygen species (ROS), phospholipids, mitochondrial functions, vesicle trafficking and apoptosis were induced under biotic as well as abiotic stresses (Ma and Bohnert 2007). Transcription factors (TFs) appear to be major orchestrators of stress crosstalk with members of WRKY, MYB, ERF, NAC and HSF displaying similar induction patterns across stress treatments (Ma and Bohnert 2007; Shaik and Ramakrishna 2013). On the other hand, another study using co-expression data to identify cis-regulatory elements (CREs) of stress responses identified distinct CREs for the response to abiotic and biotic stressors (Zou et al. 2011). In addition, a number of CREs identified for both types of stress appear to oppositely regulate the expression of downstream genes in response to abiotic or biotic stress.
A different approach, yeast two-hybrid assays targeting major regulators of rice abiotic and biotic stress response, identified proteins that are present in multiple interactomes (Seo et al. 2011; Sharma et al. 2013). These include OsMPK5, the wall-associated kinase 25 (WAK25), sucrose non-fermenting-1-related protein kinase-1 (SnRK1), and WRKY family transcription factors.

Recently, examination of the transcriptional response of different Arabidopsis thaliana accessions to combinations of abiotic and biotic stressors revealed that across the treatments on average 60% of expression changes under combinatorial stress could not be predicted by the changes in response to the individual stresses (Rasmussen et al. 2013). The functional categories enriched in the affected genes were similar to those discovered after transcriptome meta-analyses of individual stressors, i.e. stress hormone responses, ROS and MAPK signalling and regulation of hypersensitivity response. The response of many of these transcripts was cancelled or prioritized under stress combination in comparison with the individual stress pointing to potential antagonistic interactions with detrimental effects on plant adaptation under combinatorial stress. In a similar study, the increased susceptibility to a virus after simultaneous application of drought and heat stress was accompanied by down regulation of pathogenesis related (PR) genes and R-genes, which were otherwise induced under single viral stress (Prasch and Sonnewald 2013). This indicates a direct negative effect of abiotic stress on major defense executors, that adds up to the antagonistic regulation observed in other signalling pathways. These studies clearly emphasise that even though regulatory pathways overlap between stresses, combinatorial stress needs to be treated and studied as a unique condition. Further functional characterization of individual gene members playing key roles in these pathways is required to extract meaningful conclusions.

**Abiotic-biotic stress interaction interface**

As mentioned above, abiotic and biotic stress interactions can occur at multiple levels, depending on the type of the stress (osmotic, ionic), the lifestyle and infection strategy of the pathogen (biotroph/necrotroph, infection by direct penetration/through stomata etc.) as well as the pathogenesis stage. We will summarize molecular components that according to evidence mentioned above participate in stress crosstalk. We will follow the pathogenesis timeline highlighting first extracellular interactions taking place at the epidermis and the apoplast during the initial stages of pathogenesis and moving on to the interactions in the intracellular environment during pathogen colonization (Fig.2). As information under combined stress is limited, and a detailed coverage of all
potential interactions is not possible, our intention is to provide leads for future research that will aid to further dissect plant adaptive responses and tolerance under combined abiotic and biotic stress.
Figure 2. A scheme for the interaction interface and overlapping signaling pathways of abiotic and biotic stress at the cellular level. Both stress factors affect the homeostasis of chemical signals at the apoplastic space such as Ca\(^{2+}\), ROS and pH levels. Abiotic stress potentially affects the structure and properties of preformed and inducible physical barriers that function against pathogen penetration. Signaling nodes such as RBOHs and RLKs and other cell wall (CW) kinases localized at the plasma membrane, and MAPKs are shared by both stressors, with downstream signal specificity under stress combination remaining elusive. ABA signaling, central for adaptation to abiotic stress, negatively impinges on defense hormone signaling, while, pathogen dependent, positive interactions are observed for JA signaling. ABA-SA interaction is two sided, as activation of SA signaling by pathogen challenge attenuates ABA responses. ABA positively contributes to pre-invasion defense, enhancing callose deposition. Rewiring of secretory machinery under abiotic stress potentially affects its function in the exocytosis of antimicrobial compounds at the site of infection. Nuclear translocation of R-genes is negatively affected under abiotic stress. Redox state, as well as metabolite concentration such as sugars and amino acids (AA), function as drivers for post-translational modifications, modulating the activity of target proteins/transcription factors. Previously/simultaneously encountered stress effect on chromatin and DNA methylation status, potentially impacts on expression patterns of the recipient genes under stress combination. Transcription factor activation and binding to stress responsive gene promoters is a convergence point regulating the signal output under combinatorial stress with diverse outcomes.

Extracellular interface

Cuticular layer

The cuticle and cell wall constitute the first layers of defense against microbial attackers. They not only serve as physical barriers against pathogen penetration, but also as sensitive sensors for the timely activation of the intracellular and systemic defense responses. *Arabidopsis thaliana* mutants in long-chain acyl-CoA synthetase 2 (*LACS2*), a gene that is involved in cuticle biosynthesis, exhibit increased permeability of the cuticular layer which leads to increased resistance to *Botrytis cinerea* (Bessire et al. 2007). Interestingly, ABA deficiency causes similar cuticular defects and heightened resistance to *B. cinerea* through faster induction of defense responses and H\(_2\)O\(_2\) production both in Arabidopsis and tomato, indicating a link between abiotic stress signalling, cuticle structure and defense responses (Curvers et al. 2010). In the study by Xiao et al. (2004) however, *lacs2* Arabidopsis mutants show no alteration in the resistance against the necrotroph *Alternaria brassicicola* and
biotrophs, and even increased susceptibility against *P. syringae*. The latter observation points to a positive contribution of a thicker cuticle to resistance against *P. syringae*, indicating that the effects may be pathogen-specific (Tang et al. 2007). The well-documented increase in cuticular thickness under conditions of water deficiency (Kosma et al. 2009) may thus result in alteration in the deployment of the pathogen defense response. The cuticle does appear to be a sensor of the osmotic status and to be essential for the up-regulation of ABA biosynthesis genes under osmotic stress (Wang et al. 2011b) through a yet not clearly defined mechanism: cuticle disruption by pathogens may therefore affect osmotic stress acclimation.

**Cell wall-apoplastic space**

Cell walls similarly appear to be an integrated signalling component for the defense against pathogens. Changes in pectin properties and composition in the Arabidopsis powdery mildew-resistant (*pmr*) mutants *pmr5* and *pmr6* resulted in a SA, JA and ET independent increase in resistance to powdery mildew species (Vogel et al. 2004). Cellulose deficiency caused either by non-functional cellulose synthase genes or by chemical treatment enhances the synthesis of the defense hormones SA, JA and ET and signalling and results in increased resistance to pathogens (Hématy et al. 2009). Intriguingly, these responses were attenuated when plants were grown under high osmotic pressure which reduced the turgor pressure (Hamann et al. 2009), suggesting that the defense response may be initiated by sensing the increased turgor pressure as a result of cell wall weakening. Osmotic stress, which is a common component of many abiotic stresses, may therefore interfere with the ability of plants to sense damage to the cell wall, due to already reduced turgor, resulting in inadequate activation of defense mechanisms.

The above mentioned alterations in plant pathogen interactions in cell wall component biosynthesis mutants may be the consequence of the erroneous activation of integral receptor proteins such as RLKs and RLPs (receptor-like kinases and receptor-like proteins respectively) which survey the cell wall integrity and bind to MAMPs and DAMPs (Microbial and Damage Associated Molecular Patterns, respectively). Upon activation these transmembrane proteins (e.g. the receptor-like kinase family WAK), send signals for the elicitation of downstream defense responses. Changes of cell wall structure and adherence to the plasma membrane upon exposure to abiotic stresses may affect their functional integrity. This is emphasized by the observation that NDR1, an essential component of disease resistance mediated by CC-NB-LRR genes (McHale et al. 2006), is functioning in cell wall-plasma membrane
adhesion. Down-regulation of NDR1 resulted in alterations in the cell wall-plasma membrane interaction and compromised resistance to virulent *P. syringae* (Knepper et al. 2011). Abiotic stress may also affect the abundance of cell wall receptors by influencing their transcript levels. *THE1* is a member of the CrRLK1L receptor-like kinase family that is involved in cell wall damage sensing and subsequent control of the downstream accumulation of ROS, and its expression is down-regulated under abiotic stress but up-regulated after pathogen challenge (Lindner et al. 2012), while similar expression patterns are observed for the *WAK* gene family (Shaik and Ramakrishna 2013).

Pathogen recognition activates a battery of defense responses that target the apoplastic space. These include local cell wall enforcement, secretion of antifungal compounds at the site of intended penetration and up-regulation of enzymes with fungal cell wall degrading activities (Van Loon et al. 2006). These events are characterized and regulated by signature changes in pH, reactive oxygen species homeostasis and the redox state. Simultaneous exposure to abiotic stress can potentially impinge on the generation and decoding of these signatures, affecting subsequent responses. For example, apoplastic pH is transiently decreased following fungal infection (Felle et al. 2004), while an increase in pH is observed under salt stress (Geilfus and Muhling 2011). Moreover the downregulation of cell wall peroxidases under abiotic stress (Shaik and Ramakrishna 2014) can potentially dampen the production of ROS signatures that trigger defense responses (Daudi et al. 2012). Physical barriers enforcement after pathogen encounter through crosslinking of lignin monomers by ROS, which are produced by apoplastic peroxidases, NADPH oxidases and germin-like proteins, prevent pathogen penetration. Lignin content was found to be reduced under mild drought conditions to facilitate the maintenance of growth under conditions of decreased turgor pressure (Vincent et al. 2005), but severe stress resulted in increased lignin content (Lee et al. 2007a). These findings may provide insight on the mechanisms leading to differential responses under combined stress across different abiotic stress intensities.

**Vesicular trafficking and callose deposition**

Another form of inducible defense response at the site of penetration is the formation of papillae that contain callose, antimicrobial secondary metabolites such as phenolic compounds, and reactive oxygen species (ROS). Antimicrobial compounds are accumulating through vesicles originating from cellular compartments, such as the Golgi apparatus, which become polarized towards the site of infection (Underwood
and Somerville 2008). The significance of vesicle-mediated secretion in plant immunity has been demonstrated by the discovery of mutants defective in exocytosis of vesicles (with mutations in SNARE complex proteins HvROR2 and AtPEN1), which display diminished penetration resistance to powdery mildew pathogens (Collins et al. 2003). Vesicular trafficking appears to be rewired in an opposite way under salt stress, as vesicles containing Na\(^+\) are fused with the central vacuole to maximize compartmentalization of Na\(^+\) (Hamaji et al. 2009). Interestingly, knockout of different SNARE proteins resulted in increased salt tolerance (Hamaji et al. 2009), indicating possible antagonistic interactions of salt stress and pathogen infection at the level of vesicle trafficking, although further comprehensive experiments are required to substantiate this hypothesis.

Callose is a β-1,3-glucan polymer that is deposited at the sites of attempted fungal penetration in the form of papillae. It is an important inducible defense mechanism, with enhanced deposition being observed after exogenous application of priming chemicals like β-ABA. A mutant screen for plants defective in β-ABA -induced priming identified among others mutants in the ABA biosynthesis gene zeaxanthin epoxidase (ABA1) (Ton et al. 2005). These mutants failed to exhibit both β-ABA-induced callose deposition against *H. parasitica* and increased tolerance to salt stress, thereby providing a link between the induction of abiotic and biotic stress responses by β-ABA. In accordance with these observations the callose mediated increased resistance of the *ocp3* Arabidopsis mutant to necrotrophic pathogens requires ABA (Garcia-Andrade et al. 2011). Moreover *ocp3* mutants accumulate higher levels of ABA, and are more drought tolerant (Ramírez et al. 2009). Therefore *ocp3*, a homeodomain transcription factor, appears to be a convergence point for ABA and callose regulation that can be manipulated to enhance resistance under combinatorial stress.

Callose accumulation appears to be a point of convergence of abiotic and biotic signalling as variability in environmental conditions, which affect the redox state of the plant, such as light intensity, have a significant impact on the magnitude of callose deposition after pathogen elicitation (Luna et al. 2011). As callose deposition is a major component of the pre-invasion defense of plants (Ellinger et al. 2013), detailed characterization of the regulation of callose accumulation under abiotic stress may be invaluable in building combined stress tolerance in crops.

**Intracellular signalling interactions**

**Interconnections between Ca\(^{2+}\) and ROS signalling**
Changes in calcium fluxes and production of reactive oxygen species are among the earliest plant responses to abiotic stress and pathogen challenge. The decoding of both signals relies on “signature” spatiotemporal patterns and oscillations specific to the stress encountered (Dodd et al. 2010; Mittler et al. 2011). Moreover, both components are highly interconnected: Ca$^{2+}$ signalling components such as calmodulins (CaMs) and calcium-dependent protein kinases (CDPKs) regulate ROS production by NADPH-oxidases (Takahashi et al. 2011). ROS vice versa affect Ca$^{2+}$ signalling through regulation of Ca$^{2+}$ permeable channels (Demidchik et al. 2007). It is plausible that there are either unique signatures for combinations of stresses, or that there is interference between the abovementioned signals that potentially dampens or strengthens the downstream responses.

Whole genome expression analyses coupled with promoter motif identification provided further evidence that Ca$^{2+}$ orchestrates the early responses to both biotic and abiotic stress as the overrepresented motif “CGCGTT” identified in the promoters of the commonly regulated genes, contains the core “CGCG” Ca$^{2+}$ responsive cis-element (Walley et al. 2007). The investigation of mutants defective in the induction of a hypersensitive response after pathogen infection has led to the identification of genes encoding for cyclic nucleotide gated channels (CNGCs) which are non selective cation transporters (Clough et al. 2000). members of which are also involved in salt and heat stress tolerance through regulation of Ca$^{2+}$ fluxes (Finka et al. 2012; Guo et al. 2010). Furthermore, Ca$^{2+}$ downstream signalling components have been shown to mediate responses to both abiotic and biotic stress stimuli. The CAMTA3 transcription factor is important for cold acclimation of Arabidopsis by stimulating the expression of CBF1, CBF2 and ZAT12 that are also involved in adaptation to dehydration and oxidative stress (Doherty et al. 2009). Moreover, CAMTA3 negatively regulates SA accumulation and plant defenses through calmodulin (CaM) binding (Du et al. 2009). Other proteins interacting with CaM include transcription factor families like NAM, WRKY and MYB (Popescu et al. 2007) many members of which are involved in abiotic and biotic stress crosstalk.

CDPKs have a unique feature to both bind calcium and functionally decode the message by target protein phosphorylation. They appear to represent a central node in the regulation of abiotic and biotic stress responses (Schulz et al. 2013). For example, Arabidopsis CPK4 and CPK11 positively regulate ABA responses and their down-regulation renders plants salt-sensitive (Zhu et al. 2007), and are important for the oxidative burst and defense responses (Boudsocq et al. 2010). In addition, CDPKs regulate ROS production through phosphorylation-mediated regulation of RBOH activity (Dubiella et al. 2013). StCDPK4 and StCDPK5 mediated phosphorylation
increases the activity of StRBOHs and the increased ROS production results in a stronger hypersensitivity response after pathogen challenge, favouring resistance against biotrophic pathogens but compromising resistance against necrotrophic fungi (Kobayashi et al. 2012). Recently, the calcium-dependent protein kinase OsCPK12 was shown to increase salt stress tolerance and decrease blast disease resistance in rice through reduced ROS production as a result of down-regulation of RBOH expression, enhanced expression of antioxidant genes such as APX, and increased sensitivity to ABA (Asano et al. 2012).

Dissecting the spatiotemporal and molecular specificity of Ca$^{2+}$ and ROS signalling components is crucial for determining their precise functions in stress responses (Baxter et al. 2013), as is elegantly demonstrated by the identification of different Ca$^{2+}$ binding affinities regulating the activation of two soybean CaMs (Gifford et al. 2013).

**Signal relay by MAPKs**

Mitogen activated protein kinases (MAPKs) are centrally positioned in Ca$^{2+}$-ROS crosstalk and regulation as well as in the signal output after stress exposure. MAPK signalling cascades are relayed through MAPK kinase kinases (MAP3Ks) and MAPK kinases (MAP2Ks). Hydrogen peroxide (H$_2$O$_2$) has been shown to mediate activation of the three major and well-studied Arabidopsis MAPKs, MAPK3, 4 and 6, through MAP3Ks and other kinases (Rentel et al. 2004; Teige et al. 2004). These MAPKs appear to have an overlapping function in signal transduction upon abiotic stress and pathogen challenge. Activation of Arabidopsis MAPK3 and MAPK6 as well as their homologues in tobacco WIPK and SIPK (Segonzac et al. 2011) after PAMP recognition is essential for fungal and bacterial resistance (Asai et al. 2002). The importance of MAPK3 and MAPK6 in plant immune responses is highlighted by the discovery that the *P. syringae* effector HopAI1 directly interacts and inactive both, promoting virulence (Zhang et al. 2007). Additionally, MAPK6 is directly involved in regulating ethylene biosynthesis in Arabidopsis by activation through phosphorylation of ACS2 and ACS6, which results in an increase in ethylene biosynthesis (Liu and Zhang 2004). MAPK4 acts as a negative regulator of defense responses and SA accumulation by phosphorylating MEKK2, a MAP3K protein (Kong et al. 2012).

On the other hand down regulation of MAPK3 resulted in altered stomata opening patterns in response to ABA and H$_2$O$_2$ in Arabidopsis (Gudesblat et al. 2007). Moreover, the ABA-induced expression of *AtCAT1*, which is involved in H$_2$O$_2$ homeostasis, is controlled by an *AtMKK1-AtMAPK6* signalling cascade (Xing et al. 2013).
Constitutive activation of AtMAPK4 and AtMAPK6 rendered plants more tolerant to cold and salt stress (Teige et al. 2004) and CAT2 and tAPX, which are involved in H₂O₂ regulation, appear to be regulated by AtMAPK4 (Pitzschke et al. 2009). In rice OsMAPK5 appears to be a convergence point of abiotic and biotic stress responses, as its silencing results in sensitized defense responses and resistance to fungal and bacterial pathogens at the expense of salt and drought tolerance (Xiong and Yang 2003).

These examples emphasize the complexity of MAP kinase mediated defense signalling with diverse and sometimes overlapping functions of different members of the signalling pathway. Downstream targets of MAPK6 overlapped 60% with MAPK3 targets, while a 50% overlap was observed between MAPK3 and MAPK4 targets (Popescu et al. 2009). Probably, the one-dimensional overlap can be resolved by multidimensional regulation, such as different spatiotemporal transcription and protein subcellular localization, activation thresholds, feedback loops with phosphatases and scaffolding (Samajova et al. 2013; Tena et al. 2011). Many of the above-mentioned components appear to be an integral part of broad stress tolerance priming by exogenous application of chemicals (Beckers et al. 2009; Xia et al. 2009), and the detailed study of MAPK activation, localization and substrate affinity under these conditions can increase our understanding of plant responses under stress combinations.

**Hormone signalling**

Plant hormones are central to the integration of environmental stimuli in the coordination of growth under optimal and stress conditions, including the regulation of defense responses after pathogen attack. Plant hormones do not act independently, and extensive synergistic or antagonistic interaction between hormonal pathways is observed in development and stress responses after exogenous application, or through mutant analysis (Wolters and Jurgens 2009). Transcriptomic studies have aided in unveiling these interactions (Nemhauser et al. 2006), and it was recently shown that hormonal pathways can be directly connected with each other by protein-protein interactions between their signalling components (Hou et al. 2010; Zhu et al. 2011).

ABA is the major hormone that positively contributes to adaptation to osmotic stress, a major component of several abiotic stresses. Its involvement in the regulation of defense responses has been a topic of recent comprehensive reviews (Asselbergh et al. 2008; Ton et al. 2009). The consensus is that ABA negatively regulates defense responses against both biotrophic and necrotrophic pathogens through negative
interactions with SA and JA/ET biosynthesis and signalling; ABA biosynthesis mutations show sensitization of these signalling pathways after pathogen challenge (Achuo et al. 2006; De Torres Zabala et al. 2009; Sanchez-Vallet et al. 2012). Comprehensive analyses of ABA deficient mutants revealed further pleiotropic alterations that may be part of ABA-defense crosstalk such as reduced cuticle thickness and sensitized H$_2$O$_2$ production in response to B. cinerea in tomato (Asselbergh et al. 2007) and altered cell wall composition in Arabidopsis (Sanchez-Vallet et al. 2012). Moreover ABA compromised a chemically induced systemic acquired resistance (SAR) through suppression of SA biosynthesis in Arabidopsis, while genetically enhanced ABA catabolism reversed this effect (Yasuda et al. 2008).

Nevertheless, ABA signalling can positively contribute to pre-invasive defense responses and to early defense signalling against certain necrotrophic pathogens (Adie et al. 2007). ABA positively contributes to resistance against pathogens that infect through stomata, such as P. syringae (Melotto et al. 2006), as well as to other pre-invasion defense mechanisms such as callose deposition (Adie et al. 2007; Garcia-Andrade et al. 2011; Ton and Mauch-Mani 2004).

Identification of downstream regulatory nodes that channel interactions between hormonal pathways is of great importance in fine-tuning resistance to both abiotic and biotic stress. Besides transcription factors, which will be discussed in a following section, other regulators of the transcriptional machinery have been uncovered to function in stress crosstalk. RNA chaperones such as RNA helicases are shown to regulate transcription in response to various stressors (Li et al. 2008; Mazzucotelli et al. 2008). MED25, a subunit of the Mediator complex which is a component of the transcriptional machinery, is involved in the antagonistic crosstalk between ABA and JA (Chen et al. 2012). In a recent report the Arabidopsis pathogenesis-related protein 2 (PR2), which encodes b-1,3-glucanase involved in callose degradation, was shown to be down regulated in response to ABA, partly elucidating ABA mediated capacitation of callose deposition. The ahg2-1 mutant in Arabidopsis accumulates both ABA and SA and has increased expression of defense related genes, which is an indication that ABA and SA do not always act antagonistically. Transcriptome analysis of the ahg2-1 mutant revealed complex interactions between ABA and SA signalling involving altered mitochondrial and RNA metabolism (Nishimura et al. 2009), highlighting multilevel connections between the two signalling pathways that add to the complexity and hinder straightforward conclusions.

Recent research has highlighted the direct involvement of the growth hormones gibberellin, cytokinin, auxin and brassinosteroid in responses to adverse growth conditions and pathogen attack (Robert-Seilaniantz et al. 2011). For example, GA
signalling directly regulates JA signalling, mediated through direct binding of the GA repressor protein DELLA to JAZ proteins and relieving JA signalling repression (Hou et al. 2010). DELLA proteins appear to be central nodes in abiotic and biotic stress cross-talk. ABA and ET signalling promote DELLA stabilization which positively affects ROS detoxification (beneficial for acclimation to abiotic stress) through higher expression of ROS detoxification genes (Achard et al. 2008). DELLAS also sensitize JA signalling (through binding of DELLAS to JAZ) at the expense of SA signalling, enhancing resistance to necrotrophic pathogens (Navarro et al. 2008). This may provide an explanation for the often-observed positive correlation between resistance to abiotic stress and resistance to necrotrrophs (AbuQamar et al. 2009; Navarro et al. 2008; Ramírez et al. 2009).

Cytokinins were shown to positively regulate defense responses to biotrophic pathogens (Argueso et al. 2012b) via SA accumulation, and increased defense gene expression through interaction of the cytokinin response regulator ARR2 with TGA3, a TF central for defense gene activation (Choi et al. 2010). This suggests that the increased cytokinin catabolism observed under abiotic stress-induced senescence may potentially contribute to further down-regulation of SA responses and increased susceptibility to biotrophic pathogens.

The roles of auxin and brassinosteroids in stress responses and their potential participation in stress crosstalk remains elusive. Auxin signalling shows antagonistic crosstalk with SA (Wang et al. 2007), although auxin contributes to reduced senescence (Kim et al. 2011a) which may be of great importance under exposure to a stress combination. Brassinosteroid (BR) signalling positively affects abiotic stress tolerance, as is evident by both BR exogenous application (Divi et al. 2010) and genetic de-repression of the BR signalling pathway (Koh et al. 2007). BR signalling probably interacts synergistically with ABA signalling and stimulates ROS detoxification (Divi et al. 2010). BR’s involvement in defense signalling is rather complicated. In tobacco and rice exogenous application of BRs appeared to clearly enhance resistance to a wide range of pathogens (Nakashita et al. 2003). Similar results were obtained in cucumber, which showed heightened resistance to *Fusarium oxysporum* as a result of activated production of H$_2$O$_2$ by NADPH oxidase and expression of defense related genes (Li et al. 2013b). On the contrary BRs appear to be negatively regulating resistance to the root-infecting oomycete *Pythium graminicola* by antagonising SA and GA related defense responses (De Vleesschauwer et al. 2012). BR signalling shares LRR-RLK and BAK1 proteins with PAMP immune signalling (Chinchilla et al. 2009). Contradictory effects of BR signalling on immune responses
have been recently reported in Arabidopsis (Albrecht et al. 2012; Belkhadir et al. 2012; Lin et al. 2013), which require further study.

It is clear that hormonal crosstalk is extensive and occurs in multiple combinations. Further understanding of plant responses under combined stress exposure is required to dissect the multilevel responses under these conditions. As an example of the underlying complexity, both drought stress and exogenous ABA application result in an increased endogenous ABA content in tomato, but they differentially affect resistance to powdery mildew and Botrytis, with drought enhancing and ABA application compromising resistance (Achuo et al. 2006). Notably the ABA-deficient tomato mutant sitiens exhibited increased resistance similar to the effect of drought (Achuo et al. 2006). The complexity of interactions under abiotic stress is further emphasized by transcriptome analyses under abiotic stress in which up-regulation of a significant number of JA/ET-responsive genes and accumulation of their transcripts was observed (Huang et al. 2008; Walia et al. 2007). Besides the effects of direct hormonal interactions on abiotic and biotic stress tolerance mechanisms additional indirect interactions should be considered, such as the alteration of developmental programs and the regulation of senescence which may be critical for evolutionary species fitness and yield performance in crop plants (Wu et al. 2012a).

**Cellular redox state**

The cellular redox state is the sum of reducing and oxidising redox-active molecules (Potters et al. 2010) and it acts both as a sensor of environmental perturbations (as most of them impose oxidative stress) and as a buffer against these perturbations to maintain cellular homeostasis. It acts as a central integrator of ROS, energy and metabolic regulation under stress as well as optimal conditions. Its major constituents are ascorbate, glutathione (GSH), NADP(H), small proteins acting as antioxidants like thioredoxin and glutaredoxins as well as many diverse metabolites such phenolics, amino acids, carotenoids and tocopherols. The cellular redox state is dependent on both their accumulation and their reduction-oxidation state (Potters et al. 2010). Genetic manipulation of redox homeostasis results in altered hormone homeostasis and responses to pathogens and abiotic stresses (Mhamdi et al. 2010), exemplifying its significance. As abiotic and biotic stress commonly impinge on the redox status (albeit not in a similar manner (Foyer and Noctor 2005)), redox homeostasis is potentially a central orchestrator of the phenotypic response to stress combinations. Redox perturbations after imposition of a stress factor may affect responses to subsequent challenges by additional stressors, thereby shaping the
response to combined stresses. For example, a transient increase in glutathione content drives the antagonistic crosstalk between SA and JA signalling (Koornneef et al. 2008) and glutathione oxidation appears to drive the induction of both SA and JA pathways (Mhamdi et al. 2013).

Plant hormone signalling can directly perturb the redox status by modifying the expression and activities of antioxidant enzymes. ABA induces the expression of catalase, activating also at the same time the production of the ROS hydrogen peroxide through AtMAPK6 signalling (Xing et al. 2008). SA inhibits the function of catalase and cytosolic ascorbate peroxidase (Corina Vlot et al. 2009) and several glutathione transferases (Tian et al. 2012).

Programmed cell death (PCD) is a plant response to developmental and environmental stimuli (e.g. in senescence) and pathogen defense (in the form of HR) that is initiated and regulated by redox changes, like an increased oxidation ratio of GSH and ascorbate (De Pinto et al. 2012). Ascorbate peroxidase (APX) appears to be central in the redox regulation leading to PCD. Decreased activity of APX isoforms was observed in heat-induced PCD (Locato et al. 2009), and overexpression or down-regulation in Arabidopsis of a thAPX increased or decreased, respectively, sensitivity to NO-induced cell death (Tarantino et al. 2005). APX isoforms are also commonly up-regulated under abiotic stress (Miller et al. 2008). Considering the important role of APX in the drought-heat stress interaction (Koussevitzky et al. 2008) it is of great interest to explore APX enzyme regulation under combinatorial stress.

Redox status changes can directly impact protein function through post-translational modifications. One pronounced example of post-translational modifications controlling protein activity and localization is the interplay of S-nitrosylation and thioredoxin-mediated reduction in the control of the oligomeric and monomeric state of NPR1 (Tada et al. 2008), a master regulator of SA mediated defense responses and recently proposed as a SA receptor (Wu et al. 2012b). The function of many more proteins appears to be regulated by S-nitrosylation, among them ArRBOHD as mentioned above (Yun et al. 2011), SA binding protein 3 (SABP3), methionine adenosyltransferase 1, the metabolic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glycine decarboxylase (GDC), as well as metacaspase 9(Astier et al. 2011). Identification of the dynamics of post-translational modifications on these and newly identified proteins under various stress combinations will shed light on their significance for plant adaptation responses to these conditions.

NO was recently found to exhibit biphasic control over cell death triggered by pathogens and pro-oxidants in Arabidopsis. In initial stages S-nitrosothiol (SNO)
accumulation results in enhanced and accelerated cell death (Yun et al. 2011). However, constitutively high SNO levels decreased cell death through S-nitrosylation mediated reduction in AtRBOHD activity (Chen et al. 2009a; Yun et al. 2011). This differential regulation might have implications in conditions of combined abiotic and biotic stress as both result in increased NO levels. At a certain plateau concentration of NO, signalling components may be desensitized or inversely regulated, as exemplified by AtRBOHD, with detrimental effects on stress acclimation.

Redox changes and post-translational modification appear to be integral in priming for stress tolerance after exogenous application of chemicals (Tanou et al. 2009). This provides a potential explanation of the mechanism of action of diverse chemicals in plant defense sensitization. H\textsubscript{2}O\textsubscript{2} and NO priming for salt tolerance in citrus moderately increased the abundance of oxidized and S-nitrosylated proteins, which then remained relatively similar after the application of stress. Non-treated plants were more stress sensitive and exhibited increased protein carbonylation and oxidation (Tanou et al. 2012). As both compounds provide increased tolerance to both abiotic and biotic stress, further characterisation including the timing and magnitude of these post-translational modifications under different stress treatments and under stress combination may help to better understand the redox changes leading to stress cross-tolerance.

**Metabolite homeostasis and signalling**

Metabolites are the end products of gene expression and protein activities and therefore are the penultimate regulatory component for the phenotypic expression under stress conditions. As metabolites can have multiple functions such as being energy carriers, structural molecules and redox regulators or exerting direct antimicrobial activity against pathogens, uncovering their regulation and homeostasis under combined stress is of great significance.

Adaptation to both abiotic and biotic stress impinges significantly on primary metabolism homeostasis. Synthesis of antimicrobial metabolites and defense proteins is energy demanding (Bolton 2009), while abiotic stress potentially leads to energy deprivation as photosynthesis is reduced under abiotic stress (De Block et al. 2005). As a result, it is fair to assume that under stress combinations these strong antagonistic effects will result in disturbed energy balance. However, recent results challenge the carbohydrate deprivation notion under mild dehydration stress (Hummel et al. 2010) and further experimental data under combined stress are required for firm conclusions. More evidence that sugar homeostasis and signalling
drives defense responses are demonstrated by the down regulation of cell wall invertases. This results in dampening of defense responses and increased susceptibility to pathogens as a result of decreased availability of carbohydrates to fuel the defense responses at the site of infection (Essmann et al. 2008). Cell wall invertases appear to be down regulated under abiotic stress (Wingler and Roitsch 2008) and as the regulation of their activity is a convergence point of hormonal and sugar signals for stress tolerance and senescence progression (Wingler and Roitsch 2008), fine tuning of their expression might be a focal point in enhancing combined stress tolerance. The metabolic status of the host is also crucial for pathogen growth as it appears that pathogens manipulate different aspects of plant metabolism to achieve optimal conditions for their growth (Chen et al. 2010b).

The significance of amino acid homeostasis for the induction and regulation of defense responses was recently highlighted (Zeier 2013). Amino acids may function as precursors in hormone biosynthesis and affect the redox state through their chemical properties or as precursors of redox regulators such as glutathione. Amino acid abundance can impact hormone signalling through conjugation mediated regulation of hormone activity (Woldemariam et al. 2012). Amino acid concentration appears to be significantly perturbed by abiotic stress as is revealed by metabolomics studies (Obata and Fernie 2012). On the other hand a direct link between amino acid abundance and activation of SA-induced defense responses was recently demonstrated with heat-shock factor HsfB1, the translation of which is initiated under conditions of phenylalanine starvation (Pajerowska-Mukhtar et al. 2012). Phenylalanine appears to be accumulated under abiotic stress conditions (Urano et al. 2009; Widodo et al. 2009) and its potential as a molecular switch between abiotic and biotic stress responses should be explored.

Metabolic alterations under abiotic stress include the accumulation of compounds such as the raffinose family oligosaccharides raffinose and galactinol and the amino acid proline. These exhibit osmoprotective and antioxidant functions and have been positively correlated with abiotic stress tolerance (Korn et al. 2010). Galactinol overproduction was recently associated with increased resistance to necrotrophic pathogens (Mi et al. 2008). Moreover, proline metabolic regulation at the site of pathogen infection is important for both HR deployment and containment, probably through modulation of ROS levels as shown by expression and functional studies of proline dehydrogenase (Senthil-Kumar and Mysore 2012). Myo-inositol metabolic regulation appears to be a convergence point for abiotic and biotic stress responses. Myo-inositol is accumulating under most abiotic stress conditions and is positively contributing to tolerance as a compatible solute (Tan et al. 2013). A negative
relationship between myo-inositol accumulation and pathogen resistance and PCD initiation was found in Arabidopsis, with a positive correlation between myo-inositol depletion and increased SA production and cell death (Chaouch and Noctor 2010).

Analysis of mutants that exhibit qualitative and quantitative alterations in the accumulation of fatty acid metabolites demonstrated that fatty acids are not only structural components of the cellular membranes, but they also exert a multitude of signalling functions. Fatty acid release from the membranes after pathogen encounter triggers the defense response (Savchenko et al. 2010). Linolenic acid (18:3) is a precursor for the production of the major cellular signalling components JA and oxylipins (Reinbothe et al. 2009). A reduction of the levels of oleic acid (18:1) triggers constitutive defense responses that are independent of SA signalling (Kachroo et al. 2001), but dependent on NO production (Mandal et al. 2012). Fatty acid homeostasis is disturbed under abiotic stress, as membrane composition changes are vital for the maintenance of membrane rigidity and functionality. Dehydration stress is shown to result in a reduction in 18:3 and increase in 18:1 lipid levels (Upchurch 2008), and increased 18:3 levels by FAD3 or FAD8 overexpression enhanced drought tolerance in tobacco (Zhang et al. 2005). Manipulation of fatty acid composition can provide further insight into their function under stress combination.

**Transcription factors**

Regulatory modules like MAPKs-based pathways and core hormone signalling modules control the expression of a vast number of genes and therefore their manipulation in most cases have severe pleiotropic effects. Identification of downstream regulators involved in abiotic and biotic stress crosstalk such as transcription factors (TFs) is important for more targeted manipulation and adaptation of plants to multiple stresses. The appropriate fine-tuning of their expression is an important aspect towards translation of scientific knowledge in crop plant improvement (Kasuga et al. 2004).

Bioinformatics and functional analyses have demonstrated that TFs involved in stress crosstalk comprise a diverse collection of members of the largest TF families in plants, such as NAC, MYB, AP2/ERF, WRKY and others, reflecting the complexity of the genetic regulatory networks underlying stress crosstalk (Atkinson and Urwin 2012; Shaik and Ramakrishna 2014). Many members of these families are involved in regulation of leaf senescence, an integral component of both abiotic and biotic stress (Breeze et al. 2011). Moreover, in most cases the TFs identified are stress hormone-
regulated, and therefore potentially act as molecular switches for the fine-tuning of hormonal responses.

Characterization of the mechanism of action of the candidate transcription factors involved in stress crosstalk is of great importance. For example a TF with positive contribution to both abiotic and biotic stress tolerance can be directly useful for breeding combined stress tolerance. Functional characterization of several TFs has revealed various members that confer both abiotic and biotic stress tolerance. Overexpression of the rice OsNAC6 conferred tolerance to salt and dehydration stress as well as resistance to blast disease (Nakashima et al. 2007). Similarly, in wheat, overexpression of the R2R3MYB gene TaPIMP1 results in drought stress tolerance and resistance to Bipolaris sorokiniana through increased expression of abiotic stress (many of them ABA inducible) and defense-related genes (Zhang et al. 2012). Members of the AP2/ERF TF family have been shown to be positive regulators of both abiotic and biotic stress (Jung et al. 2007; Zhang et al. 2009). DREB TFs are also members of the AP2/ERF family and important contributors to abiotic stress tolerance (Liu et al. 2013a). AtDREB2A was upregulated in plants overexpressing the CC-NB-LRR gene ADR1 which conferred pathogen resistance and drought tolerance (Chini et al. 2004). Overexpression of OsDREB1B in tobacco resulted in increased resistance to abiotic stress and also virus infection (Gutha and Reddy 2008).

Overexpression of AtHSFA1b provided stress hormone independent, but H$_2$O$_2$ signalling dependent increased tolerance to drought and resistance to bacterial and oomycete pathogens (Bechtold et al. 2013). It appears that the HSF TF gene family has broad biological functions in ROS signalling and defense responses and systemic acquired resistance regulation (Miller et al. 2008; Pick et al. 2012), which can be further exploited for building broad stress tolerance into crops. Whole genome expression meta analyses can provide evidence of potential antagonistic regulation in different stress responses for a given TF, by analysing expression patterns under different stress conditions (Shaik and Ramakrishna 2014). Detailed characterization of spatiotemporal expression and cis-element binding patterns is however required for the understanding of the underlying mode of regulation. This was recently elegantly demonstrated in the characterization of OsWRKY13 which exhibits tissue specific expression and condition specific binding to cis-elements of downstream genes and thereby inversely regulated resistance to drought and bacterial infection of rice (Xiao et al. 2013)

Functional conservation of TF functions across species can be exploited to take advantage of the wealth of experimental data generated in the model plant
Arabidopsis thaliana. For example the Arabidopsis AtBOS1, an R2R3MYB TF, as well as its homologue in tomato SlAIM1 appear to regulate tolerance to abiotic and biotic stress in the same way, as mutant plants exhibit reduced tolerance to salt stress as well as to Botrytis infection (AbuQamar et al. 2009; Mengiste et al. 2003). Further similar efforts should be undertaken to accelerate the translation of experimental observations obtained in model plants species to crops.

The results obtained by the functional characterization of TFs are encouraging as many of them appear to regulate cross-resistance in a unidirectional manner, in contrast to the observations at the level of hormonal regulation that point to antagonistic relationships. Therefore, their manipulation offers many opportunities to bypass the antagonistic effects on abiotic and biotic stress tolerance observed in the more upstream regulatory nodes.

Epigenetic modifications

Epigenetic modifications such as DNA cytosine methylation and histone residues methylation and acetylation contribute to the transcriptional control of amongst others adaptive responses to environmental stimuli (Mirouze and Paszkowski 2011). A significant portion of these modifications appears to be persistent across generations and significantly contributes to phenotypic variation (Johannes et al. 2009). While cytosine methylation generally has repressive effects on gene transcription, leading to gene silencing, histone modifications can lead to transcriptional activation through local chromatin de-condensation which facilitates the accessibility of transcription factors (Liu et al. 2010). Recently, epigenetic modifications and specifically chromatin-regulated gene activation have been proposed to govern priming responses (Conrath 2011). Genome wide approaches studying DNA methylation under abiotic and biotic stress have demonstrated widespread methylation alterations (Bilichak et al. 2012; Dowen et al. 2012). It would be of particular interest to further examine the occurrence of differential alterations and their impact under combinatorial stress.

Functional studies of chromatin remodelling enzymes have revealed a functional involvement of these enzymes in the regulation of both abiotic and biotic stress responses. Histone deacetylase 19 (HDA19) mutants exhibit enhanced basal expression of many SA-responsive genes (Kim et al. 2008) but decreased expression of ABA and JA/ET-responsive genes, and the mutants are hypersensitive to salt stress (Chen et al. 2010c). The histone lysine methyltransferase ATX1 is likely to be involved in dehydration stress signalling, as atx1 mutants were sensitive to drought and ATX1
methyltransferase activity positively regulated the expression of the ABA biosynthesis enzyme NCED3 (Ding et al. 2011). Interestingly, down-regulation of the transcription factor WRKY70 during dehydration stress coincided with decreased presence of ATX1 at the WRKY70 gene locus (Ndamukong et al. 2010).

Chromatin structure can also be altered by the active deposition of variants of the canonical histones. Deposition of one of these variants, H2A.Z, is linked to transcriptional activation in response to environmental stimuli (Coleman-Derr and Zilberman 2012), and disruption of this mechanism leads to misregulated responses to both pathogens and elevated temperature (Kumar and Wigge 2010; March-Diaz et al. 2008).

It would be highly interesting to investigate how a previously imposed stress predisposes plants at the methylation and chromatin level for the encounter of a subsequent stress, (de)sensitizing subsequent responses. This type of acclimation/predisposition may even be a useful tool for preparing seeds and propagated material for stressful environments.

**R-gene resistance and systemic acquired resistance**

The plant immune system consists of successive layers counteracting suppression of defense responses by pathogens through secretion of effector proteins (Hemetsberger et al. 2012). Recognition of the effectors by corresponding R-genes belonging to NB-LRR protein family or the effect of effectors on intracellular host proteins (guarded proteins) results in effector-triggered immunity (ETI). This is usually but not always manifested by localized cell death, termed the hypersensitivity response (Coll et al. 2011). The complexity in the regulation of ETI is outlined by network analyses of individual and combined hormone mutants, which revealed compensatory interactions in contrast to synergistic interaction observed in PTI (PAMP-triggered immunity) (Tsuda et al. 2009), and which may explain the robustness of ETI to genetic perturbations. This robustness may be ideal in building tolerance to combinatorial stress through pyramiding R-genes with genes conferring abiotic stress tolerance.

However, it is becoming clear that there are multiple aspects of regulation at the NB-LRR protein level that are indispensable for the deployment of R-gene resistance (Heidrich et al. 2012). These include spatial regulation of NB-LRR accumulation in cellular compartments (e.g. the nucleus). Reduction of nuclear NB-LRR accumulation was shown to be responsible for the heat stress attenuation of disease resistance conferred by the proteins SNC1 and RPS4 in Arabidopsis(Mang et al. 2012; Zhu et al.
2010). Interestingly, mutants with reduced sensitivity to heat-induced defense inhibition were found to be based on changes in among others ABA biosynthesis enzymes, indicating that abiotic stress factors may affect R-gene compartmentation through ABA biosynthesis and signalling, although no further evidence is available. In addition, chaperone-mediated transport and folding of NB-LRR protein is important for their activity (Hubert et al. 2009a). The heat shock protein HSP90 is a component of this chaperone machinery. HSP90 is also required for the maintenance of folding of other proteins under stress conditions (Wang et al. 2004), and could potentially become limiting for proper R-gene signalling or stress protection under combined stress conditions. The recent discovery that NB-LRR protein accumulation is controlled by microRNAs (Zhai et al. 2011) adds a novel layer of regulation that would be interesting to investigate under different stress conditions (Kulcheski et al. 2011).

Initial pathogen perception and interception through PTI or ETI triggers systemic signals that prime plant defense responses to effectively counter subsequent infection attempts and limit spreading of the disease. This is referred to as systemic acquired resistance (SAR). Many compounds and genes have been identified that function in mobile signal generation and transport. Conversion of MeSA produced at the infection site to SA at the systemic tissues appears to be a prerequisite for SAR manifestation (Park et al. 2007). Additional metabolites such as pimelic acid, dehydroabietinal, azelaic acid, and glyceraldehyde-3phosphate probably function in the amplification of the signal, with no clear conclusions yet on their precise placement in the SAR circuit pathway (Dempsey and Klessig 2012). SAR has been shown to be affected by environmental conditions such as exposure to light (Griebel and Zeier 2008) and abiotic stresses such as salinity, through ABA suppression of SA biosynthesis (Yasuda et al. 2008). The further investigation of the patterns of accumulation and transport of these metabolites under conditions of combined abiotic and biotic stress may reveal potential connections between their regulation and plant phenotypic responses to combined stress.

**Approaches for gene identification and breeding for tolerance to stress combination**

In accordance with individual abiotic and biotic stressors, each abiotic stress/pathogen/host combination should be treated independently as, despite the potential universal applicability of some interactions that were characterized in Arabidopsis, many unique interactions may be crucial for the phenotypic response. As a result improving crops to these complex stress conditions first requires an extensive
phenotypic characterization at different levels of cellular regulation, i.e. transcription, translation, post-translation, and metabolites, as well as at different stages of plant development. As evidence from research on individual abiotic and biotic stress responses points to a strong dependency on developmental (Skirycz et al. 2010) as well as environmental factors (Luna et al., 2011), the environmental conditions and developmental stages of the plants should be appropriately defined before any interpretation of the phenotypic and molecular response can be done. Finally the different layers of defense can be differentially affected by abiotic stress imposition (Fig. 2); therefore, the outcome of the interaction will vary with the defense mechanisms employed and on the pathogens involved.

Breeding for resistance to combinatorial stress is challenging. However various novel approaches can aid in dissecting interactions between various types of stressors and identifying genetic components that can be breeding targets. The combination of different ~omics technologies has enabled the molecular dissection of plant phenotypes (Baerenfaller et al. 2012; Nagano et al. 2012). They provide information about the biological function of the whole gene set of an organism, and overlapping expression patterns might imply participation in common pathways (Quackenbush 2003), enabling more efficient reverse genetic approaches. Utilization of~omics in combination with forward genetic approaches like association mapping (Chan et al. 2011) may narrow down the candidate genes responsible for the observed phenotypes and provide targets for functional characterization, further manipulation and improvement of crops through breeding. As mentioned previously, currently there are limited studies on the ~omics characterization of combined abiotic and biotic stress tolerance, however functional characterization of differentially regulated genes is starting to provide interesting candidates for combined stress tolerance and their mode of action (Atkinson et al. 2013).

Manipulations that induce resistance to abiotic and biotic stress such as application of priming chemicals, followed by comprehensive phenotypic characterization can be used for candidate gene identification and molecular processes underlying stress cross-tolerance. Utilization of pre-existing chemical libraries for compounds that can prime abiotic and/or biotic stress tolerance and identification of their mode of action through chemical genetics approaches can both provide biotechnological targets for crop stress improvement and an opportunity to directly use the identified chemical in agricultural practice if no unintended side effects are observed (Hicks and Raikhel 2009; McCourt and Desveaux 2010; Okamoto et al. 2013). Moreover as the effects of chemical priming are shown to in part to be exerted through induction of phosphorylation and other post-translational modifications (Beckers et al. 2009),
probing these modifications and genetically manipulating the underlying codons to constitutively mimic them (Riano-Pachon et al. 2010) can result in altered responses under combinatorial stress.

Breeding for resistance to exposure to combined abiotic and biotic stress by incorporation of genetic components regulating the response to both stresses faces various challenges. For example, transcription factors can have thousands of binding sites across the genome (Lu et al. 2013), increasing the chance of unwanted pleiotropic effects and therefore more sophisticated deployment should be employed. Both expression regulation and binding specificity can be altered through promoter and binding domain engineering (Cox 3rd et al. 2013; Desai et al. 2009) which can be aided by comparative genomic approaches (Korkuc et al. 2014) and applied through novel site-specific mutagenesis techniques (Liu et al. 2013b). As selective and stimulus specific transcription factor binding drives stress responses regulation (Xiao et al. 2013), implementation of the above methods will aid to fine-tune downstream targets towards the desired phenotypic response. A potential drawback of transcription factor utilization is that resistance typically achieved by this approach is partial, and potentially prone to numerous antagonistic effects between stresses that cannot be predicted and can hinder efficient deployment for crop improvement to combined stresses.

Pyramiding genes that provide increased tolerance to either stress and do not (negatively) interact with each other offers an alternative route. Strong resistance mediated by R-genes, that appear to be robust to perturbations, can be pyramided with well-characterized genes conferring abiotic stress tolerance (Hu and Xiong 2013) (Kissoudis et al. unpublished data). R-gene robustness can be assessed by testing resistance responses under different abiotic stressors prior to pyramiding. The drawback of this approach is the quick breakdown of resistance due to evolving pathogens, and the fact that necrotrophic fungi resistance cannot be acquired with these genes. R-gene stacking aided by novel biotechnological approaches can reduce the risk of breakdown of R-gene mediated resistance.

Pre-invasion defense mechanisms can be exploited, especially the one that is conferred by preformed or inducible physical barriers such as callose and antimicrobial compound deposition at the site of attempted penetration. As discussed earlier, callose deposition appears to be positively regulated by ABA signaling, therefore positive or no interaction should be expected under abiotic stress. Genes such as the OCP3 transcription factor can be utilized, and for instance pyramiding abiotic stress tolerance with resistance conferred by mlo loss of function which sensitizes callose deposition at the site of infection for resistance against powdery mildew (Buschges et
al. 1997) may be a viable route (Kissoudis et al. unpublished data). However pleiotropic effects reported in mlo mutants such as compromised resistance against necrotrophic pathogens (Kumar et al. 2001) and accelerated senescence (Piffanelli et al. 2002) can have adverse consequences under stress combination.

The mechanisms through which abiotic stress tolerance is conferred can have a differential effect on disease resistance. As mentioned earlier, drought tolerance through ABA upregulation at the whole-plant level is expected to have antagonistic effects with SA signaling and therefore compromises resistance to biotrophs. Localized ABA sensitization in stomata (Bauer et al. 2013) can overcome these drawbacks and offer an advantage for resistance against pathogen that infect through stomata. Manipulation of developmental traits such as root system architecture can be beneficial for drought tolerance (Uga et al. 2013) with potentially no adverse effects on disease resistance, as they employ cell type specific signaling. Deployment of genes that have a protective function on proteins and cellular components under abiotic stress, such as dehydrins, LEA proteins or RNA chaperones (Kang et al. 2013) that apparently are downstream components of abiotic stress adaptation and mostly function through their structural properties, can minimize interaction with biotic stress signaling. Moreover, under salt stress, increased tolerance through Na$^+$ compartmentalization in the vacuoles may offer an advantage in comparison with Na$^+$ exclusion, as Na$^+$ at high concentrations can have adverse effects on pathogen feeding and development.

Approaches that result in greater antioxidant capacity such as the accumulation of flavonoids appear to confer resistance to abiotic and oxidative stress (Nakabayashi et al. 2014) while overproduction of their derivatives, anthocyanins, increase resistance to the necrotrophic pathogen Botrytis cinerea in tomato by minimizing ROS burst (Zhang et al. 2013). Therefore engineering for increased flavonoid accumulation can be promising in conferring resistance to multiple stressors, however it is unknown how it can affect the deployment of hypersensitivity response due to disturbed ROS homeostasis and thus resistance against biotrophic pathogens.

Exploitation and deployment of different strategies (Fig.3) under different abiotic stress/pathogen combinations will demonstrate their feasibility and applicability, further leading towards the goal of breeding for crops that maintain their robustness and yield performance under diverse environmental conditions.
Figure 3. Approaches for building combined abiotic and biotic stress tolerance in plants. Two strategies are proposed through either the manipulation of genetic components which potentially regulate resistance to both stresses in a preferentially unidirectional manner, or the pyramiding of genes that independently confer abiotic or biotic stress resistance and do not (negatively) interact. The selection of individual components might differ depending on the pathogen and the abiotic stress scenario.

Acknowledgements

Christos Kissoudis is supported by “Alexander S. Onassis” Public Benefit Foundation scholarship.
Chapter 3

Combined biotic and abiotic stress resistance in tomato

Christos Kissoudis\textsuperscript{1,2}, Rawnaq Chowdhury\textsuperscript{1}, Sjaak van Heusden\textsuperscript{1}, Clemens van de Wiel\textsuperscript{1}, Richard Finkers\textsuperscript{1}, Richard G.F. Visser\textsuperscript{1}, Yuling Bai\textsuperscript{1}, C. Gerard van der Linden\textsuperscript{1}

\textsuperscript{1}Wageningen UR Plant Breeding, Wageningen University & Research Centre, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands or POBox 386, 6700AJ, Wageningen, The Netherlands

\textsuperscript{2}Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

Published in Euphytica (2015)
Abstract

Abiotic and biotic stress factors are the major constrains for the realization of crop yield potential. As climate change progresses, the spread and intensity of abiotic as well as biotic stressors is expected to increase, with increased probability of crops being exposed to both types of stress. Shielding crops from combinatorial stress requires a better understanding of the plant’s response and its genetic architecture. In this study, we evaluated resistance to salt stress, powdery mildew and to both stresses combined in tomato, using the *S. habrochaites* LYC4 introgression line (IL) population. The IL population segregated for both salt stress tolerance and powdery mildew resistance. Using SNP array marker data, QTLs were identified for salt tolerance as well as Na\(^+\) and Cl\(^-\) accumulation. Salt stress increased the susceptibility of the population to powdery mildew in an additive manner. Phenotypic variation for disease resistance was reduced under combined stress as indicated by the coefficient of variation (CV). No correlation was found between disease resistance and Na\(^+\) and Cl\(^-\) accumulation under combined stress. Most genetic loci were specific for either salt stress tolerance or powdery mildew resistance. These findings increase our understanding of the genetic regulation of responses to abiotic and biotic stress combinations and can provide leads to more efficiently breeding for tomatoes and other crops with a high level of disease resistance while maintaining their performance in combination with abiotic stress.

**Keywords:** combined stress, stress interactions, crosstalk, phenotypic variation, ion homeostasis
Introduction

Crops grown in open fields encounter multiple unfavorable conditions for optimal plant growth and yield, of both abiotic and biotic origin. The ongoing climate change, accelerated by the increase in atmospheric CO$_2$ concentration (Peters et al. 2011), is resulting in an average rise in temperature and decrease in precipitation especially in regions with temperate climates (Dai 2013), which further intensifies agricultural land deterioration due to extended periods of drought and an increase in soil salinity (Munns and Tester 2008; Zhao and Running 2010). Moreover, an increase in temperature and ambient CO$_2$ concentration could directly influence plant pathogens spread and geographic distribution. While studies show that on many occasions the effects on pathogenicity are pathosystem-specific (Coakley et al. 1999), the consensus is that elevated temperatures will result in pathogen geographic expansion and enhanced fecundity, increasing the chances for host range expansion and rise of more virulent strains (Garrett et al. 2006; Harvell et al. 2002). As predictions point to increased possibilities of plants encountering abiotic and/or biotic stress, exposure to combined stresses is expected to become more frequent.

The limited data available for plant responses under abiotic and biotic stress combinations point to predominantly negative interactions at the phenotypic level (Mittler 2006; Kissoudis et al. 2014). Increased soil salt concentration results in enhanced susceptibility to soil borne diseases in tomato (Triky-Dotan et al. 2005) and other crop species (Al-Sadi et al. 2010; You et al. 2011), and similar trends are observed under water deficit (Jordan et al. 1984). Observations of the effects of abiotic stress on foliar pathogens are on the other hand mixed, with studies reporting either enhanced (Achuo et al. 2006; Wiese et al. 2004), or decreased resistance (Roubtsova and Bostock 2009; Sanogo 2004). Abiotic stress severity can affect responses to abiotic and biotic stress combinations (Soliman and Kostandi 1998), and therefore the outcome of the interaction may be dependent on the specific environmental conditions under which it occurs.

Indications for stress regulatory crosstalk can be found at the phenotypic level, and are evident as well at the gene expression level (Kissoudis et al. 2014). Recently, the transcriptome of Arabidopsis subjected to combinations of various abiotic and biotic stressors was analyzed (Atkinson et al. 2013; Prasch and Sonnewald 2013; Rasmussen et al. 2013). The striking commonality of all these studies is the unique responses observed under stress combinations that could not be predicted by the response to individual stressors. Moreover it was observed that the response of a significant number of transcripts was cancelled or prioritized under stress combinations in
comparison with the individual stress, suggesting antagonistic interactions with potential detrimental effects on plant adaptation under combined stress.

Apart from the characterization of individual genes involved in both abiotic and biotic stress (Asano et al. 2012; Ramírez et al. 2009) and the recent reports on transcriptomic characterization of the response to various stress combinations in Arabidopsis, the genetic architecture of plant response to combinatorial stress has not been investigated.

In this paper we study the interaction between salinity stress and powdery mildew (PM) infection in tomato. Tomato (*Solanum lycopersicum* L.) possesses unique properties, as it is both an economically important crop, the first vegetable in production in the world (FAOSTAT, 2011), and a model plant species, due to its diploid, relatively compact, and recently sequenced genome (Sato et al. 2012) and its large genetic and genomic resources (Ranjan et al. 2012). Tomato productivity is affected by a high incidence of increased soil salinity in the areas of cultivation (Cuartero et al. 2006). Additionally, fungal pathogens can significantly limit productivity by colonizing the foliage or the fruits. The biotrophic ascomycete *Oidium neolycopersici* (causing powdery mildew) is one of the economically most important foliar pathogens of tomato, both in the greenhouse and in open field conditions (Jones et al. 2001).

We evaluated a *Solanum habrochaites* introgression line (IL) population (accession LYC4 as the donor) in the background of cultivated tomato (cv. Moneymaker, Finkers et al. 2007b) for our study. *S. habrochaites* is native to high altitude habitats in the Andean mountains (Grandillo et al. 2011), and various accessions were used as a source for cold tolerance (Venema et al. 2008) and resistance to a wide range of fungal pathogens (Grandillo et al. 2011) including PM (Huang et al. 2000). The LYC4 population was evaluated previously for Botrytis resistance (Finkers et al. 2007b) and parthenocarpy (Gorguet et al. 2008).

The results presented in this paper show that the LYC4 IL population segregated for both salt stress tolerance and PM resistance. QTLs conferring resistance to the individual stresses were identified using a high density SNP array for accurate localisation of introgressions. In addition the effect of salt stress on the genetic factors involved in PM resistance was evaluated. Various new genetic loci contributing to tomato salt stress tolerance and PM resistance were discovered. Salt stress increased susceptibility to PM, reducing phenotypic variation for disease resistance. These results provide novel genetic resources for enhancing salt stress tolerance and PM
resistance in tomato and enhance our understanding for plant responses under abiotic and biotic stress combinations.

**Materials and Methods**

**Plant material**

The core collection of a *S. habrochaites* LYC4 introgression line (IL) population in the genetic background of *S. lycopersicum* cv. Moneymaker (MM), consisting of 31 ILs covering most of the tomato genome, was used in this study (Finkers et al. 2007b). The population was originally generated aiming at maximum coverage of the wild species genome and parts of each chromosome being present in at least three ILs as assessed with AFLP markers. Twenty nine of the ILs were genotyped using a custom made single nucleotide polymorphism (SNP) Infinium array containing 5528 SNPs, as described by (Viquez-Zamora et al. 2013). The introgressed regions according to the SNP data were visualized using the software Graphical GenoTypes 2 (Van Berloo 2008).

**Experimental conditions and treatments**

Experiments were carried out in the Unifarm greenhouse facilities of Wageningen University & Research Centre. The photoperiod regime was 16 hours light and 8 hours dark. Greenhouse air humidity was 70%. Additional lighting (100 Wm$^{-2}$) was used if the incoming shortwave radiation was below 200 Wm$^{-2}$.

Tomato seeds were sown in peat and transplanted to 3L pots filled with vermiculite. The plantlets were irrigated with half strength Hoagland’s nutrient solution every two days initially, and every day in the final week of the experiment. Due to spatial restrictions and PM containment measures the simultaneous assessment of all four treatments was not possible, therefore, two subsequent and partially overlapping experiments were carried out. In the first experiment, salt stress was applied to the population by the addition of 100 mM NaCl to the nutrient solution of three weeks old plants (5 plants per line), for 21 days. The pots were watered until leaching, to ensure uniformity of the treatment and to prevent NaCl accumulation. The concentration of NaCl in the pots was regularly monitored by measuring the Electrical Conductivity (EC) of the leachate after the completion of the irrigation.

In the second experiment, PM and combined salt stress-PM resistance were assessed as follows: 3-week old plants (4 plants per line) were watered with a solution
containing 100 mM NaCl for 1 week. Subsequently, PM was applied to both (4-week old) salt stressed and non-salt stressed plants according to (Bai et al. 2003) by spraying a suspension of 5x10^4 conidia.ml^-1 (prepared by washing conidial spores from leaves of heavily infected (sporulation stage) MM plants). The plants were grown for another two weeks after inoculation.

**Traits measured**

**Salt stress experiment**

Chlorophyll content was measured using a SPAD-502 meter (Minolta, Osaka, Japan) at the third and fourth leaf counting from the bottom, one day before harvest. Plant height and shoot fresh weight (FW) were recorded at the end of the experiment. Dry weight was determined after drying the plant tissues in a forced-air oven at 70°C until the samples reached stable weight. Salt tolerance index was calculated as the ratio of (fresh or dry) shoot biomass under salt stress and biomass under control conditions for each genotype.

**Powdery mildew and combined stress experiment**

The disease severity was expressed as disease index (DI), assessed at 12 dpi (days post inoculation). DI was expressed on a scale from 0-5, slightly modified from (Bai et al. 2003), to increase the resolution of infection incidence in order to obtain a more quantitative measure of disease resistance. The values corresponded to macroscopic observations of PM growth and sporulation where 0 = healthy plant, no visible sporulation, 1 = < 0.1-10 % of foliar area affected, slight sporulation, 2 = 10-20 % area affected, 3 = 20 – 30 % area affected, 4 = 30-50 % area affected and 5 = > 50% area affected with abundant sporulation.

**Ion chromatography**

For the ion content determination the oven-dried leaves of tomato plants were ground to fine powder using a hammer mill with 1 mm sieve. The powder was ashed at 575°C for 6 hours. Ashed samples were dissolved by shaking for 15 minutes in 1 ml 3M formic acid at 99°C and then diluted with 9 ml MilliQ water. A final 500x dilution was subsequently prepared by mixing 0.2 ml sample solution with 9.8 ml MilliQ water. The concentration of Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-, PO_4^{3-} and SO_4^{2-} of each sample was measured using Ion Chromatography (IC) system 850 Professional (Metrohm Switzerland). The anions were determined using Metrosap A 150, 150/4.0 mm column equipped with a Metrosap C5/5 Supp 4/6 Guard column and the cations with Metrosap C4 Supp 4, 250/4.0 mm column equipped with a Metrosap A Supp 4/6 Guard column.
Statistical and bioinformatics analyses

Experiments were carried out in a Split plot design with 5 replications for the salinity stress experiments and 4 replications for the PM and combined stress experiment. Statistical analyses were performed using Genstat 15th edition. Introggression lines with trait values significantly different from the recurrent parent (MM) were identified using a two-sided Dunnett test (Dunnett 1955) at a type I error rate of $\alpha = 0.05$, and the underlying introgression was assigned as a QTL. Correlations between traits were calculated using the Pearson correlation coefficient ($p \leq 0.05$). The Coefficient of Variation ($CV = s / x \times 100\%$) was used to estimate trait phenotypic variation of the population. The discovered QTLs were surveyed for underlying candidate genes with Marker2Sequence software (Chibon et al. 2012) using as input the position of the distal-most SNP markers of the S. habrochaites LYC4 introgressed region.

Results

Genotyping of ILs

The custom made, Illumina Infinium based array described in (Víquez-Zamora et al. 2013) was used for the genotyping of the S. habrochaites LYC4 ILs. 1508 SNPs out of 5528 (27.2%) were polymorphic between the S. lycopersicum and S. habrochaites LYC4 parental lines after SNP filtering with the quality control criteria (Víquez-Zamora et al. 2013, Supp. Table 1). The markers were landmarked on the genomic sequence of tomato (Víquez-Zamora et al. 2013), which facilitated precise localization of the introgressions of interest and subsequent investigation of underlying putative candidate genes located in the introgressed regions. As expected the size of the introgressions in many occasions deviated significantly on what was predicted by the genetic distances examined previously with AFLP markers (Finkers et al. 2007b). On some occasions (such as in ILs 2-3 and 8-2) introgression were revealed in different chromosomes compared to the ones originally assigned (Supp. Table 1, Supp. Fig.1). However as these are only a handful of exceptions we maintained the naming of the lines of the population as reported previously (Finkers et al. 2007b).

Variation in phenotypic traits

S. habrochaites LYC4 (LYC4) was selected from different tomato wild species and tomato breeding lines that were evaluated for salt tolerance and powdery mildew (PM) resistance. LYC4 exhibited significantly higher salt Tolerance index (calculated as the ratio of total above ground FW under salinity stress and that under control...
conditions, expressed as percentage, 74.2% compared to 56.5% of MM), and was highly resistant to PM (DI score of 0.7 compared to 4 of MM). Therefore, the *S. habrochaites* LYC4 IL population in the background of *S. lycopersicum* cv. MM as described in (Finkers et al. 2007b) was chosen for this study.

The frequency distribution of the population growth (total above ground fresh biomass) under non-stress conditions revealed a normal distribution (Shapiro-Wilk test, p=0.903) after excluding LYC4, which had significantly lower biomass than the population (Fig.1a). Similarly, relative FW under salt stress (salt tolerance index—see below) followed a normal distribution (Shapiro-Wilk test, p= 0.267) (Fig.1b). Interestingly, the majority of the population exhibited increased salt stress tolerance compared to the recurrent parent (MM).

![Graphs](image)

**Figure 1.** Frequency distribution of: a) shoot FW under control conditions, b) relative shoot FW under salt stress 100mM NaCl (salt tolerance index), c) PM resistance under control conditions, d) PM resistance under salt stress (100mM NaCl) of the 31ILs and the two parental lines of the *S. habrochaites* LYC4 population. The mean values of the two parental lines are indicated by arrows.
On the other hand, the disease index (DI) frequency distribution deviated from normality, exhibiting negative skewness. This was due to the majority of the individuals of the population being susceptible to PM, and none exhibiting the level of resistance of the donor parent Lyc4 (Fig.1c). DI frequency distribution under combined salt stress and PM was similarly negatively skewed, but with an increased degree of skewness indicating that salt stress further enhanced disease susceptibility (Fig.1d).

**Identification of QTLs for salt tolerance and ion accumulation**

FW and DW under salt stress were highly correlated (r=0.9, p<0.0001), however variation between ILs was more pronounced for FW. Therefore, salt tolerance index was determined as ratio of total above ground FW under salinity and FW under control conditions expressed as percentage, normalizing the differences in growth of the different genotypes under control conditions.

Ten ILs (IL1-2, IL1-4, IL2-3, IL3-2, IL4-2, IL8-2, IL9-1, IL10-2, IL10-3, IL10-4) exhibited higher salt tolerance index compared to the recurrent parent MM (Dunnett test, p<0.05, Fig. 2a, Table 1). Salt tolerance of those lines ranged from 67-80% maintenance of growth under saline conditions compared to 56.5% of MM, with IL8-2 exhibiting the highest degree of tolerance. Among those lines, IL2-3 and IL3-2 additionally exhibited significantly higher FW compared to the recurrent parent under salt stress (108.7g and 113.2g, respectively, compared to 89 g for MM).

Putative QTLs for salt tolerance reside in the introgressed regions of the salt tolerant lines. Several lines carried large introgressions, covering almost complete chromosomes (e.g. ILs 2-3, 3-2 and 8-2), and therefore pinpointing underlying candidate genes from the numerous genes in these introgressions is not possible. Shared introgressions between different ILs conferring salt tolerance provides a strong and more precise indication for a QTL. Both ILs 1-4 and 9-1 carried a ~4Mbp introgression at the top of Chr. 9, while a common overlapping region of ~2.7 Mbp at the bottom of Chr. 10 was shared between ILs 10-2, 10-3 and 10-4.
<table>
<thead>
<tr>
<th>Introgression lines</th>
<th>Genomic region (s)</th>
<th>References</th>
<th>Candidate genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salt tolerance index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1-2</td>
<td>N/A, AFLP data only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1-4</td>
<td>Chr2: 36411532.. 41371688, Chr9: 48774.. 4715716, Chr11: 64251736.. 51936800 (het)</td>
<td>Li et al. 2011</td>
<td>NHX3, NHX4, SOS1, Cu/ZnSOD1</td>
</tr>
<tr>
<td>IL2-3</td>
<td>Chr1: 25476.. 84030880</td>
<td>Li et al. 2011</td>
<td>NHX3, NHX4, SOS1, Cu/ZnSOD1</td>
</tr>
<tr>
<td>IL3-2</td>
<td>Chr3: 1410013.. 57499392</td>
<td>Foolad 1999</td>
<td></td>
</tr>
<tr>
<td>IL4-2</td>
<td>Chr4: 3071610.. 44938712 (het), Chr4: 46110324.. 61886216</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8-2</td>
<td>Chr7: 248378.. 58142204, Chr7: 58189028.. 60992576 (het)</td>
<td>Asins et al. 2013</td>
<td>HKT1;1, HKT1;2,</td>
</tr>
<tr>
<td>IL9-1</td>
<td>Chr9: 48774.. 3988469, Chr9: 62248928.. 67116024 (het)</td>
<td></td>
<td>APX, MDHAR, GRX, ACCox, EIN2, HSFa3, HSP70</td>
</tr>
<tr>
<td>IL10-2</td>
<td>Chr10: 53339848.. 63662428</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL10-3</td>
<td>Chr10: 53339848.. 63662428</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL10-4</td>
<td>Chr10: 60924880.. 63662428</td>
<td></td>
<td>Aquaporin, ERF1,10, Peroxidase, GST</td>
</tr>
<tr>
<td><strong>Na⁺ content (salt stress)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL12-1</td>
<td>Chr12: 161288.. 52930616</td>
<td>Huertas et al. 2012</td>
<td>SOS2</td>
</tr>
<tr>
<td>IL12-3</td>
<td>N/A, AFLP data only</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cl⁻ content (salt stress)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8-2</td>
<td>Chr7: 428378.. 58142204, Chr7: 58189028.. 60992576 (het)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL12-3</td>
<td>N/A, AFLP data only</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K⁺ content (salt stress)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL9-1</td>
<td>Chr9: 48774.. 3988469, Chr9: 62248928.. 67116024 (het)</td>
<td>K⁺ channel, Cyclic nucleotide gated channel</td>
<td></td>
</tr>
<tr>
<td>K+/Na+ ratio (salt stress)</td>
<td>Chr1: 25476.. 84030880</td>
<td>NHX3, NHX4,SOS1</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>IL2-3</td>
<td>Chr6: 40011792.. 43431840</td>
<td>H^+-ATPase</td>
<td></td>
</tr>
<tr>
<td>IL6-3</td>
<td>Chr9: 48774.. 3988469, Chr9: 62248928.. 67116024 (het)</td>
<td>K+ channel, Cyclic nucleotide gated channel</td>
<td></td>
</tr>
<tr>
<td>IL9-1</td>
<td>Chr12: 74699.. 47436216, Chr12: 48239308.. 52930616</td>
<td>Huertas et al. 2012</td>
<td></td>
</tr>
<tr>
<td>IL12-1</td>
<td>N/A, AFLP data only</td>
<td>SOS2</td>
<td></td>
</tr>
<tr>
<td>IL12-3</td>
<td>Chr3: 1410013.. 57499392</td>
<td>Powdery mildew resistance</td>
<td></td>
</tr>
<tr>
<td>IL3-2</td>
<td>Chr4: 3071610.. 44938712 (het), Chr4: 46110324.. 61886216</td>
<td>RLK, Ser/Threonine kinase</td>
<td></td>
</tr>
<tr>
<td>IL4-2</td>
<td>Chr4: 58808024.. 63693464, Chr9: 48774.. 3988469</td>
<td>RLK, Ser/Threonine kinase, PAL, PR1a, ACCox, EIN2</td>
<td></td>
</tr>
<tr>
<td>IL4-3</td>
<td>Chr6: 37310260.. 40010168, Chr7: 56595944.. 61110872</td>
<td>RLK, Ser/Threonine kinase, Peroxidase, ACCox</td>
<td></td>
</tr>
<tr>
<td>IL6-2</td>
<td>Chr9: 48774.. 3988469, Chr9: 62248928.. 67116024 (het)</td>
<td>Powdery mildew resistance (salt stress)</td>
<td></td>
</tr>
<tr>
<td>IL9-1</td>
<td>Chr9: 48774.. 5464892, Chr10: 242877.. 49064720</td>
<td>NBS-LRR, RLK, PAL, PR1a, ACCox, EIN2</td>
<td></td>
</tr>
<tr>
<td>IL10-1</td>
<td>Chr9: 48774.. 5464892, Chr10: 242877.. 49064720</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Graphical genotypes and the respective performance of ILs significantly different to the recurrent parent (MM) for: a) salt stress tolerance, b) PM resistance under control conditions, c) PM resistance under salt stress. The *S. habrochaites* *LYC4* introgressed segments are depicted as dark (homozygous) and light (heterozygous) blue squares in MM genetic background (orange).

As ion toxicity in the shoot is an important aspect of salt stress, the population was profiled for ion concentrations in shoots under both control and salt stress conditions. The genetic effects of introgressions on variation in concentrations of the macronutrients Na\(^+\), Cl\(^-\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), PO\(_4^{3-}\) and SO\(_4^{2-}\) and the consequences for salt tolerance and DI were investigated (Table 2). No significant differences in ion concentrations were identified between the ILs and MM under control conditions. Under salt stress, introgression lines IL12-1 and IL12-3 accumulated significantly less Na\(^+\) compared to MM (18.2 and 18.6 compared to 25.5mg/g dry biomass
respectively, Table 1). IL12-3 was not genotyped with the SNP array, however using the AFLP data from Finkers et al. (2007b), the introgressed region was shown to overlap with that of IL12-1. Therefore, a common genetic factor potentially underlies the reduced Na\(^+\) accumulation in those lines. IL8-2 and IL12-3 accumulated significantly less Cl\(^-\) compared to MM (30.1 and 28.2 compared to 41.6 mg/g dry biomass respectively).

Table 2. Means (n=4) and range of measured ions in the parental and introgression lines under control (C) and salt stress treatment (S).

<table>
<thead>
<tr>
<th></th>
<th>MM</th>
<th>LYC4</th>
<th>ILs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± s.d. (mg/g)</td>
<td>Mean (mg/g)</td>
<td>Range</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>8.3± 0.6</td>
<td>11.6±2.8</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>25.6±3</td>
<td>30.3±3.8</td>
<td>22.7</td>
</tr>
<tr>
<td>K(^+)</td>
<td>34.3±4.7</td>
<td>41.9±3</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>25.2±1.5</td>
<td>25.6±2.7</td>
<td>27.4</td>
</tr>
<tr>
<td>K/Na c</td>
<td>4.1±0.4</td>
<td>3.8±1.1</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>1±0.05</td>
<td>0.9±0.03</td>
<td>1.2</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>12.1±2.1</td>
<td>11.6±0.2</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>41.6±5.9</td>
<td>44.3±7.1</td>
<td>35.3</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>11.1±0.7</td>
<td>10.71±0.7</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>10.9±1.6</td>
<td>11.8±2</td>
<td>10.5</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>12.7±0.4</td>
<td>10.7±0.5</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>13.4±1.8</td>
<td>14.3±2.1</td>
<td>13.4</td>
</tr>
<tr>
<td>PO(_4)(^{3-})</td>
<td>17.7± 1.3</td>
<td>22.7±1.8</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>14.5±1.5</td>
<td>16.9±3.4</td>
<td>15.8</td>
</tr>
<tr>
<td>SO(_4)(^{2-})</td>
<td>20.6± 1.4</td>
<td>9.4±0.7</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>14.8± 1.1</td>
<td>11.4±1.9</td>
<td>15.8</td>
</tr>
</tbody>
</table>
K+ concentration was significantly higher in IL9-1 (33.0 compared to 25.2 mg/g dry biomass in MM). K+/Na+ ratio, considered to be an indicator for salt tolerance (Shabala and Cuin 2008), was significantly increased in the lines IL2-3, IL6-3, IL9-1, IL12-3 and IL12-1 compared to MM. Finally, except for SO₄²⁻ in IL3-1 which exhibited a significantly higher content, Ca²⁺, Mg²⁺ and PO₄³⁻ concentrations were not significantly different in any of the ILs under salt stress.

**Correlation analyses**

Correlation analysis (Pearson r, p<0.05) under control conditions revealed a positive correlation between FW and SO₄²⁻ concentration (r=0.61) and a negative correlation with PO₄³⁻ concentration (r= -0.43). Under salt stress only a few significant correlations were observed (Supp. Table 2). Growth under salt stress was correlated with growth under non-stress conditions, however the degree of correlation (r=0.7, p<0.001) suggests a considerable differential effect of salt stress on growth, supported by the statistically significant interaction observed between FW and stress levels in an ANOVA analysis (p<0.001). Relative growth under salt stress was strongly negatively correlated with growth under non-stressed conditions (r= -0.54, p=0.0015), indicating that large plants on average are less tolerant to salt stress than smaller plants. Relative growth under salt stress was not correlated with any of the ions measured, except for Na⁺ for which a weak positive correlation was observed (r=0.37, p=0.039).

**QTLs for powdery mildew resistance**

*S. habrochaites* LYC4 exhibited a high level of resistance against PM, with limited disease symptoms (no HR observed). Several introgression lines (IL3-2, IL4-2, IL4-3, IL6-2, IL9-1 and IL12-3) had increased resistance compared to MM, however they were considerably more susceptible than LYC4 (mean DI range 2.2-3.0 compared to 4.0 for MM and 0.7 for LYC4, Fig.2b, Fig.3, Table 1). Partial resistance in IL3-2, which carries an introgression covering a large part of Chromosome 3, was characterized by the development of necrotic (HR-like) lesions at the site of fungal spore development. All other lines exhibited quantitative resistance with no visible cell death. IL4-3 and IL9-1 both carry a ~4Mbp introgression at the top of Chr. 9. However, IL4-3 has an additional introgression at Chr. 4 that overlaps for ~3Mbp with the introgressed segment of IL4-2, a line that was also more resistant. IL6-2 carries two introgressions of ~2.7 and 4.8 Mbp on chromosomes 6 and 7, respectively. The AFLP marker data point to a large introgression in IL12-3 at the bottom arm of Chr. 12.
Correlation analysis of DI with growth and ion content revealed a positive correlation with FW (r= 0.47, p=0.006) and a negative with PO$_4^{3-}$ content (r= -0.31, p=0.047), while no significant correlation was observed between DI and chlorophyll content.

**Figure 3.** *PM resistance under control conditions (black bars) and under salt stress conditions (light grey bars) of the 31 S. habrochaites LYC4 ILs and the two parental lines. The lines are in ascending order of susceptibility under non-salt stress conditions. Asterisks indicate significant differences between the introgression lines and the recurrent parent MM (Dunnett test, p<0.05).*

**Combined salt and PM stresses**

Salt stress imposition significantly increased the susceptibility to PM of the population (mean DI of 4.45 compared to 3.65 without salt stress, p<0.001). The effect was more pronounced when only the lines with significantly greater resistance than MM under either stress condition were included (ILs 3-2, 4-2, 4-3, 6-2, 9-1, 10-1 and 12-3, mean DI of 3.93 compared to 2.78 without salt stress, p<0.001).

Three out of four ILs identified with significantly reduced DI under combined stress conditions (ILs 4-2, 4-3 and 9-1) had also significantly reduced DI under non-salt stress conditions (Fig. 2c, Fig.3, Table 1). Only IL10-1 exhibited increased resistance to PM uniquely under salt stress; it was only marginally (non-significantly) more resistant than MM under non-salt stress conditions.

To evaluate the effect of combinatorial stress on the phenotypic variation of the population for PM resistance, the coefficient of variation (CV) values for DI were
compared under PM and combined PM and salt stress. The CV under the combination of stresses was considerably lower than under PM infection (12.63% compared to 18.92%), which might indicate that the phenotypic variation for PM was reduced under salt stress (Fig.4a). This could also be a result of the population susceptibility shifting towards the maximum of the disease score scale. However, when CV was calculated for the lines that were more resistant under either conditions a similar trend was observed (14.53%, mean=3.93 (combined stress) compared to 18.56%, mean =2.78 (PM only), Fig.4b).

![Figure 4. Phenotypic variation of PM resistance under non-salt stress and salt stress conditions expressed as the coefficient of variation (CV) estimated in a) the whole IL population (31 lines) b) ILs more resistant compared to MM under either control or salt stress conditions (7 lines).](image)

No significant differences were observed between the ILs and MM for ion content under combined salt stress and therefore no QTLs could be assigned.
DI in non-salt stressed plants was significantly correlated with DI under combined stress ($r=0.77$, $p<0.0001$, Fig. 5), suggesting minor interaction of salt stress with the genotypic differences in plant susceptibility, supported by the (marginally) non-significant ($p=0.092$) interaction observed, after ANOVA analysis. No further significant correlations were identified under combined stress, except a weak positive correlation of DI with chlorophyll content ($r=0.32$).

![Figure 5](image)

**Figure 5.** Pearson correlation ($r$) between PM resistance (DI) under non-stress control conditions and under salt stress.

**Discussion**

Salt stress and powdery mildew individually pose a significant threat to tomato production, and the probability of these occurring at the same time may result in non-additive effects on plant fitness. Therefore we examined the responses of the *S. habrochaites* LYC4 IL population to both separately applied salt stress and PM and the combination of these stresses. Even though the experiments were done in the greenhouse in a controlled environment, small differences in the environmental conditions between both experiments that influence the plants' performance and the measurements could not be avoided. Therefore, direct comparisons of the trait values measured in the two different experiments need to be done with caution. Nevertheless, QTL co-localization will be discussed, as most of the QTLs identified were reproducible in subsequent experiments with the selected ILs.
Several *S. habrochaites* introgressions contributed significantly to salt tolerance, and our results indicate that under the stress conditions applied the IL lines that were larger in size were more affected by salt stress. The more vigorous (or high yielding) genotypes under non-stress conditions are often also the best-performing plants under mild stress in particular, with usually no crossover interactions (Blum 2005). The salt concentration applied in our experiments (100mM NaCl) is considered to exceed this crossover point for tomato (Maggio et al. 2007). This is supported by the significant interactions between plant biomass and salinity level and the moderate correlation between plant biomass under control and salt stress conditions. Interestingly, two ILs with higher FW than the recurrent parent MM under both control and salt stress (IL3-2 and 2-3) also exhibited a higher salt tolerance index. These properties make them valuable starting material for salt tolerance breeding, especially IL2-3 which was previously shown to out-yield, under control conditions, the parental line MM while IL3-2 had substantially lower fruit yields (Finkers 2007).

Several genomic regions of LYC4 contributed to an increased salt tolerance index. Many of the QTLs identified in this study co-localized with previously discovered stress tolerance-contributing loci in segregating populations derived from crosses with wild tomato species (Table 1). The introgressed region in Chr. 1 of IL2-3 coincides with QTLs identified in previous studies for enhanced germination, vegetative growth and fruit yield under salt stress (Foolad 1999; Villalta et al. 2007). In this region, many candidate genes reside that are involved in the regulation of ion homeostasis, such as NHX3, NHX4 and SOS1 (Gálvez et al. 2012; Olías et al. 2009), and for redox homeostasis such as Cu/ZnSOD1 (Chen et al. 2009). Notably, IL2-3 exhibited lower Na⁺ accumulation (19.9mg/g compared to 25.5mg/g dry biomass of MM), but it was marginally below the significance cut-off level and therefore no QTL was assigned. An introgressed segment from Chr. 7 present in IL8-2 had the strongest association with salt tolerance index. Two HKT genes were recently found to reside on Chr. 7, potentially being causal for a QTL controlling Na⁺ and K⁺ concentration (Asins et al. 2013). However the introgression of IL8-2 covers almost the whole Chr.7, so linking it with the HKT function should be done with caution. IL8-2 also exhibited significantly lower Cl⁻ accumulation, which was shown to contribute to salt tolerance in barley (Tavakkoli et al. 2011).

The introgressed segments on chromosomes 9, 10 and 12 found in the IL set used for this study are relatively small. IL9-1 exhibited higher levels of K⁺, as well as higher
K/Na ratio compared to MM under salt stress. A putative potassium channel, and cyclic nucleotide gated channels involved in K\(^+\) transport (Shabala and Cuin 2008) reside in the introgressed region of this line as well as other genes involved in plant stress responses. (Table 1). The introgressed region on Chr. 10 contains ethylene response factors (ERFs) involved in stress tolerance and growth regulation under abiotic stress (Cheng et al. 2013; Dubois et al. 2013) as well as aquaporins, GSTs and RLKs.

No correlation was found between salt tolerance index and ion content, except for Na\(^+\) accumulation, which was weakly positively correlated. The lack of correlation is evidenced by the limited co-localization of QTLs for salt tolerance index and ion content (Supp. Table 4), as well as the insignificant contribution to salt tolerance of a shared introgression in Chr. 12, resulting in lower Na\(^+\) accumulation, carried by ILs 12-1 and 12-3. The type and the size of the population may have limited the discovery of correlations, as usually unique introgressions are present in the different ILs, which despite the possibility of co-regulating different traits, is present in only a few individuals of the population, resulting in non-statistically significant associations. However previous studies have as well identified a non-significant correlation of Na\(^+\) accumulation with salt stress tolerance in tomato (Rao et al. 2013; Villalta et al. 2008). In fact, Na\(^+\) can be used as an osmoticum facilitating water status maintenance, as it was observed in *S. pimpinellifolium* (Bolarin et al. 2001). and this is reflected as well in our results where LYC4 is more tolerant compared to MM despite having higher Na\(^+\) accumulation, suggesting that Na\(^+\) tissue tolerance (by storing Na\(^+\) in the vacuole or older leaves) contributes to LYC4 salt tolerance. On the other hand the lack of co-localization between many salt tolerance and ion content QTLs, offers the opportunity to combine them through pyramiding and examine epistatic interactions that additionally affect salt tolerance.

*S. habrochaites* LYC4 introgression contribute to partial resistance to powdery mildew

*S. habrochaites* LYC4 exhibited a high level of resistance to PM. None of the introgression lines exhibited the same level of resistance. This phenomenon was also observed in a previous study of this IL population on resistance to *Botrytis cinerea* (Finkers et al. 2007b). It can be either a result of incomplete representation of the wild species genome in the IL population, or the breakdown of epistatic interactions between loci, which are common in plant defense signaling (Alcázar et al. 2009). Resistance to PM in LYC4 is not the result of HR. In addition LYC4 was previously found to be resistant to various pathogens and insects (Finkers et al. 2007a; Yu et al.
Therefore increased basal resistance, or secretion of secondary metabolites (antibiosis) and leaf surface structure such as the trichomes (antixenosis), may contribute to resistance (Nonomura et al. 2009), which should be further examined in this population.

Introgressions conferring PM resistance do not coincide with previously identified regions with PM resistance genes (Li et al. 2007; Li et al. 2012). All previously identified genes confer strong resistance to PM, in contrast to the QTLs reported here. Because of the size of the introgressions, no specific genes can be pinpointed for increased resistance, though several candidate genes are present (Table 1), on many occasions (such as the RLKs) in multiple copies.

**Salinity stress has a negative impact on powdery mildew resistance**

Salt stress (100mM) increased PM susceptibility in all genotypes of the population. This is in agreement with the majority of studies in literature reporting a suppressive effect of abiotic stress on defense responses and increase in susceptibility. In *Arabidopsis thaliana* abiotic stressors were shown to suppress various aspects of the defense response. Salt stress inhibited salicylic acid (SA) biosynthesis and the induction of systemic acquired resistance (Yasuda et al. 2008), while drought and heat stress suppressed basal and R-gene mediated resistance against a virus (Prasch and Sonnewald 2013). Contrarily, a previous study in tomato reported a weak positive effect of salt stress (100mM) on PM resistance (Achuo et al. 2006). These observations can be a result of the longer period the plants experienced salt stress (14 days vs 7 in our study), which may have allowed a buildup of Na⁺ and Cl⁻ concentration to toxic for the fungus levels. We have observed a significant positive impact of Na⁺ and Cl⁻ accumulation on powdery mildew resistance in experiments with varied salt stress levels, with mild salt stress increasing susceptibility while stronger salt stress is reversing the effect (unpublished results).

Powdery mildew resistance QTLs were fewer under combined salt stress than under only PM stress alone but they were mostly shared between the two treatments. This result, in conjunction with the high correlation of DI between both conditions, indicates that salinity stress had a general suppressive effect on the defense response rather than a specific interaction. Observations from molecular studies further support this conclusion, as several components of the defense signaling network appear to be down-regulated under abiotic stress (Mang et al. 2012; Prasch and Sonnewald 2013). Both the fewer QTLs identified as well the reduced phenotypic variability of PM resistance under combined stress point to a negative impact of salt.
stress on the expression of genotypic variation under these conditions, potentially altering the adaptive potential/fitness. Phenotypic plasticity is considered pivotal in the plant’s ability to adapt to changing environments (Ghalambor et al. 2007; Nicotra et al. 2010), and a reduction of this phenotypic plasticity when exposed to multiple stresses can have additional detrimental effects on plants and crop productivity. Moreover, it highlights an additional aspect that may pose a challenge for breeding for resistance to combined stresses: reduction in phenotypic variation can negatively affect selection efficiency when resistance is partial or quantitatively controlled. On the other hand, selection for increased resistance under combined stress can be more robust when resistance is controlled by a single (or few) R-genes as it can quickly eliminate R-genes that become nonfunctional under these conditions.

$Na^+$ and $Cl^-$ accumulation under salt stress can have a harmful effect on both the host and the pathogen (Blomberg and Adler 1993). However, no significant correlation between disease severity and $Na^+$ and $Cl^-$ accumulation was found in our study. It is possible that the concentration of NaCl applied did not result in the accumulation of $Na^+$ and $Cl^-$ up to levels that were toxic for the fungus (evidenced by the increased fungal growth under salt stress). The severity of the stress can influence the magnitude of impact of another stress (Soliman and Kostandi 1998). Preliminary results in our laboratory indicated that the impact of salt stress on PM growth may depend on the severity of the applied stress (unpublished results). The lack of correlation of internal salt accumulation with PM growth may therefore be explained by relatively low (subtoxic) levels of $Na^+$ and $Cl^-$. Alternatively, the genetic variability in the population for $Na^+$ and $Cl^-$ accumulation and/or disease resistance may be too limited, as evidenced by the small number of QTLs identified under salt stress alone and none under stress combination. In addition, the weak positive correlation between DI and chlorophyll content may point to the significance of nutrient availability for fungal growth, and could be indicative of the negative interaction between salt tolerance and disease under combined stress. Salt tolerance is often characterized by delayed senescence and the maintenance of chlorophyll, a nitrogenous compound, under stress can positively interact with pathogen growth by facilitating (biotrophic) pathogen nutrition (Walters and Bingham 2007).

Our data point to distinct genetic architectures for salt stress tolerance and PM resistance in the LYC4 IL population, with few QTLs shared (Supp. Table 3) and no significant correlations observed. The shared QTLs are relatively large, with possibly different genes being responsible for resistance to either stress. The introgression of IL9-1 (resistant under all stress conditions), may contribute to multiple stress tolerance. Although there are genes present in the introgressed region that have a
clear function in pathogen response and resistance (RLKs, PRs, PAL), there are also multiple genes encoding TFs, redox and ethylene signaling components that have broad functions in stress responses. Therefore, further dissection of multiple and combined stress tolerance in IL9-1 is needed, using different approaches such as fine mapping, transcriptomics analyses and reverse genetics.

In conclusion, several genomic regions were identified in the *S. habrochaites* LYC4 IL population that can contribute to salt stress tolerance and PM resistance in tomato. Salt stress predisposed plants towards increased susceptibility to PM. The reduction in phenotypic variation under stress combination may have additional implications on plant and crop performance and breeding efforts. As no correlation was observed between salt stress tolerance and PM resistance, the different components appear to not interact with each other. Therefore, a strategy of combining resistance and tolerance traits may be successful. With the availability of the tomato genome sequence and high throughput phenotypic analyses, phenotypic responses and tolerance to stress combination can be precisely associated with the genotype and breeding of combinatorial stress resilient crops may be feasible.

**Acknowledgements**

Christos Kissoudis is supported by “Alexander S. Onassis” Public Benefit Foundation scholarship.

**Supplementary material**

The supplementary data can be retrieved at DOI: 10.18174/369608 or [http://edepot.wur.nl/369608](http://edepot.wur.nl/369608)
Chapter 4

Responses to combined abiotic and biotic stress in tomato are governed by stress intensity and mechanism of resistance

Christos Kissoudis\textsuperscript{1,2}, Sri Sunarti\textsuperscript{1}, Clemens van de Wiel\textsuperscript{1}, Richard G.F. Visser\textsuperscript{1}, C. Gerard van der Linden\textsuperscript{1}, Yuling Bai\textsuperscript{1},

\textsuperscript{1}Wageningen UR Plant Breeding, Wageningen University & Research Centre, POBox 386, 6700AJ, Wageningen, The Netherlands

\textsuperscript{2}Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

Submitted
Abstract

Stress conditions in agricultural ecosystems can occur in variable intensities. Different resistance mechanisms to abiotic stress and pathogens are deployed by plants. Thus, it is important to examine plant responses to stress combinations under different scenarios. Here, we evaluated the effect of different levels of salt stress ranging from mild to severe (50, 100 and 150mM NaCl) on powdery mildew (PM) resistance and overall performance of tomato introgression lines with contrasting levels of partial resistance, as well as isogenic lines carrying the PM resistance genes \(Ol^{-1}\) (associated with slow Hypersensitivity Response; HR), \(ol^{-2}\) (a \(mlo\) mutant associated with papilla formation) and \(Ol^{-4}\) (a \(R\) gene associated with fast HR). PM resistance was affected by salt stress in a genotype and stress intensity dependent manner. In susceptible and partial resistant lines, increased susceptibility was observed under mild salt stress (50mM) which was accompanied with accelerated cell death-like senescence. On the contrary, severe salt stress (150mM) reduced disease symptoms. \(Na^+\) and \(Cl^-\) accumulation in the leaves was linearly related to the decreased pathogen growth under severe stress, suggesting a more direct role for the salt in suppressing PM growth. In contrast, complete resistance mediated by \(ol^{-2}\) and \(Ol^{-4}\) was unaffected under all treatment combinations, and was associated with a decreased growth penalty. Increased susceptibility and senescence under combined stress of the variety Moneymaker (MM) and the NIL Ol-1 was associated with the induction of ethylene and jasmonic acid pathway genes as well as of the cell wall invertase gene \(LIN6\). These results highlight the significance of stress severity and resistance type on the plant’s performance under abiotic and biotic stress combination.

Keywords: stress severity, callose, \(R\)-gene resistance, cell death, ethylene, invertase
**Introduction**

Plants in their natural environment are continuously exposed to a variety of stress factors, both abiotic and biotic, and thus have evolved a multitude of defense mechanisms in order to maintain their fitness and successfully reproduce (Mickelbart et al. 2015; Roux et al. 2014). Under natural conditions, both the timing and the intensity of the stressors can vary, thus appropriate fine-tuning of the defense responses is required to minimize detrimental effects on plant fitness (Brown and Rant 2013; Des Marais and Juenger 2010). Stress interactions between abiotic and biotic agents are projected to become more prevalent with the observed and predicted changes in global climate patterns. The average temperature increase and decrease in precipitation especially in regions with temperate climates (Cook et al. 2015; Dai 2013) can accelerate agricultural land deterioration leading to yield losses (Lobell et al. 2011). In the same way, increased temperatures can result in geographic expansion of pathogens and enhanced fecundity, increasing the chances for host range expansion and rise of more virulent strains (Garrett et al. 2006; Harvell et al. 2002).

Field crops are grown under the same variable conditions, however as they are bred and selected under relatively controlled conditions, several trade-offs might have been overlooked that can result in negative interactions under field conditions (Brown and Rant 2013; Hückelhoven et al. 2013; McGrann et al. 2014). It is thus of great importance to examine plant responses to combinations of abiotic and biotic stress factors, important variables that are relevant to crop yields (Kissoudis et al. 2014; Soliman and Kostandi 1998).

Studies aimed at elucidating interactions between abiotic and biotic stress responses are limited. The majority of these studies concludes a negative impact of abiotic stress (mostly drought and salinity stress) on pathogen resistance (Suzuki et al. 2014), however positive effects have also been reported on resistance to foliar pathogens in a plant and/or pathogen specific manner (Kissoudis et al. 2014). Plant response and performance under different stress levels is not linear (Cheng et al. 2013a; Maggio et al. 2007; Malkinson and Tielbörger 2010; Muralidharan et al. 2014) and this can significantly impact the phenotypic responses under stress combinations. An early study on maize susceptibility to smut disease (*Ustilago maydis*) under different salt stress concentrations concluded that disease severity decreased when salt stress increased to 9 dS/m and an inverse relationship between disease susceptibility and plant Cl\textsuperscript{-} content was observed (Soliman and Kostandi 1998). Resistance to pathogens can also be differentially affected by the imposition of various types of abiotic stress. For example, rice resistance to *Magnaporthe grisea* mediated by dominant resistance (R)-genes was not affected by cold stress or ABA application, in contrast to this
plant’s basal resistance without the resistance gene (Koga et al. 2004). Contrarily, heat stress was shown to negatively impact the resistance controlled by Arabidopsis R-genes, SNC1 and RPS4, in an abscisic acid (ABA) dependent manner (Mang et al. 2012). In barley, mlo-mediated recessive resistance to powdery mildew was compromised during recovery after drought stress (Baker et al. 1998).

Functional molecular studies have added pieces to the puzzle of interactions between abiotic and biotic stress signalling components with the identification of several genes and transcription factors involved in stress crosstalk (Liu et al. 2012a; Yokotani et al. 2013). ABA appears to be a central modulator of the regulatory crosstalk, directly impacting salicylic acid biosynthesis, the major regulatory hormone for defense responses against biotrophic pathogens (De Torres Zabala et al. 2009; Yasuda et al. 2008). In some cases, successful pathogenesis of a number of pathogens involves the manipulation of the ABA pathway (De Torres-Zabala et al. 2007; Kazan and Lyons 2014). On the other hand, enhanced callose deposition, a significant line of defense enhancing plant penetration resistance against pathogens, is positively regulated by the ABA pathway (Cao et al. 2011). Thus ABA-defense signalling interactions appear to be complex, and the outcome is greatly affected by the host and pathosystem as well as by the timing of infection (Chen et al. 2013; Ton et al. 2009).

Our research is focused on the response and performance of tomato under combined salinity stress and powdery mildew infection. We have previously reported a negative impact of salinity stress (100mM NaCl) on powdery mildew resistance in a tomato Introgression Line (IL) population exhibiting partial resistance to powdery mildew (Kissoudis et al., 2015). In this study we advance a step further, examining the effects of different salt stress levels representative of mild and severe stress on powdery mildew resistance. We selected the above mentioned ILs with contrasting resistance. In addition, we used near-isogenic lines (NILs) which carry monogenic resistance genes, namely Ol-1 (no gene characterized yet), ol-2 (an mlo mutant) and Ol-4 (an NBS R-gene) (Seifi et al. 2014). These Ol-genes confer resistance to powdery mildew (Oidium neolycopersici), albeit through different mechanisms (Bai et al. 2013). Our results indicated a significant interaction of powdery mildew resistance with salt stress severity that was dependent on resistance mechanism. The detailed coverage of the different variables both in terms of stress intensity and type of disease resistance gene provides significant insights on realistic scenarios of abiotic-biotic stress interactions, and potentiates efficient and targeted crop breeding for combined stress tolerance.
Materials and Methods

Plant material

Introgression lines (ILs) 2-3, 3-2, 4-2, 4-3, 6-2, 6-3, 8-2, 9-1 and 10-4 harbouring introgressions of S. habrochaites LYC4 in the genetic background of S. lycopersicum cv Moneymaker (MM) were selected on the basis of their salt tolerance and/or powdery mildew resistance (Kissoudis et al., 2015). Additionally, the NILs carrying resistance loci Ol-1, ol-2 and Ol-4 were used. The resistance conferred by Ol-1, ol-2 and Ol-4 is associated with slow HR, papilla formation and fast HR, respectively (Bai et al. 2005).

The pathogenic fungus O. neolycopersici originated from infected commercial tomato plants (Lindhout et al. 1994) and was maintained on MM plants in a greenhouse compartment at 20±3 °C with 70±15% relative humidity (RH).

Experimental conditions and treatments

Experiments were carried out at the Unifarm greenhouse facilities of Wageningen University & Research Centre. The photoperiod regime was 16 hours light and 8 hours dark. Greenhouse air humidity was 70%. Additional lighting (100 Wm⁻²) was used if the incoming shortwave radiation was below 200 Wm⁻².

Two independent experiments were carried out in two different years in Spring (April-May). Plants were grown in pots filled with vermiculite and were irrigated with half strength Hoagland’s nutrient solution till leaching of the solution at regular intervals, avoiding accumulation of nutrients and NaCl.

In the first experiment plants of all the above mentioned genotypes were evaluated for their susceptibility to powdery mildew under different salt stress regimes. Three-week old plants (4 plants per line) were watered with a solution containing different concentrations of NaCl (0· no salt stress, 50, 100 and 150 mM NaCl). Eight days after the initiation of salt treatments, plants were inoculated with powdery mildew by uniformly spraying a suspension of 5x10⁴ conidia.ml⁻¹. Plants were grown for another 25 days post inoculation (dpi) in order to observe secondary infection symptoms.

In the second experiment only NIL·Ol-1, ·ol-2 and ·Ol-4 and the recurrent parent cv. MM were evaluated. Three-week old plants (4 plants per line) were watered with 0, 50 and 150 mM NaCl. In this case, eight days after the salt treatments half of the plants were spatially isolated and were not sprayed with powdery mildew, resulting in three treatments: no salt stress/not inoculated, salt stress/not inoculated, salt stress/inoculated). Plants were grown for another 20 days after inoculation.
Plant performance evaluation under salt stress and powdery mildew infection

Chlorophyll content was measured using a SPAD-502 meter (Minolta, Osaka, Japan) at the third and fourth leaf counting from the bottom, on the fifth day after pathogen inoculation, before symptom appearance. Fresh and dry weight were measured as described previously (Kissoudis et al., 2015). The disease severity was expressed as disease index (DI) to a scale from 0 to 5, according to (Kissoudis et al. 2015), assessed at 10, 15 and 25 dpi for the first experiment and at 15 dpi for the second experiment. In addition to DI, a measure of the visual stress response was introduced to describe the accelerated senescence phenotypes observed at the later stages of infection under salt stress:

0 = healthy plant, 1 = 0.1 - 10 % of foliar area affected, 2 = 10-20 % area affected with yellowing and moderate wilting, 3 = 20 – 30 % area affected with severe wilting, 4 = 30-50 % area affected with severe wilting and moderate leaf abscission and 5 = > 50 % area affected with severe wilting and leaf abscission.

Ion content

Sampling for ion content determination differed between the two experiments. In the first experiment the 4th leaf counting from the bottom was sampled at 10dpi, shortly after symptom appearance, in order to assess the relationship between disease severity and ion concentration. In the second experiment, the top five leaves were sampled at 20dpi, the endpoint of the experiment, in order to examine differences in actively growing tissues, potentially linked to growth performance, and to avoid the dying bottom leaves of susceptible genotypes under combined stress conditions. The ion analysis included Na\(^+\), Cl\(^-\), K\(^+\), PO\(_4\)^{3-}, SO\(_4\)^{2-}, Mg\(^{2+}\) and Ca\(^{2+}\) and quantification was performed as described previously (Kissoudis et al., 2015).

Histological analyses of in situ callose deposition

Leaf disks (1.3 cm in diameter) were sampled from leaflets of the 4\(^{th}\) leaf counting from the bottom on the 3\(^{rd}\) day after pathogen inoculation, from the middle of the leaflets on both sides of the central vein. Staining was carried out in 24-well plates, with leaf disks placed with their abaxial side up. Callose deposition visualization was performed according to (Luna et al. 2011; Ton et al. 2005) with slight modifications. Leaf disks were placed in 96% ethanol to remove chlorophyll and after a 1-min wash in 0.07 M K\(_2\)HPO\(_4\) (pH=9) stained for 2 hrs in 0.05% (w/v) aniline blue in 0.07 M K\(_2\)HPO\(_4\) (pH=9) at room temperature. Leaf disks were subsequently mounted on glass slides with 70% glycerol. Callose was quantified from digital photographs as the
number of white pixels (fluorescence, callose intensity) relative to the total number of plant-derived pixels.

**Gene expression and pathogen quantification with qPCR**

Leaflets for the gene expression analyses were sampled 6dpi from the 3rd and 4th leaf counting from the bottom, before pathogen mycelium growth was visible. Leaflets for pathogen quantification were sampled 14 dpi inoculation from the 4th and 5th leaf counting from the bottom, when pathogen growth from the primary infection was highest.

RNA for gene expression analyses was isolated with the RNeasy plant mini kit (Qiagen). Plant and fungal DNA for pathogen quantification analyses was extracted with DNeasy plant mini kit (Qiagen). RNA was treated with DNAse I (Invitrogen) to eliminate residual DNA. cDNA synthesis was performed with 1μg RNA template using iScript™ cDNA Synthesis Kit (BioRAD). Quantitative real-time PCR was conducted using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad).

The reaction mix contained 5 µl 2x iQ SYBR GREEN super mix, 1 µl Forward primer (3 μM), 1 µl Reverse primer (3 μM) and 3 µl cDNA (or DNA, 20ng) template, into a final volume of 10 µl. Thermocycling conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. Primers used for fungal quantification were Fw-On-CGCCAAAGACCTAACCAAAA and Rv-On-AGCCAAGAGATCCGTTGTTG (Gao et al. 2014b). Primers for tomato elongation factor 1α (EF) were Fw-EF-GGAACCTTGAGAAGGAGCTAAG and Rv-EF-CAACACCAGCACACAGTCT. The primers used for the expression analysis of selected tomato genes are provided in Supplementary Table 1. Relative expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001).

**Statistical analysis**

Experiments were carried out in a Split plot design with 4 replications for the salt stress, powdery mildew and combined stress treatments. Statistical analyses were performed using Genstat 15th edition. Correlations between traits were calculated using the Pearson correlation coefficient (p≤0.05). The relationship between elemental concentration (independent variable) and disease severity (dependent variable) was examined with multivariate regression analysis.
Results

Effect of salt stress severity on powdery mildew resistance

To examine the effect of salinity stress intensity on powdery mildew resistance, we evaluated the response of nine *S. habrochaites* LYC4 ILs, selected from a previous study (Kissoudis et al. 2015), in which they were shown to carry introgressions for either salinity tolerance and/or powdery mildew resistance. We applied three levels of stress, which are considered to be low (50mM), intermediate (100mM) and high (150mM NaCl) salinity stress for most crops including tomato (Munns and Tester 2008).

Powdery mildew disease severity was on average the highest at 50mM NaCl, but decreased at higher salt concentrations (Fig. 1a). In particular at 10dpi the average disease index was 65% higher in plants grown at 50mM NaCl compared to no salt stress conditions. This effect of salt stress on disease index was more pronounced in ILs that exhibited a higher level of resistance e.g. ILs 3-2, 4-3 and 9-1 (Sup Fig. 1).

A unique response was observed under combined salt stress and powdery mildew infection with leaves initially exhibiting increased epinasty, even before visible pathogen growth.

![Figure 1. a) Disease index averaged across the LYC4 ILs and the recurrent parent MM under powdery mildew without salt (0mM NaCl) and in combination with 50, 100 and 150 mM NaCl, measured at 10, 15 and 25 days post inoculation (dpi). b) Senescence index across the same genotypes and treatments at 15 dpi. Statistically significant differences (P≤0.05) are designated with different letters.](image-url)
Figure 2  

a) Disease and b) senescence index of NIL·O·1, ·ol·2 and ·Ol·4 (written as Ol·1, ol·2, Ol·4 in the figure) and the recurrent parent MM under powdery mildew without salt (0mM NaCl) and in combination with 50, 100 and 150 mM NaCl, measured at 15 dpi.  

c) leaf phenotypes under different powdery mildew and combined stress treatments. Asterisks denote statistically significant differences (P≤0.05) between powdery mildew and combined stress treatments for individual genotypes.
Shortly after the mildew appearance (9-10dpi) yellowing and wilting were observed, which in more susceptible genotypes led to up to 50% leaf abscission. These accelerated senescence and leaf abscission phenotypes were expressed as a visual stress index (scale 0-5). Similarly to disease index, senescence index was highest at 50mM NaCl (Fig. 1b).

In addition to the LYC4 ILs, NIL·Ol-1, -ol-2, and -Ol-4, conferring monogenic resistance to powdery mildew through different mechanisms (Bai et al. 2005) were evaluated under the same treatment scheme. The responses under combined stress were largely disparate among the different genotypes. Resistance in NIL·Ol-1 was partially compromised at 50 and 100 mM NaCl stress, while resistance was partially restored at 150 mM NaCl stress. Additionally, NIL·Ol-1 exhibited accelerated senescence and runaway cell death, leading to leaf abscission. Resistance in NIL·ol-2 and -Ol-4, on the contrary, was not affected by salt stress at any salt stress level. No wilting or senescence symptoms were observed in either of the genotypes (Fig.2 a, b, c; Supp. Fig. 2).

**Relationship between different disease resistance responses with ion content and gene expression**

The ions Na⁺, Cl⁻, K⁺, PO₄³⁻, SO₄²⁻, Mg²⁺ and Ca²⁺ were measured at 10dpi to determine any possible relationship between leaf ion concentration and disease severity under salt stress. Both shoot Na⁺ and Cl⁻ concentrations increased linearly with increased NaCl application (Fig. 3a, b). K⁺ and SO₄²⁻ concentrations were decreased under salt stress with no differences observed between the different salinity levels, while no significant changes were observed for PO₄³⁻, Mg²⁺ and Ca²⁺ (Supp. Fig. 3).

We examined whether there was a causal relationship between the decreased disease index with increased ion contents at higher stress levels using multiple stepwise regression. We used LYC4 ILs and NIL·Ol-1 along with the recurrent parent MM, in which resistance was affected by salt stress (Fig. 1 and 2). DI under salt stress conditions subtracted from that under non-salt stress conditions (only PM infection-DI change) was used as the dependent variable, which was strongly correlated with Na⁺ and with Cl⁻ concentrations. Na⁺ and Cl⁻ concentration increase accounted for 50% and 55% of the variation in DI change, respectively (Fig. 3c, d). The addition of the rest of the ions to the model led to a slight decrease in the variance explained (46.7%), and separately (without Na⁺ and Cl⁻) these ions did not contribute significantly to variation in disease index either (p=0.068, 18% variance explained).
Correlations for the different growth, ion content and disease susceptibility traits measured were calculated within each stress level (Supp. Table 2). Under powdery mildew infection (no salt stress), DI was weakly negatively correlated with Fresh Weight (FW) and Dry Weight (DW) (r= -0.4 and -0.39 respectively) and SO₄²⁻ concentration (r= -0.52). At 50mM NaCl these correlations were no longer significant with DI, which was then negatively correlated with Ca²⁺ concentration (r= -0.47). The senescence index was positively correlated with fresh and dry weight (r= 0.5 and 0.52 respectively) and negatively correlated with PO₄³⁻ concentration (r= -0.5). No significant correlations were observed between DI and any of the traits measured at 100mM NaCl. Finally at 150mM NaCl the negative effect of Na⁺ and Cl⁻ accumulation on plant performance was apparent, with a negative correlation observed for shoot Na⁺ and (especially) Cl⁻ concentrations with fresh and dry weight (Supp. Table 2).
Examining expression markers for major hormonal and other biochemical pathways involved in resistance to stress and defense to pathogens in selected ILs (Il3-2, 8-1 and 9-1) revealed a noticeable trend in the underlying molecular responses under different intensities of salt stress and powdery mildew infection. Averaged across genotypes the expression of ACCase, encoding a biosynthetic enzyme of ethylene, was highest under the combination of mild salt stress (50mM NaCl). A similar (but less strong) response was observed for the JA biosynthesis and response genes AOS and LOXD, respectively. PR1a expression was significantly upregulated under combined stress, with MM exhibiting the greatest induction among the genotypes. On the other hand induction of NCED, an ABA biosynthesis enzyme, was modest at both mild and severe salt stress combinations with powdery mildew. (Supp. Fig. 4).

**Performance of NILs under salt stress, powdery mildew and their combination**

The above mentioned results indicated a significant effect of the genotype and the stress intensity on powdery mildew resistance under salt stress. In a second experiment we focused on the NILs carrying the Ol-genes and the response to 50 and 150mM NaCl, as they exhibited the most contrasting responses. Plants were exposed to no stress, single stress (salt or powdery mildew) as well as to combined stress and were compared to non-stressed plants.

Similar to the first experiment mild salt stress (50mM NaCl) increased susceptibility and senescence of MM and Ol-1 was observed, while at severe salt stress (150mM NaCl) this effect was reversed. The resistance of NIL-ol-2 and -Ol-4 was not affected in any of the treatments, and senescence was hardly increased. Fungal biomass was quantified and the results confirmed the visual DI scores (Fig. 4).

MM and all NILs showed decreased plant fresh weight under salt treatment (FW, Fig. 5a). Upon powdery mildew infection a reduction of 20% in terms of fresh weight was observed for MM and NIL-Ol-1, and 15% for NIL-ol-2. NIL-Ol-4 on the other hand showed no significant reduction in FW.

Under combined stresses (both salt stress and powdery mildew infection), MM and NIL-Ol-1 exhibited a further significant decrease in biomass of 15 and 12% compared to salt stress only. NIL-ol-2 and -Ol-4 were less affected: 5 and 4% decrease in biomass in 50mM NaCl with powdery mildew (significant only for NIL-ol-2), while they maintained their performance to similar levels with salt stress alone in 150mM NaCl with powdery mildew (Fig. 5a).
Figure 4. Relative Oidium neolycopersici fungal biomass in MM and NIL-Ol-1, ol-2 and Ol-4 (written as Ol-1, ol-2, Ol-4 in the figure) under powdery mildew infection alone and in combination with 50 and 150mM NaCl. Values are normalized with that of MM under powdery mildew infection (no salt stress). Asterisks denote statistically significant differences (P≤0.05) between powdery mildew and combined stress treatments for individual genotypes.

Under the assumption that ion concentration was related with growth performance of the plants, analysis, sampling for ion content analysis was carried out at the end of the experiment. The top five leaves were collected to avoid sampling of senescing (or abscised) leaves from MM and NIL-Ol-1 plants. Both Na⁺ and Cl⁻ concentrations were slightly increased under combined salt stress with powdery mildew compared to salt stress alone (Fig. 5b, c). Small differences were observed between genotypes, with the relative increase of Na⁺ and Cl⁻ concentration under combined stress being higher in NIL-ol-2 and NIL-Ol-4 compared to MM. No significant differences were observed for the other ions, except for a higher concentration of SO₄²⁻, and to a lesser extent K⁺, Mg²⁺ and Ca²⁺ in NIL-Ol-1 under powdery mildew and combined stress compared to non-stress and salt stress only (Supp. Fig. 5).
Figure 5 a) Above ground biomass (FW) of NIL-O-1, ol-2 and Ol-4 (written as Ol-1, ol-2, Ol-4 in the figure) and the recurrent parent MM under salt stress (0, 50, 150mM NaCl) or powdery mildew alone, and their combination. Level 0 for salinity stress corresponds to stress-free control conditions, while level 0 for powdery mildew combined stress corresponds to powdery mildew infection alone (no salt stress). b) Na⁺ and c) Cl⁻ concentration of Ol-lines and MM under the same treatment scheme. Asterisks denote statistically significant differences (P≤0.05) between salinity and powdery mildew combined stress for individual genotypes.
Callose deposition

*In situ* callose deposition at the leaf level was evaluated by aniline blue staining and examined by UV epifluorescence microscopy. Callose deposition, is an important penetration resistance mechanism against pathogens and powdery mildew in particular and is associated with *ol*-2-mediated resistance. Under powdery mildew infection NIL-ol-2 exhibited the highest intensity of callose deposits. Much less callose deposits were observed in NIL-ol-1 and MM and were almost absent in NIL-ol-4. Under salt stress with powdery mildew decreased callose depositions were observed in all genotypes. Callose deposition under 50 and 150mM was almost abolished in MM and NIL-ol-1 and was much lower in NIL-ol-2, especially under 150mM NaCl (Fig. 6a, b)

Gene expression analyses

In order to link the responses of MM and NILs to specific pathways, we measured expression of marker genes in defense hormonal pathways, such as ROS, antioxidant and ion homeostasis pathways. For the ABA pathway, no significant expression changes were observed for the ABA-synthesizing enzyme *NCED* under salt stress compared to control conditions. However, a reduction of *NCED* expression (2-fold for MM and NIL-ol-1) was observed under combined stress versus salt stress alone. A significant reduction in the expression of *DHN-TAS* was observed under combined stress for MM and NIL-ol-1 (ranging from 2- to 7-fold), while an induction was observed in NIL-ol-2 (8-fold under 50mM NaCl and 2-fold for 150mM NaCl with powdery mildew).

For the ethylene pathway, a dramatic expression induction of ethylene biosynthesis genes, *ACCase* and *ACCox* was observed in MM and NIL-ol-1 under combined stress (ranging from 50- to 400-fold for *ACCase*). Similarly, in MM and NIL-ol-1, the jasmonic acid biosynthesis and signalling genes *AOS* and *LOXD* were highly upregulated. The pathogenesis-related gene *PR1a* was induced up to 70- and 45 fold in MM and NIL-ol-1 respectively; the cell-wall invertase *LIN6* was induced up to 230-fold for MM and 50-fold for NIL-ol-1.

Contrasting responses were observed for the ROS signalling involved genes, *RBOHD* and *RBOHF*. On one hand, the *RBOHD* expression was increased under powdery mildew and combined stress. The induction was higher in NIL-ol-2 and ol-4 (2-fold higher compared to MM and NIL-ol-1). On the other hand, the *RBOHF* expression was reduced 2-fold under powdery mildew and combined stress in all genotypes, except NIL-ol-1 which exhibited a 2-fold induction under combined stress.
Figure 6 a). Callose deposits in leaves as visualized with UV microscopy after aniline blue staining, b) quantification of callose deposition relative to MM under powdery mildew infection (no salt stress). Asterisks denote statistically significant differences (P≤0.05) between powdery mildew and combined stress treatments for individual genotypes.
Figure 7. Expression of genes-markers for hormonal, abiotic and biotic stress signalling pathways in MM, and NIL-OI-1, -ol-2 and -Ol-4 (written as Ol-1, ol-2, Ol-4 in the figure), relative to EF1a, which was used as a housekeeping gene. Treatment and labelling scheme are the same as Fig.4. Asterisks denote statistically significant differences ($P \leq 0.05$) between powdery mildew and combined stress treatments for individual genotypes.
Differential responses were also observed among the different genotypes for the antioxidant enzymes APX and SOD. Expression of both genes was significantly reduced under powdery mildew and combined stress in MM and NIL-Ol-1 (an average of 3-fold decrease for APX and 2-fold decrease of SOD), while in NIL-ol-2 and NIL-Ol-4 their expression remained stable or was slightly increased.

In the rest of the genes examined, expression of the MLO gene (a negative regulator of disease resistance) was increased (especially in NIL-Ol-1) under powdery mildew and combined stress, while the Na⁺-H⁺ antiporter NHX3 expression was decreased under combined stress in comparison to salt stress only, with the stronger reduction (2.5-fold) observed in NIL-Ol-1.

Discussion

The results presented here address two dimensions related to the complexity of plant responses under combinatorial stress: abiotic stress intensity and resistance mechanism. Both variables are of great importance for crop cultivation practices, as plants are exposed to variable stress intensities during their lifetime and the cultivars deployed often have different mechanisms of resistance.

Mild salt stress has the most significant impact on susceptibility in partially resistant lines

In our study, the susceptible control MM and the LYC4 ILs with partial resistance to powdery mildew showed comparable responses to single and combined stresses. Under mild (50 mM) and moderate (100 mM) salt stress the observations are in agreement with many studies reporting a negative effect of abiotic stress on disease resistance (Kissoudis et al. 2014; Prasch and Sonnewald 2013; Yasuda et al. 2008). Interestingly, mild salt stress most severely promoted disease susceptibility and leaf wilting and senescence. Severe salt stress (150 mM) on the other hand partly reversed this effect, with susceptibility for some genotypes being even lower than for plants under no salt stress. This still came at the expense of overall plant performance and growth, as severe salt stress imposed a severe growth penalty. These observations are of great importance for agricultural practices and the potential threat of abiotic and biotic stress combinations for plant productivity. Mild stress conditions are the most prevalent in agricultural lands, and therefore are highly relevant (Vadez et al. 2013). The reduction of susceptibility under high salt stress has limited relevance for agricultural production because of the severe growth penalty: most of the major crops are considered glycophytes and have reduced growth and productivity by at
least 50% at salt levels of 150 mM NaCl (equalling EC values of 15-20) (Munns and Tester 2008).

Apart from its effect on powdery mildew susceptibility, the stress combination of salt and PM resulted in another unique response, i.e. accelerated leaf wilting, senescence and leaf abscission, which was not observed under salt stress or powdery mildew alone. Cell death and apoptosis are shared in the responses to the single stress factors and finely regulated (Demidchik et al. 2014; Miller et al. 2009; Petrov et al. 2015; Torres et al. 2005). The stress combination may have disrupted these balances resulting in uncontrolled cell death/senescence phenotype. Such a response is an important aspect of the negative interaction of defense pathways when plants are exposed to these stresses at the same time, and such a response can be detrimental for plant productivity (Gregersen et al. 2013).

**A direct fungal toxicity role for Na\(^+\) and Cl\(^-\)?**

A unique component differentiating salt stress from other abiotic stresses such as drought or heat is Na\(^+\) and Cl\(^-\) accumulation. This often has a toxic effect on the plant, but is toxic to the fungus as well: NaCl is known as an antifungal agent (Blomberg and Adler 1993) and it could potentially exert a direct toxic effect on fungal growth after accumulation in the plants. Our results point to a direct influence of Na\(^+\) and Cl\(^-\) on pathogenicity as observed between the different salt stress levels. This is in line with the many examples of reduction of fungal pathogenicity by metal accumulation (Fones et al. 2010; Poschenrieder et al. 2006), and a similar trend is observed for smut disease and NaCl accumulation in maize (Soliman and Kostandi 1998). The decreased susceptibility observed under severe stress conditions may therefore be a unique aspect of salt stress that cannot be extrapolated to other abiotic stresses such as drought. Yet increased drought severity also appeared to decrease susceptibility to powdery mildew in garlic mustard (Enright and Cipollini 2011) and to *Sclerotinia sclerotiorum* (a necrotrophic fungus) and *Pseudomonas syringae* pv. *tabaci* (a hemi-biotrophic bacterial pathogen) in *Nicotiana benthamiana* (Ramegowda et al. 2013).

Except Na\(^+\) and Cl\(^-\), a weak negative correlation was evident between SO\(_4^{2-}\) and Ca\(^{2+}\) concentration and increased disease resistance. Though no strong conclusions can be drawn, these observations highlight the importance of the nutritional status of the plant in the incremental build-up (or breakdown) of basal quantitative resistance. Both SO\(_4^{2-}\) and Ca\(^{2+}\) nutrition improve disease resistance (Jiang et al. 2013c; Kruse et al. 2007), and thus perturbation of their homeostasis under combined stress potentially contributes to derailing plant defenses.
Robustness and decreased fitness cost of mlo and R-gene based resistance to powdery mildew under salt stress combination.

In contrast to the relatively uniform response of LYC4 ILs under combined stress, stark differences were observed between the Ol-NILs conferring monogenic resistance through different mechanisms. While NIL·ol-2 and NIL·Ol-4 had a robust resistance phenotype under all treatments of combined stress with maintenance of (almost) complete resistance and no accelerated senescence response, resistance in NIL·Ol-1 succumbed under combination with salt stress, resembling LYC4 ILs.

Gene expression analyses reflected the phenotypic differences, similar gene expression patterns were shown in the susceptible MM, LYC4 ILs with partial resistance and NIL·Ol-1 with complete resistance to powdery mildew. The massive induction of ethylene biosynthesis genes in NIL·Ol-1 which was absent in NIL·ol-2 and NIL·Ol-4, may underlie its increased susceptibility and senescence under stress combination. Ethylene signalling has been demonstrated to be a prerequisite for symptom development after pathogen infection in tomato (O’Donnell et al. 2003). The very strong induction observed uniquely under combined stress in this study is likely to accelerate senescence and leaf abscission, potentiating the action of H₂O₂ and resulting in programmed cell death (PCD) processes (Bar-Dror et al. 2011; Sakamoto et al. 2008), in line with our observations of accelerated senescence in NIL·Ol-1 under combined stresses.

The very strong induction of the cell-wall invertase LIN6 specifically under powdery mildew and combined stress in NIL·Ol-1 may additionally contribute to the observed phenotypes. Cell wall invertases (CWI) are induced after pathogen infection (Moghaddam and Van Den Ende 2012), however their contribution to plant defense during pathogenesis is still not known. Several studies report a positive contribution of CWIs in plant resistance (Bonfig et al. 2010; Essmann et al. 2008; Sonnewald et al. 2012), however in tomato an opposite observation is reported with CWIs contributing to symptom development in response to Xanthomonas campestris pv. vesicatoria (Kocal et al. 2008). Co-silencing of Lin6 and Lin8 CWIs in tomato reduces the induction of pathogenesis related (PR-) genes together with pathogenesis symptom development (Kocal et al. 2008). In addition to the upregulation in response to pathogens, PR-proteins have been involved in processes like senescence and leaf abscission (Van Loon et al. 2006). The very high CWI induction under stress combination in NIL·Ol-1 along with PR1a (not observed in individual stress treatments) would therefore seem more likely to contribute to symptom development and the acceleration of senescence and leaf abscission.
Performance in terms of biomass was (also) significantly impacted by powdery mildew and combined stress, in line with the notion that induction of defense responses against pathogens comes at a cost (Bolton 2009). However combined stress exhibited even greater cost than powdery mildew and salt stress alone, which was most pronounced in MM and NIL·Ol-1. On the other hand NIL·Ol-4 did not show any additional fitness cost under stress combination. Fitness cost in MM and NIL·Ol-1 might be due to increased senescence and a potential photosynthesis down regulation in response to the activation of defense hormone signalling especially of ethylene and jasmonic acid (Bilgin et al. 2010). Down regulation of adaptive and protective mechanisms involved in abiotic stress tolerance such as ABA signalling (evidenced by the reduction in DHN-TAS expression) and the reduced expression of APX and SOD under combined stress, potentially contributed to decreased tolerance (Faize et al. 2011; Muñoz-Mayor et al. 2012), while the latter might also have decreased the threshold for the cell death responses observed as increased senescence (Stael et al. 2015; Yao and Greenberg 2006). Na\(^+\) and Cl\(^-\) concentrations in the leaves were slightly more increased under stress combination than under only salt stress, which may additionally contribute to the augmented growth penalty under these conditions. NIL·Ol-4 however did not show any fitness cost despite exhibiting the stronger increase in Na\(^+\) and Cl\(^-\) under combined stress compared to salt stress alone.

**What are the causal mechanisms underlying the contrasting responses of NILs with different resistance mechanisms?**

The question remains whether the alterations observed in hormone and sugar signalling (ethylene/jasmonic acid signalling and CWI induction) are the cause or the consequence for the dramatic differences observed in the differential response of NIL·Ol-1, -ol-2 and -Ol-4. Resistance of the three isogenic lines is based on completely different mechanisms. The *Ol-1* gene, likely a non NBS-LRR gene, confers incomplete dominant resistance characterized by a multiple-cell delayed cell death (slow HR, (Seifi 2011a)). The cell death in NIL·Ol-1 can retard but not completely stop fungal development (Bai et al. 2005; Li et al. 2007). The *ol-2* gene (an *mlo* mutant) confers non-race specific resistance through papillae formation at the fungal penetration sites. The *Ol-4* gene is an *Mi-1*-like gene (an NBS-LRR gene) and confers complete race-specific resistance associated with single-cell death (fast HR)(Bai et al. 2005; Li et al. 2007; Seifi et al. 2011b).

Early signalling events in both abiotic and biotic stress include Ca\(^+\) fluxes and ROS generation whose specific signatures orchestrate downstream responses (Demidchik
et al. 2014; Segonzac et al. 2011) and pre-invasive defense responses such as callose deposition. The expression of two antioxidant enzyme genes was reduced and RBOH gene expression altered in the Ol-NILs under stress combination, indicating that these signatures may be changed and the deployment of defense mechanisms may be different. These changes might have altered the ROS footprint in NIL-Ol-1 and have led to defense breakdown and accelerated cell death. Ol-1-mediated resistance is prone to breakdown when cellular homeostatic mechanisms are perturbed as shown in ALS silenced plants, while resistance conferred by Ol-4 was not affected after by same manipulation (Gao et al. 2014b)

Callose deposition was also significantly affected under combined stress. It was almost completely diminished in NIL-Ol-1 under combined stress. Although callose deposition is not the major contributor to Ol-1-mediated resistance against powdery mildew (Li et al. 2007; Li et al. 2012), the decreased callose deposits might have additionally contributed to accelerated pathogen growth under combined stresses. Callose deposits were much higher in NIL-ol-2 and became very low at higher salt stress levels (150mM NaCl). Callose deposition regulation is complex and while it has been shown to be positively regulated by ABA signalling (Ton et al. 2009), under salt stress conditions multiple factors might be affected such as altered redox status and vesicular trafficking, both important regulatory and functional components for callose formation (Hamaji et al. 2009; Luna et al. 2011).

R-gene (of the TIR-NB-LRR class) function was shown to be affected by abiotic stress, heat in particular (Mang et al. 2012) and to be regulated by proteins involved in heat stress tolerance (Hubert et al. 2009b). However, the Ol-4-mediated resistance was not affected at all by salt stress in our experiments, which may be due to the different plant response to salt stress than to heat. In addition, the Ol-4 is a CC-NB-LRR gene, which confers resistance through different routes than TIR-NB-LRR genes (Teh and Hofius 2014), such as being autophagy independent. R-gene mediated effector triggered immunity (ETI) is characterized by compensatory relationships (Tsuda et al. 2009) and its defense output is stronger and more prolonged compared to PTI (Tsuda et al. 2013), thus more robust and less prone to negative regulation from environmental or genetic factors (Cui et al. 2015).

Conclusions and breeding routes for achieving robust combined powdery mildew and salt stress tolerance in tomato.

We conclude that the impact of combined salinity and powdery mildew on tomato plants is dependent both on the salt stress severity and the mechanism of disease
resistance. Negative interactions were generally observed under mild salt stress, relevant for most agricultural scenarios, including increased powdery mildew susceptibility, leaf senescence and decreased biomass. These effects were partly reversed under severe salt stress but this significantly impacted plant biomass. HR-based disease resistance appears to be the most robust both in terms of resistance and overall plant performance under combined stress, closely followed by mlo-based disease resistance. Since R-gene resistance appears to be more stable to environmental and genetic perturbations, the additional pyramiding of salt tolerance genes to R-gene mediated resistance is expected to be more straightforward as fewer interactions can be expected (Kissoudis et al. 2014). A drawback is that pathogens can easily overcome R-gene resistance, thus pyramiding multiple R-genes is essential as well. The recessive ol-2 gene has the advantage that it is race non–specific, thus more stable over time. However, mlo-based resistance may be accompanied by increased senescence at the later stages of plant development (Piffanelli et al. 2002), and this can be further accelerated by abiotic stress. Fine-tuning of ethylene biosynthesis/response might be a key in mitigating the adverse effects of abiotic and biotic stress combination in genotypes with partial disease resistance. Ethylene biosynthesis down regulation significantly increased grain yield of maize under drought (Habben et al. 2014) and can potentially contribute to increased crop resilience under scenarios of biotic stress combinations.

The here reported results may be transferable and translated to other crops, as the core stress tolerance/defense response genetic regulation appears to be universal, despite the existence of species-specific responses. However each stress (abiotic or biotic) has some unique properties (e.g. toxic effects of Na⁺ and Cl⁻ on pathogens are unique to salt stress) that need to be taken into account. Moreover, studies should be extended to cover the entire life cycle of plants, as plant age might significantly influence the phenotypic response (senescence in particular).

Acknowledgements

Christos Kissoudis is supported by “Alexander S. Onassis” Public Benefit Foundation scholarship.
Supplementary material

The supplementary data can be retrieved at: DOI: 10.18174/369637 or http://edepot.wur.nl/369637
Chapter 5

Hormone signalling regulation of tomato response to combined biotic and abiotic stress

Christos Kissoudis\textsuperscript{1,2}, Alireza Seifi\textsuperscript{3}, Zhe Yan\textsuperscript{1}, Tanjimul Islam\textsuperscript{1}, Hanneke van der Schoot\textsuperscript{1}, Clemens van de Wiel\textsuperscript{1}, Richard G.F. Visser\textsuperscript{1}, Gerard van der Linden\textsuperscript{1}, Yuling Bai\textsuperscript{1},

\textsuperscript{1}Wageningen UR Plant Breeding, Wageningen University & Research Centre, POBox 386, 6700AJ, Wageningen, The Netherlands

\textsuperscript{2}Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

\textsuperscript{3}Biotechnology and Plant Breeding Department, Faculty of Agriculture, Ferdowsi University of Mashhad, Khorasan Razavi, P. O. Box 9177948974, Iran

Submitted
Abstract

Plant hormones are paramount to plant adaptation to changing environmental conditions and interactions with microorganisms. There is currently limited knowledge on their significance in the response to stress combination. Using near isogenic lines (NILs) that carry the $Ol$-1, $ol$-2 and $Ol$-4 gene for resistance to tomato powdery mildew caused by *Oidium neolycopersici*, this study focused on the responses of these NILs to powdery mildew and salt stress combination. In these NILs, marker genes for monitoring hormonal pathways showed differential expression pattern upon powdery mildew infection. Further by crossing these NILs with tomato mutants *notabilis* (ABA-deficient), *defenseless1* (JA-deficient) and *epinastic* (ET overproducer) the cross-talk among hormonal pathways was further investigated. Among the mutants, *epinastic* resulted in increased susceptibility of NIL-$Ol$-1 and breakdown of NIL-$ol$-2 resistance, accompanied by reduced callose deposition, effects that were more pronounced under combination with salt stress. On the other hand *notabilis*, resulting in H$_2$O$_2$ overproduction greatly reduced susceptibility of NIL-$Ol$-1 under combined stress accompanied however by heightened sensitivity to salt stress. Callose deposition reduction led to partial resistance breakdown in NIL-$ol$-2 which was reversed under combined stress. NIL-$Ol$-4 resistance remained robust across all mutant and treatment combinations. We discuss the critical role that hormone signalling appears to have for the outcome of combined stress and powdery mildew in terms of resistance and plant fitness integrating observations from physiological, histochemical and gene expression analyses. These significant insights obtained extend our understanding of hormonal regulation of combined stress responses and can aid in narrowing down targets for improving crop performance under stress combinations.

Keywords: abscisic acid, senescence, callose, ROS burst, chitinase
Introduction

Plant hormones are central to plant adaptation to changing environmental conditions as well as interactions with other pathogenic and non-pathogenic organisms. To maximize fitness under different stress scenarios, resource allocation must be precisely prioritized and thus hormonal signalling pathways are delicately interconnected and inter-regulated (Denancé et al. 2013). Understanding the underlying regulatory mechanisms of hormone crosstalk is of increased importance due to the current global climate change that is projected to further intensify unfavourable conditions for crop plant production (Challinor et al. 2014; Lobell et al. 2011; Trnka et al. 2014). A significant consequence of climate change is the increased frequency of stress combinations that plants are exposed to, especially of abiotic factors with pathogenic microorganisms (Garrett et al. 2006; Kissoudis et al. 2014; Suzuki et al. 2014). Significant progress has been made in understanding hormone cross regulation under stress. Abscisic acid (ABA) is the major orchestrator of adaptation and tolerance to abiotic stress (Yoshida et al. 2014), while interplay between salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) regulates resistance responses towards pathogenic fungi (Pieterse et al. 2012). Considerable understanding has been established about the functional relationship between hormone signalling in defense responses. In the model plant Arabidopsis thaliana, SA is the main player in responses to biotrophic pathogens, while JA and ET, antagonistically with SA, mount defense against necrotrophs (Robert-Seilaniantz et al. 2011). These distinctions in many occasions are not clear-cut, as interactions between hormonal pathways appear to be hormone concentration and timing of induction dependent (Koornneef et al. 2008; Pieterse et al. 2009). Moreover, it appears that there are species-specific responses that are distinct from those reported in Arabidopsis. For example, in barley activation of systemic acquired resistance is under the control of ERF and WRKY transcription factors, but not of SA (Dey et al. 2014). In tomato however, SA enhances resistance against necrotrophic pathogens such as Botrytis, while increasing susceptibility against biotrophs (Achuo et al. 2004).

With regard to interaction between abiotic and biotic stresses, a mostly antagonistic interaction was observed between ABA and defense signalling across many plant species. ABA was shown to negatively interact with both SA and JA/ET signalling, compromising resistance to pathogens and systemic acquired resistance (Anderson et al. 2004; Ulferts et al. 2015; Yasuda et al. 2008). Observations in ABA deficient mutants that exhibit increased disease resistance further strengthen this notion, though in many cases increased resistance is attained through pleiotropic changes occurring in ABA-depleted plants (Curvers et al. 2010; Mang et al. 2012; Sanchez-
Vallet et al. 2012). On the contrary, there are also a significant number of studies reporting on a positive role of ABA signalling especially in pre-invasive defense responses through priming for cell wall fortifications and callose deposition (García-Andrade et al. 2011; Ton et al. 2009). Thus, even though the majority of the studies indicate a negative role of ABA in defense responses, this does not preclude a potential beneficial contribution in specific pathosystems or at specific stages during pathogenesis.

As hormones are involved in the control of numerous physiological processes in plants, elucidation of hormonal interaction patterns is of significant importance to understand plants’ responses under stress combinations. Combined stress research is still in its infancy, but the results of recent studies strongly indicate the presence of non-additive interactions at both the phenotypic and the gene expression levels (Kissoudis et al. 2014; Prasch and Sonnewald 2013b; Rasmussen et al. 2013). The complexity of interactions under combined abiotic and biotic stress is further emphasized by the differential regulation of a significant number of both SA and JA/ET-responsive genes under abiotic stress (Huang et al. 2008; Walia et al. 2007). How the up-regulation of defense signalling pathways under combined stress may affect adaptation to abiotic stress has not been established yet, though there is evidence that up-regulation of SA signalling dampens ABA responses (Kim et al. 2011b).

Our research is focused on the regulation of tomato resistance responses to the combination of salt stress and powdery mildew (PM) caused by *Oidium neolycopersici*. We demonstrated that PM resistance was negatively affected by 100mM NaCl in an introgression line (IL) population segregating for partial PM resistance (Kissoudis et al. 2015). Further research indicated that salt stress has the highest impact on disease susceptibility under intermediate concentrations, while salt concentrations imposing severe stress showed an opposite effect (Chapter 4). Combined stress impacted plant performance, significantly more than the individual stresses, which was manifested by accelerated senescence and leaf abscission. However, this response was strongly conditioned by the type of resistance responses to PM as indicated by the examination of near-isogenic lines (NILs) carrying the resistance genes *Ol*-1, *ol*-2 and *Ol*-4 (Chapter 4). *Ol*-1 enhances basal defense by inducing delayed cell death (DCD) in the late stages of pathogen infection (Li et al. 2007; Seifi 2011b and 2014). The recessive gene *ol*-2, which encodes a membrane protein homologous to barley *MLO*, mediates resistance to PM by inducing callose deposition and cell wall fortification to stop PM at penetration stage (Bai et al. 2008a). *Ol*-4, is an NBS-LRR gene homologue to *Mi*-1 (Seifi 2011b) and 2014) that triggers a hypersensitivity
reaction (HR) and thereby prevents the PM colonization after formation of primary haustoria (Bai et al. 2003; Li et al. 2006). Expression analysis of selected pathway marker genes indicated a significant role of ethylene and JA, which were uniquely highly upregulated in the susceptible genotypes under combined stress (Chapter 4).

Here we evaluated the effects of three major hormonal pathways, ABA, JA and ET, on tomato resistance to powdery mildew conferred by different Ol-genes. Two complementary strategies were adopted in this work. First we monitored the expression of marker genes for different phytohormone pathways by using NILs that carry each of the different Ol-genes (Ol-1, ol-2 and Ol-4). Then we evaluated whether PM resistance in these NILs is compromised in single (either salt or PM) and combined stresses (salt and PM) when JA, ET and ABA pathways are impaired. Our results provided a better understanding of how major hormonal pathways can affect tomato resistance and plant performance under combined PM and salt stresses.

Material and Methods

Plant and fungal materials

The recessive epinastic (epi), and notabilis (not) tomato mutants and their respective backgrounds AC (Ailsa Craig), and VNF8, were obtained from the Tomato Genetic Resource Center (TGRC), University of California, Davis, California. The tomato defenseless1 (def1) recessive mutant was obtained from Dr. C.A. Ryan, Washington State University. The near-isogenic lines, NIL-Ol-1, NIL-ol-2 and NIL-Ol-4 (in the background of S. lycopersicum cv Moneymaker (MM)), which confer monogenic resistance to PM through different mechanisms, are described in (Bai et al. 2005). Each of the NILs was crossed with the epi, not, and def1 mutants, with the exception of NIL-Ol-4 crossing with not mutant. By subsequent selfing and selection for Ol-genes and the hormonal mutations (described below), F_3 and F_4 plants that were homozygous for both the Ol-gene and mutations were identified and used in the following experiments.

The pathogenic fungus O. neolycopersici originated from infected commercial tomato plants and was maintained on MM plants in a greenhouse compartment at 20±3 °C with 70±15% relative humidity (RH).

Selection for the presence of Ol-genes and hormonal mutations

Selection for homozygous Ol-genes was carried out by using gene-based or tightly-linked molecular markers for the resistance genes (Bai et al. 2005). The primers used for genotyping were: F-TGCTCTAACAAAATCACCAAAATC and R-
AAATGGTCAAACAAAGTCTATTGAG for Ol-1, F-ACCCTTAAGAAATAGGGCAAA and R- ACCATCATGAACCCATGTCT for ol-2, and F-GAACCGGATGTGCTCTTGAC and R-TTCTCCGAGACTTTGAACAAGA for Ol-4.

DNA isolation was carried out according to (Wang et al. 1993) with some modifications. About 10 mg of leaf tissue was homogenized for 5 minutes in a blender with 20 µl of 0.5 N NaOH. Then 20 µl of 100 mM Tris-HCl was added and thoroughly mixed, and 5 µl of this homogenate was diluted with 95 µl of 100 mM Tris-HCl. The PCR reaction mix contained 0.12 µl of Phire Hot Start II DNA Polymerase (Thermo Scientific), 2 µl Forward primer (5 µM), 2 µl Reverse primer (5 µM), 1 µl of the diluted leaf homogenate as a DNA template and 1 µl of PVP (10% w/v) as a chelating agent for impurities, into a final volume of 11 µl. The amplification profile was 40 cycles of 98 °C for 5 seconds, 54 °C for 5 seconds and 72 °C for 10 seconds.

Different selection approaches were used to select for plants with a homozygous hormonal mutation, depending on whether the gene and the polymorphism underlying the mutation is known. The not mutation is well characterized and is the consequence of a specific A/T base pair deletion in the coding sequence that has resulted in a frameshift mutation (Burbidge et al. 1999), indicating that it is a null mutant. Plants homozygous for the not mutation were selected based on sequencing an amplicon containing the location of the A/T mutation at position 597 of the ORF (primers used not-F- GTTCGAAACGGAGCTAACCC, not-R- AACAAGTCCGAAGAGCCCA). The gene mutation causing the epi phenotype is not known, but mutant seedlings carrying the epi mutation are significantly shorter when germinated in the dark compared to wild type plants (Barry et al. 2001). Thus, seeds were germinated in the dark and plants showing no etiolation were selected as homozygous plants carrying the epi mutation. Selection for the def1 mutant was done on the basis that this mutation affects JA biosynthesis (Howe et al. 1996). Thus, the mutants are unable to synthesize the hormone under conditions normally inducing JA biosynthesis and signalling, such as wounding. To test this, we pierced a single leaflet of the wild type, JA deficient parental lines and evaluated the induction of the JA marker leucine aminopeptidase A (LapA) 24hr after wounding with primers; F- ATCTCAGGTTTCTCTGGTTAAGGA, R-AGTTGCTATGCGAGCGAGCAGAG. RNA isolation was performed with a MagMAX™-96 Total RNA Isolation Kit in a KingFisher™ Flex Magnetic Particle Processor according to manufacturer’s instructions, and expression of the LapA gene was evaluated. An average of 100-fold difference in expression was observed between wild type (wt) plant and homozygous def1 mutant, thus it could be used safely as a qualitative marker for selecting the mutant.
Experimental conditions and treatments

Experiments were carried out with a photoperiod regime of 16 hours light and 8 hours dark. Greenhouse air humidity was 70%. Additional lighting (100 Wm\(^{-2}\)) was supplied if the incoming radiation was below 200 Wm\(^{-2}\). Plants were grown in pots filled with vermiculite and were irrigated with half strength Hoagland’s nutrient solution at regular intervals till leaching of the solution, in order to avoid accumulation of nutrients and NaCl.

The experiments were carried out twice in different years (2013, 2014) in the period of April-May. In the first experiment, homozygous plants (4 plants per line) of all resistance genotypes x mutant combinations (e.g. Ol-1xdef plants, etc) were evaluated for their susceptibility to PM or in combination with salt stress, along with plants selected from the segregating population that carry the Ol- genes but not the hormonal mutations. For each combination, 6 to 8 plants were tested. Three-week old plants were watered with half strength Hoagland solution containing either zero (no salt stress) or 50 mM NaCl (mild salt stress). Eight days after the initiation of salt treatments, plants were inoculated with PM by uniformly spraying a suspension of \(5 \times 10^4\) conidia*ml\(^{-1}\) prepared by washing conidial spores from leaves of heavily infected (sporulation stage) MM plants. Plants were further grown for 20 days after inoculation.

In the second experiment, all resistance genotypes x mutant combinations were similarly assessed (excluding the genotypes from the population that carry the Ol- genes but not the hormonal mutations). In addition, we included a non-PM treatment (only salt stress). Half of the plants from Ol-1xepi or Ol-1xnot (selected based on their explicit phenotypes) were spatially isolated eight days after the salt treatments and were not sprayed with powdery mildew, which allowed to have plants receiving all possible treatment combinations (no salt stress/not inoculated, no salt stress/inoculated, salt stress/not inoculated, salt stress/inoculated). Plants were further grown for 20 days after inoculation.

Plant performance evaluation under salt stress and powdery mildew

The disease severity was assessed at 10, 15 and 25 days post inoculation (dpi) for the first experiment, and at 15 dpi for the second experiment as disease index (DI) on a scale from 0 to 5 as described before (Kissoudis et al. 2014). In addition to DI, a measure of the extent of senescence (senescence index (SI)) was introduced to describe the accelerated senescence phenotypes observed at the later stages of infection under salt stress: 0 = healthy plant, 1 = 0.1 - 10 % of foliar area affected, 2 = 10-20 % area
affected with yellowing and moderate wilting, 3 = 20 – 30 % area affected with severe wilting, 4 = 30-50 % area affected with severe wilting and moderate leaf abscission, and 5 = > 50 % area affected with severe wilting and leaf abscission.

**Ion content analysis**

The five youngest leaves (counting from the top of the plant) were sampled at 20dpi, the endpoint of the second experiment, in order to examine differences in actively growing tissues, potentially linked to growth performance, and avoid the dying bottom leaves of susceptible genotypes under combined stress conditions. The concentration of Na\(^+\), Cl\(^-\), K\(^+\), PO\(_4\)\(^{3-}\), SO\(_4\)\(^{2-}\), Mg\(^{2+}\) and Ca\(^{2+}\) was measured with ion chromatography as described previously (Kissoudis et al. 2015).

**In situ histological analyses of H\(_2\)O\(_2\) accumulation and callose deposition**

Leaf disks (1.3 cm in diameter) were sampled from leaflets of the 4\(^{th}\) leaf counting from the bottom on the 3\(^{rd}\) day after pathogen inoculation. To ensure uniformity, leaf disks were taken from the middle of the leaflets on both sides of the central vein. Staining was carried out in 24-well plates, where leaf disks were placed with the abaxial side up. For H\(_2\)O\(_2\) visualization, leaf disks were stained in 1 mg/ml DAB (3-3’-diaminobenzidine), pH =3.7 for 16 h in the dark and were subsequently transferred to 96% ethanol for 24h to remove chlorophyll according to (Martinez De Ilarduya et al. 2003). Leaf disks were mounted on glass slides with 70% glycerol. DAB staining intensities were quantified from digital photographs by the number of dark-brown DAB pixels relative to total pixels corresponding to plant material.

Callose deposition visualization was performed according to (Luna et al. 2011; Ton et al. 2005) with slight modifications. Leaf disks were initially placed in 96% ethanol to remove chlorophyll and after a 1-min wash in 0.07 M K\(_2\)HPO\(_4\) (pH=9), were stained for 2 hrs in 0.02% (w/v) aniline blue in 0.07 M K\(_2\)HPO\(_4\) (pH=9) at room temperature. Leaf disks were mounted on glass slides with 70% glycerol. Callose was quantified from digital photographs by the number of white pixels (fluorescence, related to callose intensity) relative to the total number of pixels covering plant material using Adobe Photoshop.

**Gene expression and pathogen quantification analyses with qRT-PCR**

For the time course expression study on hormonal marker genes in the NILs with only PM challenge, the same time series that was used previously for cDNA-AFLP profiling (Li et al. 2007) was used in this experiment. In brief, this time series
included cDNAs from plants of MM, NIL-Ol-1, NIL-ol-2 and NIL-Ol-4. Non-inoculated (mock) and PM-inoculated leaves (2nd and 3rd) of three plants per line were collected at 1, 3, 5, 7 and 9 dpi. For each line, the cDNAs from mock samples from different time points were mixed and used as calibrator for qRT-PCR analysis.

To evaluate the expression of stress, defense and hormone marker genes under salt and PM stresses, the 3rd and 4th leaf counting from the bottom was sampled at 6 dpi, when pathogen mycelium growth is not visible. Sampling of the 4th and 5th leaf counting from the bottom for pathogen quantification was carried out 14 dpi, when pathogen growth from the primary infection had reached its peak. For each genotype, 3 to 5 plants were used.

RNA template for gene expression analyses was isolated with the RNaseasy plant mini kit (Qiagen), while plant and fungal DNA for pathogen quantification analyses was extracted with DNeasy plant mini kit (Qiagen). RNA was treated with DNase I (Invitrogen) to eliminate residual DNA. cDNA synthesis was performed with 1 μg RNA template using iScript™ cDNA Synthesis Kit (BioRAD). Quantitative real-time PCR was conducted using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). The reaction mix contained 5 μl 2X iQ SYBR GREEN super mix, 1 μl Forward primer (3 μM), 1 μl Reverse primer (3 μM) and 3 μl cDNA (or DNA, 20 ng) template, in a final volume of 10 μl. Thermocycling conditions were 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 30 seconds. Primers used for fungal quantification were Fw-On-CGCCAAAGACCTAACCAA and Rv-On-AGCCAAGAGATCCGTTG. Primers for tomato elongation factor 1α (EF) were Fw-EF-GGAACTTGAGAAGGAGCCTAAG and Rv-EF-CAACACCAACAGCAACAGTCT (Gao et al. 2014b). The primers used to monitor the expression of tomato genes are provided in (Supplementary Table 1). Relative expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001).

Results

Time course expression analysis of hormonal pathways in NILs.

To monitor changes in JA, SA, ET, and ABA pathways, the expression levels of marker genes or these pathways were measured in the NILs and MM, in a time course from 1 to 9 dpi with PM. Significant differences were observed in the expression patterns and in the magnitude of induction for some of these pathway related genes in the NILs and MM (Fig 1).
SA induces expression of a group of pathogenesis-related genes (PR genes) in Arabidopsis including PR-2, which is often used as a marker gene for SA pathway (Uknnes et al. 1992). The tomato PR-2 gene (Domingo et al. 1994) was induced in response to Phytophthora infestans as well as in response to Benzothiadiazole (BTH, an analog of SA) (Beyer et al. 2001). Therefore, we used PR-2 as a marker gene for the SA pathway in this study (see SlPR-2 in Supplementary Table 1).

![Figure 1](image)

**Figure 1.** Expression of a) PR-2, b) Chitinase9, c) LOXD and d) rd22 (markers for SA, ET, JA, and ABA pathways, respectively) in MM, and NIL-Ol-1, -Ol-2, and -Ol-4 in a time-course after inoculation with PM. Second and third leaves were sampled at 1, 3, 5, 7, and 9 days post inoculation (dpi) from powdery mildew-inoculated and non-inoculated (control) plants (n=4).

At 1 dpi, there was an induction in the PR-2 expression in NIL-ol-2 and NIL-ol-4, but very little induction in NIL-ol-1 and MM. At the last time point (9 dpi), the PR-2 expression was increased in MM, NIL-ol-1 and NIL-ol-4, with the highest level in NIL-ol-1 (approximately 9-fold induction compared to non-inoculated plants).

The ET pathway signalling was monitored through the expression of the Chitinase9 (Chi9) gene, which has been used as a marker gene for ET pathway in tomato (Barry et al. 2001). The expression level of Chi9 did not show great fluctuations across genotypes and time points with the exception of NIL-ol-1 in which a marked up-
regulation was observed in the later time points (4.5- and 8-fold induction at 7 and 9 dpi, respectively).

The lipoxygenase D (LOXD) gene has been shown to be induced by JA in tomato (Heitz et al. 1997), thus we used this gene as a marker for the JA pathway. Its expression was relatively stable or slightly down-regulated across all genotypes till 7dpi, but a marked up-regulation was observed in all genotypes at 9 dpi, which was strongest in MM and NIL-ol-2 (increase of approximately 6 and 4-fold, respectively, compared to control conditions).

In Arabidopsis, rd22 is an ABA-responsive gene (Shinozaki et al. 2003). By performing TBLASTN in tomato Unigene database (http://solgenomics.net) the homologues of these genes in tomato (EU679376.1) were retrieved and used as the tomato rd22 orthologue. Similar to the JA marker LOXD, the rd22 expression was relatively stable among genotypes in the time points from 1-7dpi and was significantly up-regulated at 9dpi. MM showed the highest expression, 8.5-fold increase compared to control conditions, while all NILs exhibited a 4-fold upregulation.

**Effects of hormonal mutants on the PM resistance in the NILs under combined stresses**

In order to evaluate the effect of hormones on tomato PM resistance conferred by different Ol-genes (Ol-1, ol-2 and Ol-4), we crossed the NILs with the tomato hormonal mutants def1 (JA-deficient) (Howe et al. 1996), not (ABA-deficient) (Burbidge et al. 1999) and epi (ET overproducer) (Fujino et al. 1988). F3 and/or F4 plants that were homozygous for each Ol-gene (Ol/Ol) and mutation (m/m) were selected for each cross combination and evaluated for PM resistance/susceptibility under no stress and salt stress condition. 50mM NaCl was applied which represents a mild salt stress that is representative for agricultural production conditions and has been shown to greatly affect resistance in combination with PM (Chapter 4). The mutants and their background lines, as well as plants from the crosses that are homozygous for individual Ol-genes but do not carry the hormone mutations (null segregants) were evaluated for PM susceptibility. The hormone mutants and their background lines were all susceptible to PM, and not significantly different from the susceptibility of MM. The null hormone mutant segregants however were as resistant as the NILs, suggesting that the resistance conferred by the Ol-genes was not affected by the genetic background crosses (data not shown).
Similar to our previous results (Chapter 4), application of 50mM NaCl significantly increases the PM susceptibility of MM and NIL-Ol-1, while the resistance level of NIL-ol-2 and NIL-Ol-4 was not affected (Fig. 2). The resistance conferred by Ol-1 and ol-2, but not Ol-4 was significantly affected when combined with the hormone mutants (Fig. 2, Supp. Fig. 1).

Resistance conferred by the Ol-1 gene was compromised in plants carrying the epi mutation without salt stress (e.g. average DI of 3.2 for Ol-1xepi compared to 0.6 for NIL-Ol-1), and susceptibility of Ol-1xepi plants was further increased under salt stress (e.g. DI of 4.3 for Ol-1xepi with salt compared to 3.2 without salt, Fig. 2a). The significant increase in susceptibility of the Ol-1xepi plants was accompanied by almost complete abolishment of the accelerated senescence and cell death symptoms under salt stress observed in NIL-Ol-1 (senescence index-SI of 0.6 compared to 2.8 for NIL-Ol-1, Fig. 2b). The Ol-1xnot plants showed a level of resistance similar to NIL-Ol-1 plants without salt stress. However, ABA deficiency markedly increased the compromised Ol-1-conferred resistance under salt stress (DI of 0.7 for Ol-1xnot compared to 2.4 for NIL-Ol-1, Fig. 2a), and additionally reduced the accelerated senescence and leaf abscission phenotype (SI of 1 compared to 2.8 for NIL-Ol-1, Fig. 2b). JA deficiency impacted senescence in PM treated Ol-1xdef plants with increased yellowing and older leaves abscission (Supp. Fig. 1a,c).

For the ol-2-mediated resistance, increased susceptibility was observed in ol-2xdef, ol-2xepi and ol-2xnot plants. Under salt stress, this susceptibility was significantly further increased for ol-2xepi plants (DI of 1.2 with salt compared to 0.8 without salt), while it was significantly decreased for ol-2xdef and ol-2xnot plants (DI of 0.3 and 0.5 with salt compared to 1 and 1.5 respectively without salt). No significant differences were observed for senescence under either PM infection or combined stress between NIL-ol-2 and any of the ol-2x hormone mutant plants.
Figure 2. a) Disease and b) senescence index of NIL-Ol-1, -ol-2, -Ol-4, the recurrent parent MM and their crosses with different hormone mutants under powdery mildew individually (no salt stress) and in combination with 50 mM NaCl measured at 15 dpi. Error bars depict standard error (n=6). Asterisks denote statistically significant differences (P≤0.05) between powdery mildew and combined stress treatments for individual genotypes.

PM quantification by means of qPCR was in line with the visual scoring, in many occasions revealing even greater differences between genotypes or treatments. Only for NIL-Ol-1 plants and Ol-1xdef plants under combined stress the qPCR results revealed a smaller difference compared to what our visual scoring suggested, potentially due to the senescence symptoms leading to an overestimation of visual disease score (Fig. 3).
Relative Oidium neolycopersici fungal biomass (calculated as the ratio of fungal ITS gene amplification in comparison with tomato EF1a and normalized with the values of MM under powdery mildew infection (no salt stress)) in MM and NIL-Ol-1, -ol-2, and -Ol-4 and their respective mutants under powdery mildew infection alone and in combination with 50 mM NaCl. Asterisks denote statistically significant differences ($P \leq 0.05$) between powdery mildew and combined stress treatments for individual genotypes.

**Figure 3.**

**Performance and fitness cost of NIL-Ol-1 and NIL-ol-2 crosses with the epi and not mutants under combined stress**

Explicit phenotypes were observed in Ol-1xepi, Ol-1xnot, ol-2xepi, ol-2xnot plants, which were studied in more detail under control conditions, salt stress (50 mM) only, PM only, and combined salt stress and PM, allowing a comparison of growth performance cost under the different stress conditions. These Ol-gene and mutant combinations are particularly interesting as ABA is the major hormone orchestrating abiotic stress responses in plants (Yoshida et al. 2014), while ET signalling was shown to be crucial for plant susceptibility and senescence responses under combined stress (Chapter 4).

The Ol-1xepi and ol-2xepi plants had reduced biomass under conditions without stress compared to the respective NIL line, but had increased biomass under salt stress relative to biomass under control conditions. In contrast and as expected, ABA
deficiency conferred by the not mutation significantly increased reduction of biomass under salt stress relative to control conditions (Fig. 4). PM resulted in a decrease in aboveground fresh weight in all Ol-gene x mutant combinations. The combination of salt and PM imposed an even greater growth penalty than salt stress alone. While the reduction in performance between salt stress only and combined salt stress-PM was lower in the not mutants crosses, the growth reduction under salt stress per se was far greater in the ABA deficient plants compared to the NILs (Fig. 4).

Figure 4. Aboveground biomass (FW) of MM, NIL-O-1, ol-2, and their crosses with the hormone mutants under control conditions (0) and salt stress (50mM NaCl) on the x-axis, and with or without powdery mildew (black vs. light grey). Level 0 for salinity stress corresponds to stress-free control conditions, while level 0 for powdery mildew-combined stress corresponds to powdery mildew infection alone (no salt stress). Asterisks denote statistically significant differences (P≤0.05) between salinity and powdery mildew-combined stress for individual genotypes.

Ion content, and especially Na\(^+\) and Cl\(^-\) concentration was shown to impact PM susceptibility (Chapter 4). The Ol-1epi and Ol-1xnot plants accumulated a higher amount of Na\(^+\) and Cl\(^-\) under salt stress compared to the respective parental NILs (Suppl. Fig.2). However, the Ol-1xepi and ol-2 x epi plants exhibited a significant reduction in the concentration of Na\(^+\) and Cl\(^-\) under combined PM and salt stress compared to salt stress only, while the Ol-genexnot combinations had increased Na\(^+\) and Cl\(^-\) concentrations under these conditions. K\(^+\) content was significantly higher in Ol-1xepi and ol-2xei plants under all conditions examined.
Histological analyses of callose deposition and H$_2$O$_2$ accumulation

Callose deposition at the sites of attempted pathogen penetration increases plant resistance and is involved in ol-2-mediated resistance (Bai et al. 2008b; Ellinger et al. 2013).

Figure 5. a) Callose deposits in leaves of MM, NIL-Ol-1 and ol-2 and their respective crosses with epi and not mutants as visualized with UV microscopy after aniline blue staining. b) Quantification of callose deposition relative to MM under powdery mildew infection (no salt stress).
As shown previously (Chapter 4), NIL-ol-2 exhibited increased callose deposits compared to NIL-ol-1 and MM upon PM infection, and additional salt stress decreased callose deposits in all genotypes (Fig.5).

Examination of hydrogen peroxide generation with DAB staining indicated slightly higher ROS production in NIL-ol-1 compared to MM after PM infection, while no significant differences were observed for NIL-ol-2. A massive H₂O₂ increase was observed in both Ol-1xnot and ol-2xnot plants. The epi mutation on the other hand did not have a considerable impact on H₂O₂ accumulation (Fig.6a and b).
Figure 6. \(H_2O_2\) visualization after DAB staining in MM, NIL-Ol-1 and ol-2 and their respective crosses with epi and not mutants under a) control and salt stress, b) powdery mildew and combined stress.

**Expression analyses**

Gene expression of additional marker genes for the biosynthesis and signalling of major hormonal pathways, ROS, antioxidant and ion homeostasis pathways involved in abiotic and biotic stress responses of tomato were determined a day prior to powdery mildew symptom development (Fig.7). The expression of the ABA biosynthesis gene \(NCED\) was either reduced (in \(Ol-1\times epi\) plants) or stable (in \(ol-2\times epi\) plants) under combined stress compared to salt stress only. In the not mutant this gene contains a mutation that causes a frameshift mutation. It may be transcribed but does not code for a functional enzyme. ABA deficiency in not is in line with the modest expression levels (significantly lower compared to NILs) of the ABA catabolic gene, \(ABAOH\), and the dehydrin gene, \(DHN-TAS\) under all conditions. Dehydrin expression was highly induced in in \(Ol-1\times epi\) and \(ol-2\times epi\) plants under salt stress.
Figure 7. Expression of genes-markers for hormonal, abiotic and biotic stress signalling pathways in MM, NIL-ol-1 and ol-2 and their respective crosses with epi and not mutants, relative to EF1a, which was used as a housekeeping gene. Treatment and labelling scheme are the same as Fig.4. Asterisks denote statistically significant differences (P≤0.05) between powdery mildew and combined stress treatments for individual genotypes.
(50- and 6-fold, respectively), but this response was completely abolished under combined stress, while it was exceptionally induced under combined stress in NIL-ol-2.

Under combined stress, an induction of ET biosynthetic genes \textit{ACCase} and \textit{ACCox} (\textit{ACO1}) was observed in NIL-Ol-1, accompanying the increased susceptibility and senescence response. This induction was significantly reduced \textit{Ol-1xepi} and \textit{Ol-1xnot} plants. On the other hand, \textit{CHI9} was vastly induced in both \textit{Ol-1xnot} and \textit{ol-2xnot} plants (up to 20-fold compared to NIL-Ol-1 and NIL-ol-2) and this was maintained in all the (stress) treatments.

Expression levels of \textit{AOS} and \textit{LOXD}, nodes of the JA pathway, were significantly reduced in \textit{ol-2xepi} and \textit{ol-2xnot} crosses under salt and combined stress treatments (reductions up to 6-fold). \textit{PR1a} induction observed in NIL-Ol-1 after PM and combined stress (25- and 70-fold higher, respectively, compared to non-stress conditions) was greatly reduced in both \textit{Ol-1xepi} and \textit{Ol-1xnot} plants, despite the higher basal expression in these plants. The strong induction of invertase \textit{Lin6} observed in NIL-Ol-1 under combined stress (see also Chapter 4) was greatly reduced in \textit{Ol-1xepi} and \textit{Ol-1xnot} plants.

**Discussion**

Plant hormones are central modulators of plant responses to environmental stress and pathogen attack. Hormonal regulation is therefore important for adaptation to both abiotic and biotic stresses (Peleg and Blumwald 2011; Robert-Seilaniantz et al. 2011). Our results showed differential response of the major hormonal pathways involved in abiotic (ABA) and biotic stress (JA, ET) in response to PM, salt stress and PM and salt stress combined in tomato NILs carrying different genes for resistance to PM (Fig.8), as exemplified by growth response, histochemical development of disease and by gene expression of key genes involved in different signaling pathways.

**The epi mutation compromises the Ol-1- and ol-2-mediated PM resistance**

The \textit{Ol-1} gene confers incomplete PM resistance by inducing delayed cell death (DCD) at the late stage of PM infection (Li et al., 2007; Seifi et al. 2011a). The gene is not cloned yet, but it likely is a non NBS-LRR gene enhancing basal defense. The \textit{ol-2} gene is a \textit{mlo} mutant and mediates resistance to PM by inducing callose deposition to stop PM at penetration stage (Bai et al. 2005).
The PM resistance mediated by the \textit{Ol-1} and \textit{ol-2} gene was compromised by the \textit{epi} mutation, suggesting a negative role of ethylene signaling in \textit{Ol-1} and \textit{ol-2}-mediated resistance to PM. In addition, salt stress had an additional negative effect on the PM susceptibility of \textit{Ol-1epi} and \textit{ol-2epi} plants, indicating an additive effect of this abiotic stress and ethylene in compromised \textit{Ol-1} and \textit{ol-2}-mediated resistance. This is in line with the established role of ethylene in susceptibility for biotrophic pathogens in \textit{Arabidopsis} and several other plant species through negative interaction with salicylic acid signaling (Pieterse et al. 2009). Ethylene signaling appears to be involved in disease symptom development against \textit{Xanthomonas campestris} pv.
vesicatoria in tomato, which additionally requires the sequential induction of jasmonates and SA (O’Donnell et al. 2003).

The *epi* mutant has been shown to overproduce ethylene (Fujino et al. 1988). This can be reflected by the expression of ACCase which showed an approximate 10-fold induction compared to WT. No differences were observed in *ACO1* expression, the final enzyme in ethylene biosynthesis. This is in accordance with previous studies that showed that ACC (the product of ACCase and the rate-limiting compound for ethylene synthesis) is increased in the *epi* mutant (Fujino et al. 1988).

However, *epi* is a mutant with pleotropic phenotypic effects severely affecting plant morphology, such as reduced growth and leaf epinasty (Barry et al. 2001; Fujino et al. 1988). There is a striking difference between the increased senescence and cell death observed in NIL-Ol-1 and the complete absence of senescence and cell death in *Ol-1xepi* plants under combined stress, which seems to contradict the known promotion of senescence by ethylene (Penfold and Buchanan-Wollaston 2014). Ethylene overproduction is also shown to stimulate ROS production and the accompanying symptoms (Bartoli et al. 2013). However this did not occur in *epi* plants, in accordance with the absence of senescence symptoms. In NIL-Ol-1, increased susceptibility under combined stress was accompanied by an induction in the expression of ethylene biosynthesis and response genes *ACC* and *ACO1*, but not for *CHI9*. In *Ol-1xepi* plants, this increase was only modest for *ACC* and *ACO1*, thus ethylene biosynthesis might have not exceeded a certain threshold to impact senescence.

Similar observations on the lack of important ethylene-induced symptoms such as increased senescence were reported previously for this mutant (Barry et al. 2001). Additional pleiotropic alterations at the cellular level have been observed for *epi*, especially changes of the epidermal cells which are different from the wild type by having a more round shape and being swollen (Barry et al. 2001). These changes can be functionally significant for biotic stress responses through a potential effect on the cytoskeleton dynamics and the secretion and deposition of anti-fungal compounds. Manipulation of these processes resulted in a significant compromise of exocytosis mechanisms which are linked to the transport of antifungal compounds at the site of infection and increased susceptibility in PAMP-mediated resistance, but had no impact on HR-mediated resistance (Hardham et al. 2007; Henty-Ridilla et al. 2014; Miklis et al. 2007). This shares significant similarity with our findings of reduced callose deposition in *Ol-1xepi* and *ol-2xepi* plants while the HR based resistance conferred by *Ol-4* was unaffected by the *epi* mutation.
The *not* mutation influences differently the PM resistance mediated by the *Ol*-1 and *ol*-2 gene

The *not* mutation induces ABA deficiency and had both positive and negative impacts on disease resistance conferred by the *Ol*-genes, depending on the *Ol* gene and the combination with salt stress. It slightly but significantly increased susceptibility of NIL-*ol*-2 after PM infection only, while no significant changes were observed for NIL-*Ol*-1. Under combined stress, the increased susceptibility and senescence of NIL-*Ol*-1 was significantly alleviated in the *Ol*-1*not* plants. In *ol*-2*not* plants a slight decrease of susceptibility was also observed under combined stress. These results indicate a complex interaction between ABA signaling and disease resistance as pointed out by numerous previous studies (Audenaert et al. 2002; Curvers et al. 2010; De Torres Zabala et al. 2009; Mang et al. 2012) and the addition of salt stress adds one more layer of complexity.

Both ROS production (increased) and callose deposition (decreased) were significantly affected in *Ol*-1*not* and *ol*-2*not* plants and might underlie the differential resistance responses between the resistance-hormone combinations under different treatments. A ROS-induced oxidative burst has been considered as a means of defense against pathogens and was shown to contribute to defense against *Botrytis cinerea* in the tomato ABA deficient mutant *sitiens* (Asselbergh et al. 2007). However, recent findings support a minimal effect on pathogenicity for the ROS-induced oxidative burst (Samalova et al. 2014). In *ol*-2*not* plants, reduced callose deposition may have allowed increased PM penetration, with the further growth of the pathogen overriding the effect of increased ROS levels. The addition of salt stress partially decreased disease symptoms in *ol*-2*not* plants, accompanied by increased callose deposition. This increased callose deposition potentially results from the partial restoration of ABA signaling by exposure to stress, positively affecting callose deposition. The *not* mutant has 10-15% ABA compared to WT, and the addition of salt stress may have resulted in induction of additional tomato *NCED* genes, as evidenced by the 10-fold induction of the ABA marker *DHN-TAS*. *Ol*-1*not* exhibited higher callose deposition under combined stress compared to PM only. The elevated levels of Na$^+$ and Cl$^-$ concentration under combined stress might add to salinity-induced increased resistance; the levels observed in the *not* mutants at 50mM NaCl, were similar to the levels observed in MM plants under 150mM NaCl, and this was shown to reduce disease progression in our previous study (Chapter 4).

*Not* mutants exhibited a unique increase in the expression of *CHI9*, which is considered as a component of ET signaling in tomato (Wu and Bradford 2003) and has direct antifungal properties (Hong and Hwang 2006).
The most pronounced effect of the *not* mutation under combined stress was the marked attenuation of senescence and leaf abscission in *Ol-1xn* plants. This occurred despite the very high levels of ROS observed, which are known to be associated with senescence (Gregersen et al. 2013), although H$_2$O$_2$ alone was insufficient in triggering cell death in tobacco in response to bacteria (Mur et al. 2005). Our results indicate that ABA induces senescence, with recent studies supporting these findings (Yang et al. 2014b). Uncontrolled cell death and senescence under combined stress may therefore be mediated through the control of the ABA pathway. Ethylene signaling regulation might be also important for this phenotypic response as the expression of ethylene biosynthesis and response genes was reduced in the *not* crosses with *Ol*-genes. Literature describes both synergistic and antagonistic regulation of ABA and ET, though under abiotic stress ABA appears to enhance ethylene levels (Albacete et al. 2009). ABA deficient *not* and *sitiens* mutants have lower ET content compared to WT plants (Nitsch et al. 2012), thus the effect of ABA deficiency might be mediated by ethylene signaling.

**Concordance of hormonal pathway induction during PM pathogenesis with phenotypes of Ol-gene and mutant crosses**

Ethylene signaling is induced in NIL-*Ol*-1 compared to MM and other NILs at 7 and 9 dpi (Fig. 1), and the *epi* mutation might be disrupting this pattern resulting in increased susceptibility. Stress induced ABA signaling appears to contribute to susceptibility in NIL-*Ol*-1 and is induced in the susceptible MM in response to PM infection, which agrees with the restoration of the compromised *Ol*-conferred resistance in *Ol-1xn* plants.

JA signaling is induced in the resistant NIL-*ol*-2 challenged with PM, but disruption of JA signaling in the *ol-2xdef* mutant results in partial breakdown of resistance. This information on synagonistical interaction of abiotic stress with defense pathways can be of great significance for the maintenance of resistance of *ol*-2 under combined stress.

**Fitness cost and benefit NIL- Ol-1 and NIL- ol-2 crosses with epi and not under combined stress**

Despite its positive effect in decreasing senescence under combined stress, ABA deficiency had a severe plant performance cost in terms of fresh weight under salt and combined stress. The ABA pathway appears to be a major node in the negative crosstalk of adaptation to abiotic and biotic stress (Sanchez-Vallet et al. 2012; Yasuda et al. 2008). Thus ABA signaling should be studied in more detail under combined
stress, including examination of downstream signaling components such as transcription factors and kinases to identify nodes that enhance disease resistance but do not affect abiotic stress adaptation and vice versa (Garcia-Andrade et al. 2011). Ethylene overproduction in the *epi* mutant crosses resulted in better relative growth performance under salt as well as combined stress, despite the increase in PM susceptibility. However, the growth penalty of the *Ol-gene x epi* plants under control conditions should be taken into account when considering the *epi* mutation for improving stress tolerance of commercially grown tomato under multiple stress conditions. Nevertheless, the potential of adapting ethylene signaling for improving crop resilience is further supported by several studies identifying a positive contribution of ethylene signaling components in adaptation to abiotic stress (Cheng et al. 2013b; Jiang et al. 2013b; Peng et al. 2014).

ABA, JA and ET pathways have no influence on the resistance mediated the *Ol-4* gene

In contrast to *Ol-1* and *ol-2*, the resistance mediated by *Ol-4* was unaffected under all treatment and with mutant combinations. *Ol-4* is a homolog to the *Mi-1* gene coding NBS-LRR protein (Seifi 2011b). It triggers HR in a single epidermal cell where the fungal growth can be stopped completely (Bai et al. 2003; Li et al. 2006). R-gene resistance is based on effector triggered immunity (ETI) which is characterized by compensatory relationships between its different signalling components and its defense output is stronger and more prolonged compared to PAMP triggered immunity (PTI) (Tsuda et al. 2009), thus it is more robust and less prone to negative regulation from environmental or genetic factors. Since resistance conferred by R genes is not affected by large genetic perturbations disrupting whole hormonal pathways, it has the potential to be stable in combination with larger changes in hormone signaling pathways conferring abiotic stress tolerance.

In conclusion ethylene appears to be central in the responses under combined stress, increasing susceptibility despite being beneficial for plant salt tolerance. ABA and JA role on the other hand appears to be more complicated as their effect was dependent on the type of resistance and the co-occurrence of salt stress. ABA deficiency appears to limits senescence symptoms, with however significant trade-offs on plant salt tolerance and growth. Thus a more delicate approach should be carried to identify specific components of ABA with fewer pleiotropic effects, to be effectively implemented in increased combined stress tolerance in crops. Further research is required to delineate the synergonistic and antagonistic relationships between signalling components under combined stress and to implement them with precision.
breeding. Alternatively the stacking of robust R-genes, like Ol-4 with well-established abiotic stress tolerance inducing genes and loci can be followed providing robust resistance under abiotic and biotic stress combination, with the prerequisite that no negative interactions on the underlying signalling pathways occur.

**Acknowledgements**

Christos Kissoudis is supported by “Alexander S. Onassis” Public Benefit Foundation scholarship.

**Supplementary material**

The supplementary data can be retrieved at DOI: 10.18174/369639 or http://edepot.wur.nl/369639
Chapter 6

Roles and contribution of tomato WRKY genes to salt stress and powdery mildew resistance

Christos Kissoudis¹,², Dongli Gao¹, Dewi Pramanik¹, Mengistu Birhanu¹, Clemens van de Wiel, Richard G.F. Visser, Yuling Bai, C. Gerard van der Linden

¹Wageningen UR Plant Breeding, Wageningen University & Research Centre, P.O. Box 386, 6700AJ, Wageningen, the Netherlands

²Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands
Abstract

WRKY is a transcription factor family unique to plants with diverse functions in defense pathways, abiotic stress tolerance and developmental programs. Family members are characterized by the conserved WRKY domain and significant sequence variation in the remainder of the protein, which is translated into distinct functions even for closely related genes. We utilized the extensive functional characterization of the *Arabidopsis thaliana* WRKY family to identify tomato homologues of Arabidopsis WRKY genes that are involved in defense responses (*AtWRKY 11, 29, 48, 70 and 72*). In total 13 tomato WRKY homologues were identified for these genes, of which 9 were successfully over-expressed, and 12 stably silenced via RNAi in transgenic tomato lines. The transgenic lines were evaluated for their response to salt stress, powdery mildew resistance and the combination of these stresses. Lines overexpressing *SlWRKY11* and *SlWRKY23*, and RNAi lines of *SlWRKY7* and *SlWRKY9* showed both increased biomass and improved salt tolerance. For *SlWRKY11* and *SlWRKY23* overexpression (OE) lines, this was accompanied by a moderate increase in oxidative stress tolerance. The *SlWRKY6*OE line showed strongly improved salt stress tolerance, but a growth penalty under control conditions. Exceptional phenotypes were observed for the *SlWRKY10*OE line (stunted growth) and the RNAi line *SlWRKY23*RNAi (necrotic symptoms), but these phenotypes were partly restored to normal under salt stress. Both these lines exhibited increased resistance to powdery mildew, but this was compromised when the plants were put under salt-stress as well. Important functions for tomato WRKY genes were revealed in both the abiotic and biotic stress response and several genes should be further explored to elucidate their downstream regulatory functions that lead to increased stress tolerance.

**Keywords:** salt stress tolerance, oxidative stress, ROS burst, lesion mimic, growth-defense trade-off
Introduction

Transcription factors play crucial roles in regulating stress-inducible gene expression, which leads to adaptation of plants to biotic and abiotic stresses (Mizoi et al. 2012; Puranik et al. 2012; Singh et al. 2002). The plant-specific WRKY family was initially shown to function in plant defense responses, but was later found to be involved in the regulation of diverse functional processes such as growth, development, hormone-mediated pathways, and abiotic stresses (Bakshi and Oelmuller 2014). Members of this gene family are therefore candidate genes for contributing to crop resilience against numerous stress conditions.

WRKYs are a superfamily of transcription factors carrying the highly conserved WRKY domain, a 60 amino acid region at the N-terminal end, which contains the highly conserved WRKYGQK sequence at the N-terminus followed downstream by a Cx4–5Cx22–23HxH or Cx7Cx23HxC zinc-finger motif (Rushton et al. 2010). Based on the number of binding domains and features of the zinc-finger-like motif, WRKY families are grouped into three major groups (Rushton et al. 2010). Several NB-LRR proteins carry WRKY domains (Rushton et al. 2010) that may act as negative regulators of downstream defense signalling (Noutoshi et al. 2005).

The WRKY superfamily comprises numerous members in all plant species studied to date, including 74 in Arabidopsis, 182 in soybean, 102 and 98 in indica and japonica rice, respectively, and 81 in tomato (Bencke-Malato et al. 2014; Huang et al. 2012; Ross et al. 2007; Ulker and Somssich 2004).

WRKY protein function is mediated by the binding of the WRKY domain to W-boxes in the promoters of downstream regulated genes. Its specificity varies depending on the sequence variation of the W-box (Franco-Zorrilla et al. 2014), the amino acid variation of the WRKY-binding domain (Brand et al. 2013), interactions with other proteins and transcription factors (Hu et al. 2013; Lai et al. 2011) and post-translational modifications (Ishihama and Yoshioka 2012). WRKYs are not only transcriptional activators but many members have repressor functions (Yokotani et al. 2013a) and are involved in regulatory cascades and loops including self-regulation or cross-regulation between family members (Cheng et al. 2015; Yan et al. 2013).

Numerous expression and functional studies, primarily in Arabidopsis and rice but also at an accelerating pace in other crop species, outline the involvement of WRKY genes in different aspects of plant biology and stress and defense responses (Bakshi and Oelmuller 2014). These studies indicate that different WRKY family members
can have either positive or negative impact on plant stress tolerance, thus both overexpression and silencing studies are essential to uncover their functions.

The importance of WRKY TFs in resistance to pathogens is evidenced by the fact that pathogen effectors target WRKYs to dampen defense responses (Sarris et al. 2015). Furthermore, overexpression of a number of WRKYs has led to primed defense responses and disease resistance (Dang et al. 2014; Yu et al. 2012; Zheng et al. 2006). Several other WRKY genes however have been shown to function as negative regulators of defense responses (Journot-Catalino et al. 2006; Liu et al. 2014) indicating complex relationships in the WRKY regulatory web to optimize the plant’s defense response.

WRKY members are also directly involved in abiotic stress signaling and tolerance. Expression of ABA-responsive genes is altered in AtWRKY40 or AtWRKY40 / AtWRKY18 knockout lines (Shang et al. 2010). MAP kinase-mediated activation of OsWRKY30 confers drought tolerance in rice (Shen et al. 2012). ThWRKY4 mediates abiotic stress tolerance in the halophytic species Tamarix hispida by modulating stress responses involving ROS through enhanced peroxidase activity (Zheng et al. 2013). WRKY members can be regulators of transpiration (efficiency) under drought stress by modulating stomatal aperture (Ding et al. 2014; Li et al. 2013a). The broad functions of WRKYs are highlighted in functional studies where single WRKY genes affect resistance to a number of abiotic stresses and phytopathogens (Dang et al. 2013; Sun et al. 2015). A desirable outcome for breeding would indeed be increased resistance to multiple stress factors with a single gene (Qiu and Yu 2009). However, opposite effects on abiotic and biotic stress from a single gene have also been reported (Yokotani et al. 2013). WRKYs were shown to be significant regulators of an important component of crop performance under multiple stress factors, i.e. senescence programming (Besseau et al. 2012).

Tomato WRKY genes have rarely been studied and only recently a genome-wide bioinformatics analysis was carried out revealing 81 putative WRKY genes (Huang et al. 2012). In this study, we utilized information on functional characterization of Arabidopsis WRKYs 11, 29, 48, 70 and 72, which are involved in defense responses, to identify and clone tomato WRKY genes with putative functions in stress response. Bioinformatics and in silico analysis of the tomato genes indicated that these are strongly regulated both under biotic and abiotic stress. Taking this information into account, WRKY overexpression (OE) and silencing (RNAi) transgenic tomato lines were examined for their tolerance to salt stress, powdery mildew infection as well as a combination of both stresses. The results confirmed the broad functions of this tomato
WRKY gene set, with several genes contributing to increased salt tolerance or powdery mildew resistance.

Materials and Methods

WRKY gene cloning and generation of transgenic plants
The sequences of the Arabidopsis WRKYs 11, 29, 48, 70 and 72 were queried for homologous sequences in tomato using BLAST. Thirteen tomato WRKY genes exhibited high sequence homology to the above-mentioned genes (Table 1). The full gene sequence was cloned for nine of the thirteen genes, and these were used to create tomato WRKY overexpression lines. For twelve out of thirteen genes, except for WRKY81, RNAi constructs were designed from gene specific regions (Supplementary Table 1). Transformation, transformant selection and plant regeneration was carried out as described in (Gao et al. 2014a; Huibers et al. 2013).

Plant material and growth conditions
Transgenic tomato (Solanum lycopersicum L. cv. Moneymaker, MM) plants of the T1 generation carrying either overexpression or RNAi constructs for the different WRKY genes (Table 1) were evaluated in this study. At least two independent transformation events (lines) per gene were evaluated (4 plants per line).

The seeds of overexpression T0 WRKY tomato lines and MM were disinfected and selected in vitro for the presence of the transgene. For this, seeds were washed in ethanol (95%) for one minute and rinsed once with sterile water. The seeds were subsequently disinfected in 1.5% NaOCl solution for 15 minutes followed by three washes in sterile water. The disinfected seeds were sown in MS medium (pH 5.8, 0.8% agar) supplemented with the antibiotic kanamycin (100 mg/l) for the selection of plants carrying the transgene (except for MM). Every 3-4 weeks, plantlets were propagated on MS medium supplemented with 20 mg/l of cefotaxime to avoid bacterial infections. Rooted explants with uniform size were used in the greenhouse experiments. For all treatments, the plants were transplanted into 3l pots containing vermiculite in the greenhouse and watered with ½ Hoagland medium.

Salt stress treatment and evaluation of plant performance
The transplanted in vitro plants were grown in the greenhouse for 2 weeks to acclimatize and subsequently treated with 100 mM NaCl for 4 weeks. Salt solution was applied until saturation of the solution (until leaching was observed from the pots) to maintain an appropriate and uniform salt concentration.

Growth parameters, shoot length and fresh weight, were measured at the end of the experiment (4 weeks after salt treatment), samples were taken for electric leakage,
and samples were dried for ion analysis (see below). Chlorophyll content was measured 3 weeks after salt treatment using Minolta chlorophyll meter, SPAD-502. Three measurements per plant were made on fully expanded mature leaflets and the average value was taken for each individual plant.

**Electrolyte leakage**

To assess the level of oxidative stress tolerance, electrolyte leakage of leaf disks immersed in paraquat solution was measured. Twelve (12) leaf disks (~7 mm in diameter) were cut from leaflets of each line by cutting with a metal leaf borer and were subsequently put in a tube containing 20 ml of MQ ultrapure water supplemented with 1 µM paraquat solution. The samples were incubated overnight under dark for 12 hrs. Then, the tubes were transferred to the light. The electrical conductivity of the solution was measured twice, i.e. at 24 hrs and 48 hrs after transfer to the light, using an EC meter (Cond 315i, WTW, Germany). Then, samples were autoclaved at 121˚C for 5 min and the final electrical conductivity was measured after cooling down. The electrolyte leakage (EL%) was calculated in percentage as described in the study by (Dionisio-Sese and Tobita 1998):

\[
EL = \frac{EC_i}{EC_f} \times 100
\]

Where \( EC_i \): initial electrical conductivity (24 or 48 hrs); \( EC_f \): final electrical conductivity (after autoclaving).

**Ion content analysis**

Dried leaves and stems were used for ion content analysis (Na⁺, K⁺, Ca²⁺, Mg²⁺, SO₄²⁻, PO₄³⁻, and Cl⁻). The dried samples were ground with a mill with 1 mm mesh. Approximately 30 mg mg of the resulting powder was ashed in an oven for 6 hrs at a maximum temperature of 575˚C. The analyses were performed as described previously (Kissoudis et al. 2015).

**In situ H₂O₂ accumulation histological analysis**

Leaf disks (1.3 cm in diameter) were sampled from leaflets of the 4th leaf counting from the bottom 2 weeks after the initiation of salt stress treatment from only salt-treated plants. To ensure uniformity, leaf disks were taken from the middle of the leaflets on both sides of the central vein. Staining was carried out in 24-well plates, with leaf disks placed with the abaxial side up. For H₂O₂ visualization, leaf disks were stained in 1 mg/mL DAB (3,3’-diaminobenzidine), pH =3.7, for 16 h in the dark and...
the disks were subsequently transferred to 96% ethanol for 24h to remove chlorophyll according to (Martinez De Ilarduya et al. 2003). Leaf disks were mounted on glass slides with 70% glycerol.

**Powdery mildew and combined stress treatments**

The Wageningen isolate of powdery mildew *Oidium neolycopersici* was applied on 4-week old plants (for combined stress eight days after the start of the salt treatment of 100mM NaCl) by uniformly spraying a suspension of 5x10⁴ conidia.ml⁻¹. Plants were evaluated 10 days after inoculation. The disease severity was expressed as disease index (DI) on a scale from 0 to 5, according to (Kissoudis et al. 2015).

**Gene expression analysis**

**Primer design**

To monitor *WRKY* expression with qPCR, gene-specific primers were designed using the web-based primer design program ([http://www.ncbi.nlm.nih.gov/tools/primer-blast/](http://www.ncbi.nlm.nih.gov/tools/primer-blast/); Supplementary Table 2). Primers were designed outside the RNAi construct towards the 3’ end of the sequence region. Multiple alignments were made of each of the *WRKY* genes against all the tomato *WRKYs* to identify non-conserved regions of the coding sequence to be used for primer design.

**Examining expression of stress related genes**

The expression of putative candidate genes indicative for hormonal biosynthesis, ion homeostasis, cell death regulation, NADPH-oxidase, and ROS-scavenging was analysed with real-time qPCR (Supplementary Table 3). Elongation factor (EF1a) was used as reference (housekeeping) gene.

**RNA isolation and cDNA synthesis**

Leaf samples were taken from the second leaf counting from the top (first to be moderately expanded) of salt treated and control plants 2 weeks after the initiation of salt stress. The samples were immediately frozen in liquid nitrogen and stored at -80°C before RNA extraction. The leaf samples were ground thoroughly in liquid nitrogen with mortar and pestle. RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instruction. The concentration and quality of the isolated RNA was checked with a nanodrop spectrometer and 2% agarose gel electrophoresis, respectively. One µg of total RNA was treated with RNase-free DNase I (Invitrogen), and DNase I was inactivated with 1µl of 25 mM EDTA solution. The RNA was then reverse transcribed using iScript™ cDNA synthesis kit following the manufacturer’s instruction.
Quantitative real-time PCR (qRT-PCR)
After cDNA synthesis, real-time qPCR was used to examine and quantify the expression level of the WRKY genes targeted for overexpression or silencing in each of the WRKY lines under control conditions. The reaction mix containing 5 µl SYBR GREEN super mix, 1 µl forward primer, 1 µl reverse primer, and 3 µl template cDNA was prepared with a final volume of 10 µl. The PCR amplification was set as initial denaturation at 95°C for 5 minutes, followed by 39 cycles of 94°C for 10s, 59°C for 30s followed by a melt curve analysis. Relative expression of the genes was determined from the difference in cycles observed to reach the threshold (ΔΔCt) between the target genes and reference gene. Elongation factor (EF1a) was used as a reference gene in each PCR reaction. Each sample analysis was performed with two biological and two technical replicates. The expression level relative to the reference gene expression level was calculated using the formula: $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001).

Promoter sequence analysis and other bioinformatics analyses
The 1000bp sequences of upstream promoter regions of WRKY genes were downloaded from SGN (http://solgenomics.net/). The predicted cis-element motifs in the promoter regions were discovered using plant PAN (http://plantpan.mbc.nctu.edu.tw/) and plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases.

The mRNA, protein and promoter sequences were aligned with MAFFT (http://mafft.cbrc.jp/alignment/server/) and phylogenetic trees were constructed with the Neighbour Joining method. Conserved domains were identified with NCBI online tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Statistical analysis
The experiment was carried out in a split plot design with four replications. Data were evaluated statistically with GenStat 16th edition. Significance was determined by analysis of variance (ANOVA). The treatment means were compared by Fisher protected least significance difference (LSD) test with a significance level of p<0.05.
Results

Identification of WRKY homologues in tomato

Multiple homologous tomato WRKY genes were identified for each of the Arabidopsis genes (WRKYs 11, 29, 48, 70 and 72) queried. Thirteen genes (Table 1) were further evaluated.

Table 1: Tomato WRKY genes examined. Unigene codes, SGN annotation and information on their expression pattern according to literature (Huang et al. 2012) and tomato microarray repositories (http://ted.bti.cornell.edu/) are provided. Similarity with Arabidopsis WRKY genes based on the protein sequence (protein-protein BLAST) is provided.

<table>
<thead>
<tr>
<th>Tomato Unigene</th>
<th>SGN annotation</th>
<th>BLASTp (Arabidopsis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGN-U565155</td>
<td>S\textit{WRKY8} (Moderately expressed, induced drought/salt/pathogen)</td>
<td>15,11,17,39,74</td>
</tr>
<tr>
<td>SGN-U565159</td>
<td>S\textit{WRKY25}</td>
<td>27,29,65,16,22</td>
</tr>
<tr>
<td>SGN-U571282</td>
<td>S\textit{WRKY6} (Induced under drought)</td>
<td>42,6,31,72</td>
</tr>
<tr>
<td>SGN-U587314</td>
<td>S\textit{WRKY22} (repressed under drought/increased under biotic)</td>
<td>27,22,29,65,16</td>
</tr>
<tr>
<td>SGN-U602602</td>
<td>S\textit{WRKY23}</td>
<td>27</td>
</tr>
<tr>
<td>SGN-U578656</td>
<td>S\textit{WRKY81}</td>
<td>62,46,53,63</td>
</tr>
<tr>
<td>SGN-U563809</td>
<td>S\textit{WRKY11} (highly expressed, induced salt/drought, mixed response to pathogens)</td>
<td>11,17,39,74</td>
</tr>
</tbody>
</table>
Messenger RNA and protein sequence analysis confirmed that all of them carried the invariable WRKYGQK amino acid sequence as part of the WRKY domain (Fig.1a). Variation was observed outside of the core sequence with amino acid variants of different hydrophilicity and structure (Figure 1b, c) potentially altering the binding specificity of the proteins. Outside of the WRKY domain, the sequences were highly variable between the identified WRKY genes.

Neighbor Joining (phylogenetic) analysis of both mRNA and protein sequence displays the high variability of the different SIWRKY genes with SIWRKY81 being the most distant. Yet several highly similar genes were identified as well, such as SIWRKY 10 and 11. All genes had a single WRKY domain, and SIWRKY7, 8, 10 and 11 carried a Zn-finger DNA binding domain as well (Fig. 2a). Protein lengths varied from 203 to 570 amino acids.

Promoter sequence alignment and analysis revealed even greater variability between the WRKY genes that was not following the pattern of relatedness at the protein level. For example promoters of the highly similar SIWRKY 10 and 11 exhibited weak similarity, potentially indicating distinct mechanisms of regulation at the level of transcription (Fig. 2b).

<table>
<thead>
<tr>
<th>Tomato Unigene</th>
<th>SGN annotation</th>
<th>BLASTp (Arabidopsis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGN-U576890</td>
<td>SIWRKY10</td>
<td>11,74,15,17,19</td>
</tr>
<tr>
<td>SGN-U577936</td>
<td>SIWRKY48</td>
<td>71,28,57,68,43</td>
</tr>
<tr>
<td>SGN-U571278</td>
<td>SIWRKY73</td>
<td>72,61, 9, 6, 47, 42</td>
</tr>
<tr>
<td>SGN-U571280</td>
<td>SIWRKY74</td>
<td>72,61, 9, 6, 47, 42</td>
</tr>
<tr>
<td>SGN-U573117</td>
<td>SIWRKY7</td>
<td>7,11,17</td>
</tr>
<tr>
<td>SGN-U581993</td>
<td>SIWRKY9</td>
<td>9,72,61,31</td>
</tr>
</tbody>
</table>
Figure 1. Protein sequence alignment of the WRKY DNA-binding domain and visualization of amino acid properties with Jalview: a) ClustalX similarity colour scheme b) hydrophobicity properties of amino acids with the most hydrophobic residues coloured red and the most hydrophilic ones coloured blue c) amino acid burial propensity with the highest depicted with blue while the lowest is green.
Figure 2. Neighbour Joining phylogenetic tree of a) WRKY protein sequence (the presence and position of WRKY and Zinc-finger domains are depicted) and b) the 1000 bp upstream promoter sequence of each gene.
The gene promoters were further queried for the presence of transcription factor-binding cis-acting elements. Several biotic stress-related cis-acting regulatory elements were found in the promoter regions. In addition, several abiotic stress responsive elements were present in all promoters, indicating that tomato WRKYs may respond to multiple signals and might be involved in adaptation to various stress conditions. Numerous homeobox binding domains for Homeodomain (HD) transcription factors were present in all gene promoters, as well as WRKY-binding W-box domains, possibly indicating feedback regulation or cross regulation between different WRKY TFs. Most of the promoters carried the ABA and abiotic stress-responsive ABRE and ACGT domains. Putative ethylene responsive elements (ERE) were found in the SIWRKY7 promoter region. In addition, a putative heat stress responsive cis-acting element (HSE) was found in the SIWRKY23 and SIWRKY9 promoter region. Finally guard cell-specific expression elements were found in SIWRKY6 and SIWRKY23 promoters.

The publicly available microarray databases (http://ted.bti.cornell.edu/) and the recent bioinformatics and expression analysis of the WRKY family in tomato (Huang et al. 2012) provided further information on WRKY expression patterns and response to abiotic and biotic stress factors. The consensus of these data pointed to highly induced expression of SIWRKY6 under drought. SIWRKY11 and SIWRKY10 exhibited contrasting expression profiles with SNWRKY11 being highly and constitutively expressed and even further stimulated by drought and salt stress, with variable expression patterns in response to pathogens, while SIWRKY10 was expressed at low levels under normal conditions and induced only after pathogen infection. SIWRKY7 exhibited contrasting expression patterns in response to abiotic or biotic stress, being induced by salt and drought stress, but repressed after pathogen infection (Table 1).

The WRKY genes used in this study showed a wide range of expression under control conditions in cultivar Moneymaker (MM) (Fig. 3). Most of the genes were expressed at low levels compared to the housekeeping gene EF1a, (SIWRKY48, SIWRKY23, SIWRKY74 and SIWRKY10), while high expression levels were observed for SIWRKY7, SIWRKY11 and SIWRKY8.

**Generation and validation of transgenic plants**

For 9 SIWRKY genes transgenic overexpressor plants were successfully obtained and for 12 SIWRKY genes RNAi lines were made, with the exception of SIWRKY81. All T0 plants produced fruits and seeds, but for SIWRKY10 the stunted phenotype was accompanied by a very low number of fruits and seeds.
The expression of the overexpressed or silenced WRKY genes in the transgenic lines was evaluated and compared to that of the native genes in MM (Table 2). Among the overexpression lines the highest expression compared to the native gene was observed for SiWRKY48 lines (~300-fold highest expression) and high expression levels were also measured in SiWRKY10 (~120-fold) and SiWRKY6 (30-40-fold), while SiWRKY11 exhibited only moderate overexpression (3.5-fold). No overexpression was observed for SiWRKY22 lines and only one line of SiWRKY8 showed significant upregulation (Table 2). Silencing was successful for many but not all target genes and ranged from 2 to 10-fold relative to the native gene expression levels (Table 2). Lines that did not show considerable overexpression or silencing were not analysed further (SiWRKY22 and -81 OE lines and WRKY8, -25, -6, -22, -81, -73, 74 RNAi lines).

Most of the lines (especially the RNAi lines) did not differ significantly from MM in terms of growth. Few lines exhibited increased growth relative to MM especially in terms of plant height, such as SiWRKY23-OE, SiWRKY11-OE, SiWRKY7-RNAi and SiWRKY9-RNAi (Fig. 4a,b, Supp. Fig. 1)
Table 2. Fold difference in the expression of the tomato WRKY genes in the respective overexpression or RNAi (S) lines compared to MM. Only lines significantly different than MM (p≤0.05) are included.

<table>
<thead>
<tr>
<th>WRKY OE Lines</th>
<th>G.E. Relative to MM ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRKY 6-1</td>
<td>38.35 ± 3.21</td>
</tr>
<tr>
<td>WRKY 6-3</td>
<td>29.07 ± 4.65</td>
</tr>
<tr>
<td>WRKY 8-3</td>
<td>6.42 ± 0.87</td>
</tr>
<tr>
<td>WRKY 10-1</td>
<td>118.1 ± 7.32</td>
</tr>
<tr>
<td>WRKY 11-1</td>
<td>3.39 ± 0.34</td>
</tr>
<tr>
<td>WRKY 11-3</td>
<td>3.87 ± 0.45</td>
</tr>
<tr>
<td>WRKY 23-1</td>
<td>21.71 ± 2.34</td>
</tr>
<tr>
<td>WRKY 23-2</td>
<td>17.39 ± 3.45</td>
</tr>
<tr>
<td>WRKY 23-3</td>
<td>67.66 ± 4.32</td>
</tr>
<tr>
<td>WRKY 25-2</td>
<td>2 ± 0.27</td>
</tr>
<tr>
<td>WRKY 48-2</td>
<td>314.1 ± 17.86</td>
</tr>
<tr>
<td>WRKY 48-3</td>
<td>295.4 ± 21.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WRKY RNAi Lines</th>
<th>G.E. Relative to MM ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRKY 7-1S</td>
<td>0.52 ± 0.17</td>
</tr>
<tr>
<td>WRKY 7-2S</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>WRKY 9-2S</td>
<td>0.16 ± 0.051</td>
</tr>
<tr>
<td>WRKY 9-3S</td>
<td>0.29 ± 0.064</td>
</tr>
<tr>
<td>WRKY 11-1S</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>WRKY 10-1S</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>WRKY 10-2S</td>
<td>0.52 ± 0.14</td>
</tr>
<tr>
<td>WRKY 23-2S</td>
<td>0.4 ± 0.15</td>
</tr>
<tr>
<td>WRKY 23-3S</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>WRKY 48-1S</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>
On the other hand, severe negative (pleiotropic) effects were observed for *SlWRKY10*-OE with stunted growth, *SlWRKY6*-OE had reduced growth and increased branching and *SlWRKY23*-RNAi demonstrated increased cell death symptoms and reduced growth (Supp. Fig. 2).

**Figure 4.** Growth of tomato WRKY transgenics under control conditions: a) plant height and b) shoot fresh weight. WRKY genes with significant differences in either of the traits are presented. WRKY naming ending in “S” refers to RNAi lines. Asterisks denote significant differences (*P* ≤ 0.05) compared to MM.

**Performance of WRKY transgenics under salt stress**

Transgenic lines were evaluated for their response to salt stress, with a focus on lines that had significantly different performance compared to MM. Lines *SlWRKY23*-OE, *SlWRKY11*-OE, *SlWRKY7*-RNAi and *SlWRKY9*-RNAi showed both higher absolute and relative growth (ratio of growth under saline conditions and normal conditions in %) under salt stress compared to MM (ranging from 68 to 72% compared to 63% of
MM, Supp. Fig. 1). *SIWRKY8*-OE and *SIWRKY6*-OE showed high relative salt tolerance (77-86%), though for *SIWRKY8*-OE only a single line with significant overexpression was recovered thus additional lines need to be examined to confirm this result. Exceptional phenotypes were observed for *SIWRKY23*-RNAi and *SIWRKY10*-OE under salt stress. The cell death symptoms in *SIWRKY23*-RNAi observed under control conditions were relieved by salt stress, leading to significantly better performance than MM (Supp. Fig. 2). *SIWRKY10*-OE exhibited even higher growth under salt stress compared to control conditions (106% relative salt tolerance), indicating that salinity counteracts the negative pleiotropic effects of *SIWRKY10* overexpression to some extent (Fig. 5a).

**Figure 5.** Abiotic stress tolerance of tomato WRKY transgenics: a) relative salt tolerance expressed as the percentage of fresh weight under salt stress divided by fresh weight in control conditions for the same genotype, b) relative electrolyte leakage after paraquat treatment as a proxy for membrane damage and oxidative stress tolerance. Asterisks denote significant differences (P≤ 0.05) compared to MM.
Oxidative stress tolerance and ion contents under salt stress

Determinants of salt tolerance such as oxidative stress tolerance and regulation of Na\(^+\), Cl\(^-\) and other ion concentrations were further examined. Lines *SIWRKY23-OE* and *SIWRKY11-OE* exhibited reduced electrolyte leakage compared to MM, while for the other lines exhibiting salt tolerance (*SIWRKY6-OE, SIWRKY7-RNAi* and *SIWRKY9-RNAi*) this was not significantly different from MM. The highest electrolyte leakage was observed for *SIWRKY10-OE* (89.3%), followed by *SIWRKY23-RNAi* (83-87%), both higher than MM (72%) (Fig. 5b). No significant differences were observed for leaf Na\(^+\), Cl\(^-\) and K\(^+\) content between all the transgenic lines examined and MM under salt stress, but for *SIWRKY10-OE* leaf Na\(^+\) and Cl\(^-\) concentrations were significantly higher (45.7 and 46.4 compared to 32.6 and 36 mg/g for MM, respectively) (Supp. Fig.3).

Cell death reduction of *SIWRKY23-RNAi* and *SIWRKY10* under salt stress and ROS burst

The exceptional phenotypes of *SIWRKY23-RNAi* and *SIWRKY10-OE* led us to further explore ROS presence and activity in these lines. Under control conditions both *SNRKY23-RNAi* and *SIWRKY10-OE* had high levels of ROS production in leaves as indicated by DAB staining (Figure 7), with *SIWRKY23-RNAi* having high targeted ROS production leading to necrotic spots formation, while *SIWRKY10-OE* leaves had uniform high ROS production. Under salt stress, DAB staining was significantly reduced in *SIWRKY23-RNAi* with disappearance of the ROS foci, while *SIWRKY10-OE* DAB staining was reduced to a lesser extent (Fig. 6a). Electrolyte leakage data of leaf disks in distilled water supported the results from DAB staining with leakage under salt stress that was even lower than under control conditions for *SIWRKY23-RNAi* despite the presence of higher concentrations of Na\(^+\) and Cl\(^-\) electrolytes, indicating that membrane damage in this line under control conditions is very high and is strongly suppressed under salt stress. *SIWRKY23-OE* and MM showed the opposite response, with *SIWRKY10-OE* displaying more electrolyte leakage under control compared to MM, and less increase under salt stress (Fig. 6b).
Figure 6. a) $H_2O_2$ visualization after DAB staining in MM, WRKY23OE and RNAi and WRKY10OE under control and salt stress (100mM NaCl), b) relative electrolyte leakage of leaf disks sampled from control and salt treated plants in MQ ultrapure water. Asterisks denote significant differences ($P \leq 0.05$) compared to MM for the same treatment. Error bars depict standard error ($n=4$).
Gene expression analysis under salt stress

Several stress response expression markers were analysed in genotypes and lines that exhibited significant differences in salt tolerance (Fig. 7 a, b). *SiWRKY6*OE showed both high expression and further induction of *ACCase* (marker for ethylene biosynthesis) under salt stress and a similar pattern was observed for *LOXD* (a node in the JA pathway). *MCA1* expression was the highest compared to the other genotypes. On the other hand *SiWRKY6*OE exhibited relatively low expression of *NCED1* that was not further induced under salt stress.
Figure 7. Expression analysis genes-markers for hormonal, abiotic and biotic stress signalling pathways, relative to EF1a, in selected a) overexpression and b) RNAi WRKY lines. Error bars depict standard error (n=4).
Expression patterns of most of the examined genes in *SIWRKY11*-OE were similar to MM, with the exception of *RBOHD*, which was 1.8-fold upregulated in *SIWRKY11*-OE under salt stress compared to a 2-fold downregulation in MM.

In *SIWRKY10*-OE the expression of *ACCase, RBOHD* and *MCA1* was downregulated under salt conditions (5-, 3-, and 4-fold respectively). *SIWRKY10*-OE exhibited the lowest expression of *NCED1* of all lines under both conditions.

The RNAi lines *SIWRKY7*-RNAi and *SIWRKY9*-RNAi exhibited increased expression of *APX1* under both control and salt stress. The observed stress-mediated reduction of cell death in *SIWRKY23*-RNAi was reflected in expression patterns of genes related to ROS and cell death: the elevated expression of *ACO3, LOXD, MCA1*, and *RBOHD* under control conditions (the highest among the lines examined) was greatly reduced under salt stress (Fig. 7b).

**Resistance to powdery mildew and combined stress**

The majority of the lines exhibited susceptibility levels to powdery mildew and to combined stress that were similar to MM (Fig. 8). However, contrasting phenotypes were observed in *SIWRKY23*-OE and RNAi lines. *SIWRKY23*-OE was highly susceptible to powdery mildew (higher than MM) and its susceptibility was further increased under combined stress. In contrast, *SIWRKY23*-RNAi exhibited increased PM resistance accompanied by cell death, especially line *SIWRKY23*-3RNAi, correlating with the higher level of silencing of the *WRKY23* gene in this line (Fig. 9). Combined stress significantly reversed this effect, increasing susceptibility to powdery mildew. *SIWRKY10*-OE exhibited increased resistance to PM, with the addition of salt stress counteracting this effect and increasing susceptibility (Fig. 9). *SIWRKY8*-OE also showed higher resistance compared to MM, though not to the same extent as *SIWRKY23* RNAi and *SIWRKY10*-OE. However, this resistance was not decreased under combined stress.
Figure 8. Disease index of overexpression and RNAi WRKY lines 10 days after inoculation with powdery mildew alone or in combination with 100mM NaCl. Asterisks denote significant differences (P≤ 0.05) compared to MM for the same treatment. Error bars depict standard error (n=4).

Discussion

WRKY transcription factors were originally discovered as important regulators of plant defense responses (Rushton et al. 2010) and were later shown to be part of an intricate signalling web, modulating multiple aspects of plant growth and development and adaptation to environmental conditions (Bakshi and Oelmuller 2014). In this paper we demonstrate that several tomato WRKY genes play important roles both in salt stress tolerance and powdery mildew resistance, and can be important nodes in the crosstalk between abiotic stress and defense signalling pathways.

In this study, the strongest effects on plant morphology were generally observed for the WRKY overexpression lines rather than the WRKY RNAi lines, probably as a result of genetic redundancy. The increased growth especially in terms of plant height of SlWRKY23-OE, SlWRKY11-OE, SlWRKY7-RNAi and SlWRKY9-RNAi indicates that the first two are positive regulators of growth while the last two are probably negative regulators. All genes carried homeobox and light regulated elements in their promoters indicating that they may be involved in regulation of developmental processes (Alabadí and Blázquez 2009).
Figure 9. Leaf phenotypes of MM, WRKY23OE and RNAi and WRKY10OE after 10 days of powdery mildew (PM) inoculation alone or in combination with 100mM NaCl.
Growth penalties were observed for *SIWRKY6*-OE and *SIWRKY10*-OE. *SIWRKY6* is homologous to *AtWRKY6*, of which overexpression in Arabidopsis resulted in small, stunted transgenic plants that showed altered leaf morphologies and changes in flowering time (Robatzek and Somssich 2002). Expression analysis of genes involved in key regulatory pathways in both lines provided information on possible mechanisms underlying the growth penalties. The very high induction of ethylene biosynthesis genes indicated an involvement of ethylene, which is implicated in growth retardation (Kim et al. 2012). The growth penalty of *SIWRKY23*-RNAi lines on the other hand was uniquely accompanied by increased cell death, which was reflected in the expression data by significantly higher expression of cell death and ROS marker genes *MCA1* and *RBOHD* compared to MM.

With respect to salt tolerance, lines that showed increased growth compared to MM (*SIWRKY23*-OE, *SIWRKY11*-OE, *SIWRKY7*-RNAi and *SIWRKY9*-RNAi) exhibited better growth compared to MM not only in absolute but also in relative growth (compared to their biomass under control conditions). Differences were in the range of 6-10%, but this relatively moderate effect on growth and salt tolerance was confirmed in another experiment (data not shown). Thus, manipulation of *WRKY* expression can be a viable means of increasing salt tolerance without negative pleiotropic effects. Several *WRKY* genes from different species have been shown to be able to confer abiotic stress tolerance either through overexpression (Wang et al. 2013; Xiong et al. 2010; Zhou et al. 2008) or loss of function/silencing (Jiang and Deyholos 2009; Ren et al. 2010). None of the four tomato *WRKY* genes mentioned above (*SIWRKY23, SIWRKY11, SIWRKY7* and *SIWRKY9*) exhibited high homology with previously characterized genes involved in abiotic stress tolerance. However, both the presence of abiotic stress responsive elements in their promoters and analysis of co-regulatory networks of their closest Arabidopsis homologues (http://string-db.org/) indicated the possibility of interaction with other abiotic stress response components such as HSPs, HSFs and calmodulin for *SIWRKY11*-OE as an example.

*SIWRKY6*-OE lines were among the most salt tolerant of all the *WRKY* transgenics (with *SIWRKY8*-OE), but the plants had considerably less biomass than MM. Irrespective of the negative effect on growth, *SIWRKY6* appeared to be an important modulator of abiotic stress tolerance, being significantly induced under drought as indicated by data in the tomato transcriptomics repository (Table 1). The presence in the *SIWRKY6* promoter of a cis element for guard cell-specific expression might be an indication of its involvement in stomatal behaviour and regulation. The homologous Arabidopsis gene *AtWRKY6* was originally discovered as a positive regulator of senescence (Robatzek and Somssich 2002). A similar effect on senescence was
observed for *SIWRKY6*-OE alongside the increased expression of gene expression markers for ethylene production. Subsequent research revealed the involvement of *AtWRKY6* in nutrient acquisition. It enhances sensitivity to phosphate deficiency (Chen et al. 2009b) but is a critical component in adaptation to boron deficiency (Kasajima et al. 2010). Thus, further study of *SIWRKY6*-OE binding targets in tomato may provide insights in adaptive responses to other abiotic stressors in addition to salt stress.

The regulation of salt tolerance involving the above-mentioned *WRKY* genes was not a result of altered Na\(^+\) and Cl\(^-\) concentrations in the plants. Therefore, ion uptake and transport appeared to be unchanged, and not under the control of these *WRKY* genes. The increased salt tolerance of *SIWRKY8*-OE, *SIWRKY23*-OE and *SIWRKY11*-OE might be the result of improved oxidative stress tolerance as compared to MM. *WRKY* TFs were shown to regulate the antioxidant (defense) response by reducing $H_2O_2$ and enhancing peroxidase activity (Miao et al. 2004; Zheng et al. 2013). Our expression data however did not provide conclusive results on which signaling pathways may be involved for the tomato *WRKY*s examined and more comprehensive examination of the antioxidant status of these lines should be undertaken determination and quantification of enzyme and antioxidant activity.

Exceptional responses to salt stress were observed for the lines that exhibited remarkable significant pleiotropic effects under non stress conditions: *SIWRKY10*-OE and *SIWRKY23*-RNAi. *SIWRKY10*-OE responded to salt stress with a surprising increase in biomass as compared to control conditions, while *SIWRKY23*-RNAi displayed a significant reduction of cell death and necrotic spots. The increased resistance to powdery mildew of these lines compared to MM was decreased with the additional exposure to salinity. Under control conditions, high expression levels of defense-related genes such as cell death and ROS markers *MCA1* and *RBOHD*, and the ethylene pathway were observed, but all of these genes were downregulated under salt stress. We conclude that both these *WRKY* genes are important regulators of tomato pathogen defense responses; *SIWRKY10* is a positive regulator (increased tolerance found with overexpression) and *SIWRKY23* is a negative regulator (increased tolerance with silencing), and that both these regulatory functions are counteracted by salt stress. The negative role of *SIWRKY23* in disease resistance is further supported by the ultra-susceptible phenotype of the over-expressor line *SIWRKY23*-OE. Knockout mutants of the closest homologue of *SIWRKY23* in Arabidopsis, *AtWRKY27*, showed delayed symptoms in response to the bacterial pathogen *Ralstonia solanacearum* (Mukhtar et al. 2008), providing an additional indication of functional convergence for *WRKY* genes of the two genetically distant
species. *SlWRKY10* is a close homologue of *AtWRKY74*, which appears to be co-regulated with receptor-like and serine/threonine protein kinases involved in defense (http://string-db.org/). Assuming that *SlWRKY10* shares functional similarity with *AtWRKY74*, a constitutive upregulation of defense signalling could be underlying the increased resistance to PM in the *SlWRKY10* OE overexpression line.

The primed defense responses of *SlWRKY10* OE and *SlWRKY23* RNAi and their compromised growth indicate that both *SlWRKY10* and *SlWRKY23* are nodes in the widespread phenomenon of growth-defense tradeoff observed in plants (Huot et al. 2015), with resources being invested in defense and thus compromising biomass accumulation. This phenomenon was found to be under hormonal control (Denancé et al. 2013; Shyu and Brutnell 2015), which agrees with our results of higher expression of the ethylene synthesis pathway. The increased growth of *SlWRKY10* OE and the cessation of cell death in *SlWRKY23* RNAi under salt stress and the decreased resistance to powdery mildew under combined stress are indicative of a negative interaction of the salt stress response with the pathogen defense response in these transgenic lines. This is further evidenced by the reversal of the upregulation of defense pathway genes under salt stress. These observations are in line with studies in Arabidopsis and tomato (De Torres Zabala et al. 2009; Kim et al. 2011c; Kissoudis et al. 2015; Prasch and Sonnewald 2013; Yasuda et al. 2008) where negative interactions have been observed between components of abiotic and biotic stress responses, mediated by hormonal pathways, and agree with *SlWRKY23*’s role as a significant node balancing abiotic and biotic responses.

*SlWRKY11* and *SlWRKY10* have highly similar sequences in the DNA-binding domain with only a few polymorphic amino-acids (Fig.1), but showed extremely different morphological features. This exemplifies that even slight changes in protein sequence may have a significant effect on TF binding to downstream promoter sequences (Brand et al. 2013) and needs to be further explored.

In conclusion, the functional characterization of tomato homologues of selected Arabidopsis defense-related *WRKY* genes revealed functions that go beyond defense responses, and that include growth control, salt stress tolerance and cell death control. Among those genes, *SlWRKY23* stood out as an important node in the cross-regulation of abiotic and biotic stress tolerance and regulation of cell death, that should be further studied with its downstream targets should be identified. *SlWRKY6* appears to be part of abiotic stress adaptation, senescence and ethylene signalling. *SlWRKY10* functions at the crossroads of defense/growth control and appears to be a component in relaying the negative impact of abiotic stress on defense. Further functional characterization including interactions with other proteins, cis-element
targets and identification of downstream target genes for transcriptional regulation may reveal the mechanisms and pathways of which these WRKYs are an intricate part (Inoue et al. 2013; Lozano-Durán et al. 2013; Xiao et al. 2013). The presence of W-box genes in all WRKY gene promoters examined indicates the significant contribution of cross and self-regulation among WRKY genes, forming transcription networks and feedback loops which is demonstrated in numerous WRKY genes (Chen et al. 2010a; Cheng et al. 2015).

In addition to further detailed studies on signalling cascades, lines such as SlWRKY11-OE and SlWRKY7-RNAi that showed increased salt tolerance with apparently no cost in plant performance under control conditions are promising candidates for increased stress tolerance without unintended side-effects in tomato.

**Acknowledgements**

Christos Kissoudis is supported by a “Alexander S. Onassis” Public Benefit Foundation scholarship.

**Supplementary material**

The supplementary data can be retrieved at DOI: 10.18174/369640 or http://edepot.wur.nl/369640
Chapter 7

General discussion
General discussion

Plant responses to abiotic-biotic stress combinations are underlined with high complexity due to the multitude of interactions expected between two living organisms - the plant and the pathogen - and the additional dimension of abiotic stress exposure. We chose to study the interaction between tomato and powdery mildew under salt stress in an effort to gain insight of plant responses to two major factors limiting plant productivity, which can be representative of a wider array of abiotic stresses and pathogenic fungi. We uncovered specific responses to combined salt stress and powdery mildew that were dependent on both stress intensity and pathogen resistance mechanism. Explicit physiological and hormonal responses were observed with exaggerated senescence and induction of the ethylene pathway that potentially contribute to the fitness cost under combined stress. In addition, members of the WRKY transcription factor family were identified that regulate powdery mildew resistance and cell death in a salt stress-dependent manner. Our results point that tolerance to salt stress and powdery mildew combination can be effectively realized by pyramiding genes/loci conferring salt tolerance with R-gene resistance genes, as the Ol-4 R-gene exhibited the highest robustness under combined stress.

Can combined salt stress and powdery mildew challenge provide a representative view of abiotic-biotic stress interactions

It is of great importance, especially in exploratory studies, to elucidate interactions that can be representative of a variety of pathogens and stress conditions in order to increase the translation potential to crop performance under field conditions.

Salinity stress incorporates components that are shared with other abiotic stresses, such as osmotic and oxidative stress and the induction of autophagy and senescence (Liu et al. 2009). Under field conditions salinity stress is frequently affecting plants throughout their lifetime, (although dynamic alterations might occur due to changing ground water level that can bring salt towards the soil surface by capillary forces). Therefore, pathogen infection in most occasions follows salt stress. This contrasts drought or heat stress, which can occur at various timepoints and can be either preceding or following pathogen infection, adding more layers of complexity to the outcome of these interactions.

The unique component of salt stress is ion imbalance imposed by high Na⁺ and Cl⁻ concentrations which might interfere with early signaling in pathogen defense, such as changes in ion fluxes (Yoshioka et al. 2006). It may also affect cell death with
leakage of K⁺ ions (Demidchik et al. 2014), and can thus have a significantly higher impact on these processes than other abiotic stresses. Vesicular trafficking under salt stress appears to be rewired with vesicles containing Na⁺ fused with the central vacuole to maximize compartmentalization of Na⁺ (Hamaji et al. 2009), which contrasts with vesicular movement during pathogen infection that becomes polarized towards the site of infection carrying antimicrobial compounds (Underwood and Somerville 2008). High Na⁺ and Cl⁻ accumulation during salt stress can uniquely act as protective mechanism to pathogen feeding, due to the toxicity of these ions, similar to the protective effect of heavy metals in heavy metal hyper accumulators (Boyd 2007).

Powdery mildew is both economically important for tomato productivity and can be considered a model for biotrophic pathogens for tomato (Seifi et al. 2014). Biotrophic pathogenesis involves suppression of immune responses through manipulation of host defenses, in contrast to necrotrrophic pathogenesis where secretion of phytotoxins and cell wall degrading enzymes promote host tissue necrosis prior to colonization (Laluk and Mengiste 2010). Therefore there are active interactions between the host cellular machinery and the pathogen during biotrophic pathogenesis that are potentially more prone to be impacted by a concurrent abiotic stress. Additionally, resistance to biotrophic pathogens is characterized by different mechanisms. A first line of defense responds to extracellular molecular patterns of pathogens with the production of antimicrobial compounds, pathogenesis-related proteins and structural barriers such as callose, which hamper pathogenesis penetration and usually do not culminate in a hypersensitivity response (HR). A second line of defense is triggered by the recognition of pathogen effectors resulting in a similar but significantly amplified response leading to HR and localized cell death (Cui et al. 2015; Dodds and Rathjen 2010; Laluk and Mengiste 2010; Tsuda and Katagiri 2010). Tomato genetic resources resistant to powdery mildew have been identified and characterized that cover the abovementioned mechanisms (Li et al. 2007; Li et al. 2012), offering a unique opportunity to identify mechanism-specific effects of abiotic stress on disease resistance.

Resistance to biotrophic pathogens appears to exhibit more antagonistic relationships between abiotic stress signaling and signaling to necrotrrophic pathogens, as evidenced by the negative interactions of ABA and SA signaling (Kim et al. 2011c; Yasuda et al. 2008) and the frequently opposing phenotypes in relation to abiotic and biotic stress tolerance after single gene manipulations (Asano et al. 2012; Campo et al. 2012). In contrast there are numerous reports of resistance to necrotrrophic pathogens accompanied by abiotic stress tolerance (AbuQamar et al. 2009; Navarro et al. 2008;
Ramírez et al. 2009; Zhu et al. 2014). Therefore, despite some exceptions, abiotic stress-biotrophic pathogen interactions can provide significant insights towards the elucidation of negative interactions between abiotic and biotic stress signaling and this may lead to the identification of molecular nodes that bypass this antagonism and that have a great potential for achieving combined stress tolerance in crops.

What have we learned from tomato salt stress and powdery mildew interactions

Additive negative effects of salt stress on powdery mildew susceptibility in partially resistant *S. habrochaites LYC4* population.

Identification of genotypes that exhibit multi-stress tolerance is of significant importance in delineating the (co-)regulation of abiotic and biotic stress adaptation and tolerance and understanding their genetic architecture by forward genetic studies. *S. habrochaites* LYC4 carried these attributes, being resistant to both high salinity and powdery mildew, thus offering the unique opportunity to examine the segregation of both traits and other related secondary traits in an introgression line population.

We show in Chapter 3 that salt stress (100mM NaCl) had a universal suppressing effect on powdery mildew resistance across all ILs, with no significant genotype x treatment interactions, indicating that the effects are additive. These observations translate to a general suppressive effect of salt stress on quantitative, partial disease resistance, resembling the antagonistic interactions observed at the gene expression level, with the opposite regulation of many genes involved in abiotic stress response compared to the biotic stress response (Rasmussen et al. 2013; Zou et al. 2011), and the suppressing role of ABA on SA signaling (De Torres Zabala et al. 2009). However, these results should be interpreted under the prism of potential breakdown of additive and epistatic interactions in the IL population, as individual lines carry single introgressions altering the trans-regulation of signaling pathways. Epistatic interactions are of great importance in plant defense responses with signaling cascades that include protein-protein interactions and modifications via phosphorylation (Adachi et al. 2015; Chinchilla et al. 2007), and these may have been disrupted in the individual introgression lines. Investigation of different segregating population types such as RIL populations that can incorporate epistatic interactions, can increase the detection power of genotype x treatment interactions under multiple stress conditions (Landers and Stapleton 2014).
Despite the issues above, IL populations offer significant advantages when examining the presence of favorable allelic variance for agronomic traits in wild relatives, limiting aberrant phenotypes that may mask the effect of useful alleles. *S. habrochaites* Lyc4 is an important source of allelic variation for several traits, with QTLs being identified for *Botrytis cinerea* (Finkers et al. 2007) and fruit parthenocarpy (Gorguet et al. 2008) which were further expanded in the Chapter 3 study on salt stress tolerance and powdery mildew resistance.

Despite the fact that in many occasions the introgression is relatively large, there are several likely candidate genes in the introgressed regions of IL lines with increased salt stress resistance. These include the NHX transporters on chromosome 1 and the SOS2 kinase on chromosome 12, which could be evaluated for allelic variation and potential advantageous functionality under salt stress.

Although no strong powdery mildew resistance was identified in the ILs, the partial powdery mildew resistance observed in IL9-1 co-segregated with salt tolerance. Its short introgression houses genes involved in ethylene signaling and redox control, as well as multiple receptor like kinase (RLK) genes. These are all potentially important regulators in stress crosstalk, with the latter being increasingly recognized as nodal points of ABA and defense signaling (Hok et al. 2014; Paparella et al. 2014), and can be the building blocks for both understanding and achieving combined stress tolerance.

**Stress severity and resistance type dependent responses to combined stress: ethylene and ABA as significant modulators of susceptibility and fitness.**

Perhaps not surprising, tomato responses to combined salt stress and powdery mildew were highly affected by the severity level of the salt stress, with increased susceptibility under mild stress, which was reversed at higher salt stress levels (Chapter 4). Plant physiological and biochemical responses under different stress levels are not linear (Cheng et al. 2013a; Maggio et al. 2007; Malkinson and Tielbörger 2010; Muralidharan et al. 2014) which is also evidenced by the strikingly small overlap between transcriptome profiles under mild and severe stress in arabidopsis (Clauw et al. 2015; Harb et al. 2010).

NaCl accumulation in the leaf tissues under salt stress can impede pathogen feeding from the host. NaCl is toxic to the pathogen, similar to the negative effect of heavy metal accumulation on pathogen growth (Fones et al. 2010). However this might not be the only reason for the limited pathogen growth under severe stress conditions. Additional structural and physiological changes might take place, such as changes in
the cell wall structure, wax composition and the leaf microenvironment. For example lignin content was found to be reduced under mild drought conditions to facilitate the maintenance of growth under conditions of decreased turgor pressure (Vincent et al. 2005), but severe stress resulted in increased lignin content (Lee et al. 2007a). Wax depositions increase under osmotic stress to restrict water loss (Cameron et al. 2006) and have a significant role in pathogen perception and defense. Responses however appear to be pathogen-specific with high wax increasing susceptibility to P. syringae and the necrotroph Sclerotinia sclerotiorum (Bourdenx et al. 2011), but positively contributing to resistance to cereal powdery mildew Erisyphegramminis in wheat and Lolium (Carver et al. 1990; Kader et al. 1995).

Tomato NILs NIL-ol-2 and NIL-ol-4 were not impacted by the imposition of any salt stress concentration, implicating that mlo-mutant and R-gene resistance are robust under salt stress and exhibit small fitness costs in comparison to salt stress individually. An interesting question is whether this is a salt specific effect, or can similar responses be expected under different abiotic stress conditions such as drought, heat and nutrient deficiency.

NIL-ol-2 maintained its resistance despite the observed decrease in callose depositions at higher salt stress levels (Chapter 4). Callose depositions are positively regulated by ABA signaling (García-Andrade et al. 2011) and this is evidenced by the reduction of callose and increase in susceptibility in the ol-2xnotabilis ABA deficient mutant. Yet we observed significant reduction in callose development with increased salt stress concentrations. It appears that under salt stress multiple signaling components are affected, including the redox state and structural changes in the cytoskeleton that might interfere with the production or transport of callose to the sites of infection (Luna et al. 2011; Miklis et al. 2007; Wang et al. 2011a). Callose might not be the only mechanism of action by which the mlo mutation confers powdery mildew resistance (Lipka et al. 2010). However, considering that decreased resistance in ol-2xnotabilis was accompanied reduction in callose deposition, we can hypothesize that Na+ and Cl\textsuperscript{−} toxicity contributed to NIL-ol-2 maintenance of resistance at high salt concentrations despite loss of callose deposition. That would imply that the observed NIL-ol-2 robustness might be salt stress-specific, which would agree with the partial resistance breakdown observed under drought stress (Baker et al. 1998).

Ol-4 R-gene mediated resistance exhibited the highest robustness, being stable across treatments and genetic perturbations (Chapter 4). Compared to PTI and basal resistance (characterized by compensatory relationships, (Tsuda et al. 2009)) R-gene effector triggered immunity (ETI) defense output is stronger and more prolonged
compared to PTI (Tsuda et al. 2013), thus more robust and less prone to negative regulation from environmental or genetic factors (Cui et al. 2015). However, heat stress was shown to negatively impact the resistance controlled by Arabidopsis R-genes SNC1 and RPS4 (in an abscisic acid (ABA) dependent manner (Mang et al. 2012)) and Mi-1 (which is considered a homologue of Ol-4 (Seifi et al. 2011b)), hampering nematode resistance (Marques de Carvalho et al. 2015). In all cases heat interferes with R-gene function posttranslationally and in Arabidopsis it reduces their accumulation in the nucleus (Mang et al. 2012). It would thus be of great interest to further evaluate the stability of Ol-4 under different stress scenarios.

The highest impact of salt stress and powdery mildew combination was observed in genotypes with partial disease resistance. The stress exaggerated susceptibility, senescence and reduced growth and was accompanied by strong induction of hormonal pathways, in particular ethylene and jasmonate, and a steep induction of cell wall invertase expression (Chapter 4). The same induction patterns are observed under powdery mildew infection only, albeit at a lower level, thus differences between the two conditions appear to be quantitative rather than qualitative and unsettle cellular and whole plant homeostasis. Similarly to other signaling molecules such as ROS, quantitative differences in hormone signal output can result in vastly different phenotypic responses ranging from stress acclimation to cell death (Brosché et al. 2014; Mittler et al. 2011).

Manipulations that entail the inhibition induction of entire signaling pathways, such as in our approach with the hormone mutants in Chapter 5, lack the depth to precisely delineate interactions dependent on the relative expression levels of the different signaling components. Therefore, despite the strong phenotypic effects of the ethylene-overproducing mutant and its increased susceptibility (Chapter 5), it cannot be simply concluded that attenuation of ethylene signaling will enhance combined stress tolerance. Ethylene signaling significantly contributes to enhanced salt stress tolerance through regulation of ROS detoxification and of the K+/Na+ ratio (Amjad et al. 2014; Jiang et al. 2013b; Peng et al. 2014). Like ethylene, jasmonates positively contribute to senescence, but are additionally involved in abiotic stress adaptation and stomata closure (Savchenko et al. 2014; Zhao et al. 2014) as well as callose deposition in response to pathogens (Scalschi et al. 2015). This would explain the increased susceptibility of ol-2 when crossed with the jasmonate deficient mutant defenseless (Chapter 5). ABA signaling appears to have a catalytic role in exaggerating senescence and susceptibility under combined stress. In the ABA-deficient notabilis mutant senescence and susceptibility were attenuated, but the plant growth was severely compromised by the salt stress.
Delineation of hormone signaling pathways and detailed characterization of cross regulatory nodes by targeted genetic manipulation appears to be quintessential for achieving combined stress tolerance with small or no fitness cost, or unintended side effects. Hormone profiling under combined stress and comparison with abiotic stress only (Albacete et al. 2008; Ghanem et al. 2012) is an approach that can identify signature organ-specific profiles.

Hormone-regulated transcription factors that integrate ABA and/ or ethylene signaling and that are known to contribute to abiotic and biotic stress tolerance, such as members of ERF, NAC and WRKY TF families (Chen et al. 2013; Dang et al. 2013; Park et al. 2001; Yi et al. 2004) are starting points in identifying synergistic and antagonistic branching points of hormone signaling.

**WRKY transcription factors at the crossroads of abiotic and biotic stress tolerance**

WRKY transcription factors are at the core of both biotic and abiotic stress responses of plants. WRKYs have been repeatedly and independently identified in transcriptome meta-analyses as nodal points in stress cross-regulation (Ma and Bohnert 2007; Shaik and Ramakrishna 2014). WRKYs have recently been shown to be targets of pathogen effectors for the suppression of plant immunity (Le Roux et al. 2015; Sarris et al. 2015) and to be directly involved in ROS burst following pathogen attack by promoting RBOH transcriptional activation (Adachi et al. 2015). On the other hand, three Arabidopsis WRKYS (AtWRKY18,-40 and -60) are part of the core ABA signaling machinery cooperatively repressing the ABA transcriptional activators ABI4 and ABI5 (Liu et al. 2012b).

The striking and contrasting phenotypes of *SIWRKY23* overexpression and silencing lines described in Chapter 6 suggest that this gene is a non-redundant node in abiotic and biotic stress crosstalk in tomato. Overexpression lines exhibited significantly higher salt tolerance than the parental lines, but the most explicit differences were observed for powdery mildew resistance, with hyper-susceptibility in the *SIWRKY23* overexpression lines and resistance accompanied by cell death in the RNAi lines. Cell death was even more pronounced under conditions without infection or stress, resembling the phenotype of lesion mimic mutants (Bruggeman et al. 2015). These observations indicate that *SIWRKY23* may be involved in the control of ROS equilibrium under stress. It is however puzzling that salt stress, which enhances ROS production as well, suppresses the *SIWRKY23* RNAi phenotypes. Perhaps *SIWRKY23* is a branch in the ABA signaling relay and a node in the ABA-SA antagonistic relationship. Thus its loss of function might shift the equilibrium towards SA
signaling, enhancing defense and inducing cell death under control conditions. Enhancement of ABA signaling by salt stress potentially results in the dampening of SA signaling, suppressing the pleiotropic effects of \textit{SIWRKY23} silencing. The identification of the downstream targets of \textit{SIWRKY23} and potential association with R-genes or RLKs can shed additional light on its function.

Genetic redundancy and compensatory relationships might have contributed to the mild phenotypic changes as demonstrated by most of the WRKY transgenics (especially the RNAi lines). Notable exception was \textit{SIWRKY10} overexpression, which resulted in powdery mildew resistance and reduced growth and salt stress imposition enhancing susceptibility and restoring growth (Chapter 6). Thus, \textit{SIWRKY10} might be a node of defense and growth trade-offs in tomato similarly to \textit{WRKY40} in Arabidopsis (Lozano-Durán et al. 2013). \textit{AtWRKY40} is a negative regulator of defense and associates with the brassinosteroid-regulated transcription factor BZR1 to promote growth at the expense of immune signaling.

Remarkably, \textit{SIWRKY11} exhibits high sequence similarity with \textit{SIWRKY10} in the DNA binding domain but overexpression of \textit{SIWRKY11} translates to opposite phenotypic responses with slightly increased growth and salt tolerance. These observations indicate that even slight changes in protein sequence of the DNA-binding domain may have a significant effect on TF binding to downstream promoter sequences (Brand et al. 2013). This phenomenon was observed in other (even allelic) pairs of transcription factors with high sequence similarity (Du et al. 2014; Tao et al. 2011; Tao et al. 2009), and can be further explored to understand transcription factor binding specificity.

Thus, expression manipulation of specific (but not all) WRKY transcription factors can be a viable means of increasing salt tolerance apparently without negative pleiotropic effects. Alleviating redundancy by combining multiple \textit{WRKY} genes might lead to even stronger effects.

**Directions for future research**

**Different stresses, different adaptive responses and tolerance mechanisms**

Abiotic stress factors such as drought, cold, heat and salt stress share similar properties such as the imposition of secondary oxidative stress but these also possess unique features. Calcium signature responses during early stress perception depend on the type of stress (Whalley and Knight 2013). Gene expression profiles are significantly different under different stress factors (Rabbani et al. 2003), and unique
and often opposing physiological and biochemical responses are observed, such as the contrasting plasma membrane lipid remodelling during heat and cold stress (Ruelland and Zachowski 2010) and opposing stomata responses during drought and heat stress (Zhao et al. 2013). Stomatal opening for example is beneficial under heat stress, however it might increase the pathogenicity of stomatal invading pathogens, while drought induced stomatal closure might decrease pathogenicity. Heat stress in many occasions results in the breakdown of R-gene mediated resistance (Marques de Carvalho et al. 2015; Zhu et al. 2010). Conclusions however are not straightforward, as elevated temperatures (and low temperatures as well) can significantly impact the physiology of the pathogen, restricting its growth and pathogenicity (Peduto et al. 2013).

Plant nutritional status and potential nutritional imbalances or deficiencies can be detrimental for plant health as abundance of most nutrients (including K⁺, PO₄³⁻, SO₄²⁻, Fe³⁺ and Mg²⁺) in many (but not all) occasions have been shown to reduce susceptibility to various pathogens (Amtmann et al. 2008; Huber and Jones 2013; Walters and Bingham 2007; Ye et al. 2014). Nitrogen though is a notable exception. When abundantly available it increases the nutritional value of the plant tissues for the pathogens, increasing virulence and susceptibility (Fagard et al. 2014), though several exceptions have been observed especially in the Solanaceae (Hoffland et al. 2000; Veresoglou et al. 2013).

Tolerance to abiotic stresses can be achieved through different physiological and biochemical routes, each one with potentially distinct impacts on pathogenesis. For example drought stress tolerance can be acquired via whole plant potentiation of ABA signaling (Okamoto et al. 2013), ROS detoxification (Lee et al. 2007b), reduction of transpiration via increased synthesis of epicuticular wax (Bourdenx et al. 2011), cytokinin overproduction resulting in senescence alleviation (Reguera et al. 2013) as well as by deeper rooting allowing greater soil water extraction and stress avoidance (Uga et al. 2013). Potentiated ABA signaling or increased ROS detoxification might negatively interfere with SA signaling and cell death/hypersensitivity response respectively (Cao et al. 2011; De Pinto et al. 2012; De Pinto et al. 2006). Cytokinin manipulation can be beneficial in combating senescence induced under combined stress and to augment defense responses (Argueso et al. 2012a; Jiang et al. 2013a), but this might be a double-edged sword as maintenance of green tissue can be optimal for biotrophic pathogen feeding (Walters and McRoberts 2006). Stress avoidance strategies mediated by increased water uptake through improved root anatomical features and/or aquaporin expression and reduced water loss via stomata might be the smoother strategy to limit interaction between abiotic and biotic stress adaptation.
Stress avoidance strategies by limiting Na⁺ and Cl⁻ uptake however might not be the preferred strategy under salt stress, as we showed that Na⁺ and Cl⁻ accumulation in the leaves can be toxic and limiting fungal growth (Chapter 4). Strategies aiming at ion compartmentalization in the above ground organs inside the vacuoles (He et al. 2005) might therefore be more beneficial under combined stress.

The length of the period at which the plants are exposed to abiotic stress can significantly affect the outcome under combinatorial stress. Stress can be persistent, a common occurrence for salinity and nutrient deficiencies, and of variable length, intermittent or terminal for instance with drought, heat and cold. Plant adaptation to prolonged stress was shown to vary significantly from short-term stress, with altered hormonal interactions (Yang et al. 2014a), cell wall anatomical changes such as increased lignification under prolonged drought (Moura et al. 2010) and significant build-up of Na⁺ and Cl⁻ under salt stress (Yer et al. 1991). Therefore the outcome of abiotic and biotic stress combination under prolonged stress might considerably resemble our observations of short term severe abiotic stress.

**Different pathogens, different pathogenicity mechanisms and lifestyles**

Biotrophic and necrotrophic pathogens employ distinct virulence strategies that can translate in significantly disparate outcomes when the plants are also exposed to abiotic stress factors. The majority of reports indicate an increase of susceptibility under abiotic stress, similar to biotrophic pathogens (Al-Sadi et al. 2010; You et al. 2011) Necrotrophs actively produce H₂O₂ and induce an oxidative burst in the host to facilitate tissue death and maceration (Choquer et al. 2007) and the increased levels of ROS correlate with fungal growth and cell death (Laluk et al. 2011; Łaźniewska et al. 2010). Capacitation of the plant antioxidant machinery has been shown to increase tolerance to necrotrophs (Plazek and Zur 2003) and therefore can be a common ground in improving tolerance to both abiotic and biotic stress from necrotrophs. Overexpression of wheat ERF1 did result in tolerance to cold and *Rhizoctonia cerealis* (Zhu et al. 2014).

Infection by pathogens that penetrate the root/vascular tissue can be directly affected by the changes in soil moisture and salt concentrations, although soil-borne fungi generally can withstand lower water potentials than plants (Cook and Papendick 1972). While salt stress appears to enhance root rot disease caused by *Fusarium oxysporum f. sp. radicis-lycopersici* and *Phytophthora sojae* in tomato and soybean respectively, drought stress reduces the incidence of vascular tissue pathogen *Verticillium albo-atrum* in alfalfa, possibly because of reduced xylem flow as a result
of lower transpiration (Pennypacker et al. 1991). In an apparent mutualistic relationship *Verticillium* infection enhances drought tolerance in Arabidopsis via increased cambial activity resulting in xylem hyperplasia (Reusche et al. 2012). On the other hand negative interactions for plant fitness were observed in *Phaseolus vulgaris*, which exhibited increased shoot Na\(^+\) and Cl\(^-\) content after concurrent salt stress and infection by the root pathogen *Macrophomina phaseolina* (You et al. 2011). Thus boosting antioxidant defense might be a viable route in achieving resistance to abiotic stress combinations with necrotrophic pathogens while the pathogen or stress type specific interactions observed for root pathogens might require pathosystem specific approaches.

**Deep dive into molecular crosstalk of early abiotic and biotic stress adaptive responses**

Unravelling the differences in early signaling in response to stress combinations in comparison with individual stresses can be key in rewiring signaling pathways and achieving combined stress resilience.

Early patterns of ion fluxes, such as calcium waves, are signatures for stress specific responses (Stephan and Schroeder 2014). Components involved in ion flux changes are to a significant degree shared between abiotic and biotic stress. Several ion channels appear to function in both salinity adaptation and signaling for defense responses induction and cell death, such as CNGCs (Clough et al. 2000), K\(^+\)-permeable channels (Demidchik et al. 2014; Shabala et al. 2006) as well as Na\(^+\) and Cl\(^-\) transporters (NHX and CLC respectively) (Chen et al. 2014b; Guo et al. 2014). NHX1 Na\(^+\)-H\(^+\) antiporter activity was shown to be involved in regulation of vacuolar pH and cellular oxidation for the optimal induction of plant defense (Chen et al. 2014b). Investigation of the function of ion transporters and channels under stress combination may provide clues about agonistic or antagonistic interactions especially considering that numerous members of these transporter families are involved in ion homeostasis during salt stress and contribute to salt stress tolerance (Gálvez et al. 2012; Guo et al. 2008).

Redox status is a regulator of the activity of many proteins via post translational modifications (Spoel and Loake 2011; Yang et al. 2015). Central redox regulators such as ascorbate peroxidase (APX) and glutathione peroxidase (GPX) crucially contribute to plant environmental adaptation, with increased expression and/or activity of both enzymes shown to be beneficial for abiotic stress tolerance, but also to increase pathogen susceptibility (Gou et al. 2015; Herbette et al. 2011). Reduced APX activity
on the other hand resulted in lower ROS detoxification capacity, potentiating cell death (de Pinto et al. 2013). Thus, fine tuning redox status in response to environmental variation and pathogen challenge is crucial for plant fitness. This was elegantly demonstrated in wheat where moderate reduction of APX increases *Puccinia striiformis f. sp tritici* resistance, while further reduction results in increased programmed cell death and senescence (Gou et al. 2015). This is in line with our observation that the increase in programmed cell death and senescence under combined stress is coinciding with reduction in APX expression (Chapter 4). Further monitoring of redox state kinetics under combined stress and in comparison with individual stress conditions might elucidate functional relationships between antioxidants concentration, enzyme activity and phenotypic responses.

Cellular component recycling upon stress mediated by autophagy responses is critical for optimal resource allocation in response to abiotic stress and cell death initiation (but also containment) under pathogen attack (Liu and Bassham 2012). Autophagy execution is dependent on the function of ATG genes, and their manipulation has provided significant insights on their importance in development and for stress adaptation (Lv et al. 2014). Autophagy initiation positively contributes to abiotic stress, nutrient deficiency tolerance and defense against necrotrophic pathogens (Lenz et al. 2011). Remarkably, it can have either pro-survival (restricting cell death) or pro-death functions (runaway cell death) against biotrophic pathogens, depending on the pathogen (Liu and Bassham 2012). Interestingly cell death initiation by TIR-NB-LRR genes requires a functioning autophagy pathway, while no such requirement was observed for CC-NB-LRR genes (Hofius et al. 2009). The gene underlying the Ol-4 locus is a CC-NB-LRR gene, potentially explaining its robustness as potential autophagy miss-regulation under salt stress would not affect its functions in immunity. Another dimension of complexity especially important under stress combinations is the observation that the outcome of autophagy is dependent on stress intensity, as the Arabidopsis *atg5* mutant exhibited enhanced stay-green phenotypes under mild osmotic, salt and oxidative stress, but reversal of this phenotype under severe stress resulting in cell death (Sakuraba et al. 2014).

Protein-protein interactions and their functional associations are important for relaying the stress signal especially in response to pathogen infection (Gassmann and Bhattacharjee 2012; Inoue et al. 2013) and in many occasions the formation of R-gene complexes with chaperones such as HSP proteins is essential for their function (Chen and Shimamoto 2011). It has been proposed that heat stress reduces the availability of HSPs to form chaperones with R-genes (Lee et al. 2012), explaining in part many
observations of R-gene resistance breakdown under heat stress. Resistance protein trafficking and endocytic recycling regulates receptor localization and defense signaling (Frescatada-Rosa et al. 2015). Similar processes are induced under abiotic stress and increased endocytic recycling appears to enhance salt tolerance and reduce ROS production (Tian et al. 2015). Thus it would be interesting to monitor intracellular trafficking responses under stress combinations.

Environmental stress conditions might alter gene expression patterns via condition-specific cis-element binding of the same transcription factor. This was recently demonstrated in the characterization of OsWRKY13 which exhibits tissue-specific expression and condition-specific (drought in comparison to Xanthomonas oryzae infection) binding to cis-elements of downstream genes and thereby inversely regulating resistance to drought and bacterial infection in rice (Xiao et al. 2013). Similarly Arabidopsis ERF1 in response to biotic stress was bound to GCC boxes exclusively, while under abiotic stress there was specific binding to DRE elements (Cheng et al. 2013b). It would be intriguing to investigate if differential binding is occurring with the tomato WRKY transcription factors investigated in Chapter 6 (especially WRKY10) in relation to the effects of salt stress in combination with powdery mildew.

**Implications and applications for crop improvement to stress combinations**

Achieving robustness to stress combinations in crops and designing breeding strategies to maximize efficacy and efficiency to achieve this goal is highly challenging. The innumerable possibilities of abiotic stress-pathogen combinations along with the in many occasions quantitative differences of the same regulatory pathways differentiating resistance or susceptibility, require precise fine-tuning of genetic regulation.

We propose that tomato performance improvement strategies under powdery mildew and salt stress combination should be adapted to abiotic stress intensity and type of resistance (Fig.1). Under mild stress priority should be given to senescence alleviation, while when the target environments are characterized by high salt concentrations, salinity tolerance should be prioritized, preferably by tissue Na+, Cl− and oxidative stress tolerance to minimize the growth penalty imposed by salt. Whether these approaches are applicable to other abiotic stresses (e.g. drought) and pathogen combinations remains to be elucidated by future research.
Uncovering the interactions in the ethylene-invertase-cytokinin regulatory triangle is critical to manipulate and confine the increased senescence/cell death/leaf abscission phenotypes that we characteristically observed under combined stress (Chapter 4 and 5) and that can significantly hamper plant performance and reproductive potential (Shinozaki et al. 2015). We suggest that quantitative differences due to the excessive induction of both ethylene and cell wall invertases under combined stress are causal for the phenotypes observed, disturbing senescence programming and source-sink relations. Each in isolation can positively contribute to stress tolerance (Albacete et al. 2014; Peng et al. 2014; Rivero et al. 2009; Taizin and Giardina 2014). Inter-regulatory relationships were shown by overexpression of cell wall invertase which lowered ethylene and increased cytokinin concentrations in tomato (Albacete et al. 2014). Further understanding of cytokinin/invertase-ethylene interactions under combined stress can enable approaches to effectively control their signaling and activity output, for instance by manipulation of ERF and/or NAC transcription factors involved in ethylene control of senescence (Kim et al. 2014; Koyama et al. 2013).

Receptor-like kinases (RLKs) have emerged as major regulatory hubs in abiotic and biotic stress signaling inter-regulation especially in relation to ABA signaling (Hok et al. 2014; Paparella et al. 2014). Their implementation might be ideal to restrict ABA signaling effects on potentiation of senescence and dampening of defense signaling.

Our observation that R-gene resistance by the CC-NB-LRR gene OI-4 was not affected by abiotic stress and hormonal perturbations is promising for achieving combined stress tolerance by pyramiding abiotic stress tolerance genes with such an R-gene (Fig.1). It will be important to examine if this stability is maintained under other stress conditions, and if it applies to R-genes of both the TIR- and CC-NB-LRR class (discussed earlier). mlo-mutant-mediated resistance exhibited a similar high robustness under salt stress. However resistance breakdown under drought has been reported (Baker et al. 1998) as well as pleiotropic effects such as increased senescence at the later stages of plant development (Piffanelli et al. 2002), or compromised resistance to other pathogens (Kumar et al. 2001; McGrann et al. 2014), which might hinder its utilization in breeding. Pleiotropy is frequently observed when plant susceptibility genes (S-genes) are knocked down (Hückelhoven et al. 2013) thus identification of mlo or other S-gene allelic variants with minimal fitness cost can enable their effective utilization.
Figure 1. Proposed strategies for achieving combined salt stress and powdery resistance configured in relation to resistance type and abiotic stress severity. ET:ethylene, CK:cytokinin, TF: transcription factor, RLK: receptor-like kinases

Gene stacking is greatly facilitated by genetic engineering and a combination of multiple R-gene stacking along with genes conferring tolerance to various abiotic stress conditions (Li et al. 2014) may significantly improve crop resilience to stress combinations. R-gene deployment is not applicable for resistance to necrotrophic pathogens and other routes should be followed such as the expression of protease inhibitors and chitinases (Chen et al. 2014a).

Minimizing antagonistic interactions when pyramiding abiotic and biotic stress tolerance genes is cornerstone. In addition to tissue- and condition - regulation of expression, the utilization of rootstocks (where applicable) conferring tolerance to soil-related abiotic stresses such as salt, drought or nutrient deficiencies (Albacete et al. 2015; Estañ et al. 2005) and/or soil-borne pathogens (Guan et al. 2012), in combination with resistance factors in the scion can limit unintended interactions (although systemic signaling to some extent my still occur (Haroldsen et al. 2012)).

Exploitation of natural variation is increasingly facilitated by the advancement of sequencing, molecular marker and genetic engineering technologies (Bolger et al. 2014), and wild species can be an ideal source of allelic variation for stress tolerance as they grow and reproduce in marginal habitats (Ortiz 2015). For example S. habrochaites LYC4, exhibiting both abiotic and biotic stress tolerance, is ideal starting material for allele mining, especially genes present in IL9-1, with numerous
RLKs in the introgressed region (Chapter 3). The information on allelic variation and its phenotypic impact can the starting point of superior allele generation by targeted gene editing (Rinaldo and Ayliffe 2015) accelerating improvement and limiting pleiotropy and fitness cost.

Implementation of high throughput phenotyping such as thermal imaging for transpiration and fluorescence imaging for monitoring stress severity, nutrient deficiencies and diseases (Furbank and Tester 2011) along with high resolution recording of the environment (Campbell et al. 2015; Nagano et al. 2012) can provide unequivocal genotype-phenotype associations across environmental variables, greatly facilitating improvement for highly complex traits such as resistance to stress combinations.
References


American tomato accession is caused by loss of mlo function. Molecular Plant-Microbe Interactions 21: 30-39


Genome-wide annotation of the soybean WRKY family and functional characterization of genes involved in response to *Phakopsora pachyrhizi* infection. BMC Plant Biol 14: 236


Cameron KD, Teece MA, Smart LB (2006) Increased Accumulation of Cuticular Wax and Expression of Lipid Transfer Protein in Response to Periodic Drying Events in Leaves of Tree Tobacco. Plant Physiology 140: 176-183


172


DiLeo MV, Pye MF, Roubtsova TV, Dunniway JM, MacDonald JD, Rizzo DM, Bostock RM (2010) Abscisic acid in salt stress predisposition to Phytophthora root and crown rot in tomato and chrysanthemum. Phytopathology 100: 871-879


Domingo C, Conejero V, Vera P (1994) Genes encoding acidic and basic class-III beta-1,3-glucanases are expressed in tomato plants upon viroid infection. Plant Molecular Biology 24: 725-732.


identification of QTLs for resistance to Botrytis cinerea. Theoretical and Applied Genetics 114: 1071-1080


Gregersen PL, Culetic A, Boschian L, Krupinska K (2013) Plant senescence and crop productivity.  82: 603-622


Heitz T, Bergey DR, Ryan CA (1997) A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiology 114: 1085-1093


Herbette S, De Labrouhe DT, Drevet JR, Roeckel-Drevet P (2011) Transgenic tomatoes showing higher glutathione peroxydase antioxidant activity are more resistant to an abiotic stress but more susceptible to biotic stresses. Plant Science 180: 548-553


Kissoudis C, van de Wiel C, Visser RGF, Van Der Linden G (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. Frontiers in Plant Science 5


Koga H, Dohi K, Mori M (2004) Abscisic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of Magnaporthe grisea. Physiological and Molecular Plant Pathology 65: 3-9


Li PF, Chen L, Zhou YH, Xia XJ, Shi K, Chen ZX, Yu JQ (2013b) Brassinosteroids-Induced Systemic Stress Tolerance was Associated with Increased Transcripts of Several Defence-Related Genes in the Phloem in Cucumis sativus. PLoS ONE 8


Martinez De Ildruya O, Xie Q, Kaloshian I (2003) Aphid-induced defence responses in Mi-1-mediated compatible and incompatible tomato interactions. 16: 699-708


Seifi A (2011a) Characterization of tomato genes for resistance to Oidium neolycopersici. PhD thesis Wageningen University, Wageningen, The Netherlands


192


Trnka M, Rotter RP, Ruiz-Ramos M, Kersebaum KC, Olesen JE, Zalud Z, Semenov MA (2014) Adverse weather conditions for European wheat production will become more frequent with climate change. 4: 637-643


193


Whalley HJ, Knight MR (2013) Calcium signatures are decoded by plants to give specific gene responses. New Phytologist 197: 690-693


stresses through regulation of defence- and stress-related genes. New Phytologist 196: 1155-1170


Summary

Projections on the impact of climate change on agricultural productivity foresee prolonged and/or increased stress intensities and enlargement of a significant number of pathogens habitats. This significantly raises the occurrence probability of (new) abiotic and biotic stress combinations. With stress tolerance research being mostly focused on responses to individual stresses, our understanding of plants’ ability to adapt to combined stresses is limited.

In an attempt to bridge this knowledge gap, we hierarchized in chapter 1 existing information on individual abiotic or biotic stress adaptation mechanisms taking into consideration different anatomical, physiological and molecular layers of plant stress tolerance and defense. We identified potentially crucial regulatory intersections between abiotic and biotic stress signalling pathways following the pathogenesis timeline, and emphasized the importance of subcellular to whole plant level interactions by successfully dissecting the phenotypic response to combined stress. We considered both explicit and shared adaptive responses to abiotic and biotic stress, which included amongst others R-gene and systemic acquired resistance as well as reactive oxygen species (ROS), redox and hormone signalling, and proposed breeding targets and strategies.

In chapter 3 we focused on salt stress and powdery mildew combination in tomato, a vegetable crop with a wealth of genetic resources, and started with a genetic study. *S. habrochaites* LYC4 was found to exhibit resistance to both salt stress and powdery mildew. A LYC4 introgression line (IL) population segregated for both salt stress tolerance and powdery mildew resistance. Introgressions contributing to salt tolerance, including Na\(^+\) and Cl\(^-\) accumulation, and powdery mildew resistance were precisely pinpointed with the aid of SNP marker genotyping. Salt stress (100mM NaCl) combined with powdery mildew infection increased the susceptibility of the population to powdery mildew in an additive manner, while decreasing the phenotypic variation for this trait. Only a few overlapping QTLS for disease resistance and salt stress tolerance were identified (one on a short region at the top of Chromosome 9 where numerous receptor-like kinases reside). Most genetic loci were specific for either salt stress tolerance or powdery mildew resistance indicating distinct genetic architectures. This enables genetic pyramiding approaches to build up combined stress tolerance.

Considering that abiotic stress in nature can be of variable intensities, we evaluated selected ILs under combined stress with salinity ranging from mild to severe (50, 100
and 150mM NaCl) in chapter 4. Mild salt stress (50mM) increased powdery mildew susceptibility and was accompanied by accelerated cell death-like senescence. On the contrary, severe salt stress (150mM) reduced the disease symptoms and this correlated with leaf Na\(^+\) and Cl\(^-\) content in the leaves. The effects of salt stress on powdery mildew resistance may be dependent on resistance type and mechanisms. Near Isogenic Lines (NILs) that carry different PM resistance genes (Ol-1 (associated with slow hypersensitivity response, HR), ol-2 (an mlo mutant associated with papilla formation) and Ol-4 (an R gene associated with fast HR) indeed exhibited differential responses to combined stress. NIL-Ol-1 resembled the LYC4 ILs response, while NIL-ol-2 and NIL-Ol-4 maintained robust resistance and exhibited no senescence symptoms across all combinations, despite the observed reduction in callose deposition in NIL-ol-2. Increased susceptibility, senescence and fitness cost of NIL-Ol-1 under combined stress coincided with high induction of ethylene and jasmonate biosynthesis and response pathways, highly induced expression of cell wall invertase LsLIN6, and a reduction in the expression of genes encoding for antioxidant enzymes. These observations underlined the significance of stress intensity and mechanism of resistance to the outcome of salt stress and powdery mildew combination, underscoring the involvement of ethylene signalling to the susceptibility response under combined stress.

To examine the significance of hormone signalling in combined stress responses we evaluated crosses of tomato hormone mutants notabilis (ABA-deficient), defenseless1 (JA-deficient) and epinastic (ET overproducer) with NIL-Ol-1, NIL-ol-2 and NIL-Ol-4 in chapter 5. The highly pleiotropic epinastic mutant increased susceptibility of NIL-Ol-1, but decreased the senescence response under combined stress, and resulted in partial breakdown of NIL-ol-2 resistance, accompanied by reduced callose deposition. The effects of ET overproduction on susceptibility were more pronounced under combined stress. ABA deficiency in notabilis on the other hand greatly reduced susceptibility of NIL-Ol-1 under combined stress at the expense of stronger growth reduction, and induced ROS overproduction. Partial resistance breakdown in the ol-2xnotabilis mutant accompanied by reduced callose deposition was observed, and this was restored under combined stress. Jasmonic acid deficiency phenotypic effects in defenseless mutants were subtle with modest increase in susceptibility for NIL-Ol-1 and NIL-ol-2. For NIL-ol-2 this increased susceptibility was reverted under combined stress. NIL-Ol-4 resistance remained robust across all mutant and treatment combinations. These results highlight the catalytic role of ET and ABA signalling on susceptibility and senescence under combined stress, accentuating concomitantly the importance of signalling fine tuning to minimize pleiotropic effects.
The potential of exploiting transcription factors to enhance tolerance to multiple stress factors and their combination was investigated in chapter 6 through the identification and functional characterization of tomato homologues of \textit{AtWRKYs 11, 29, 48, 70 and 72}. Thirteen tomato WRKY homologues were identified, of which 9 were overexpressed (using transformation with \textit{A. tumefaciens}) and 12 stably silenced via RNAi in tomato cultivar Money Maker (MM). \textit{SiWRKY11-OE} and \textit{SiWRKY23-OE} and RNAi lines of \textit{SiWRKY7} and \textit{SiWRKY9} showed both increased biomass and relative salt tolerance. \textit{SiWRKY6-OE} exhibited the highest relative salt stress tolerance, but had strongly decreased growth under control conditions. Exceptional phenotypes under control conditions were observed for \textit{SiWRKY10-OE} (stunted growth) and \textit{SiWRKY23-RNAi} (necrotic symptoms). These phenotypes were significantly restored under salt stress, and accompanied by decreased ROS production. Both lines exhibited increased resistance to powdery mildew, but this resistance was compromised under salt stress combination, indicating that these genes have important functions at the intersection of abiotic and biotic stress adaptation. \textit{SiWRKY23} appears to have a key regulatory role in the control of abiotic stress/defense and cell death control.

Experimental observations are critically discussed in the General Discussion with emphasis on potential distinctive responses in different pathosystems and abiotic and biotic stress resistance mechanisms as well as genetic manipulations for effectively achieving combined stress tolerance. This includes deployment of individual common regulators as well as pyramiding of non-(negatively) interacting components such as \textit{R}-genes with abiotic stress resistance genes, and their translation potential for other abiotic and biotic stress combinations. Understanding and improving plant tolerance to stress combinations can greatly contribute to accelerating crop improvement towards sustained or even increased productivity under stress.
Samenvatting

Voorspellingen over de gevolgen van klimaatverandering voor de landbouw laten zien dat de stress langduriger en het stressniveau hoger wordt. Ook wordt verwacht dat de leefgebieden van verschillende pathogenen groter zullen worden. Dit heeft tot gevolg dat de kans groter wordt dat gewassen worden blootgesteld aan (nieuwe) combinaties van zowel abiotische stress (o.a. droogte, hitte, zilte gronden) als biotische stress (ziektes en plagen). Onderzoek aan stress tolerantie van planten richt zich nu hoofdzakelijk op het weerstaan van één stress tegelijk. Kennis en begrip over de mogelijkheden van de plant zich aan te passen aan meerdere stress factoren tegelijk nog beperkt.


In hoofdstuk 3 wordt in een genetische studie ingezoomd op de combinatie van zout stress en gewone meeldauw (*Oidium lycopersicum*) infectie in tomaat. *S. Habrochaites* Lyc4 bleek zowel tolerant voor zoutstress als resistent tegen gewone meeldauw, en een Lyc4 populatie van introgressie lijnen (IL) splitsde uit voor deze eigenschappen. Introgressies die bijdragen een zout tolerantie, zoals voor Na⁺ en Cl⁻ ophoping in het blad, konden genetisch worden gekarteerd met behulp van SNP merkers. Zout stress (in dit geval blootstelling aan 100mM NaCl) gecombineerd met meeldauw infectie resulteerde in een additieve verhoging van de gevoeligheid voor meeldauw en een vermindering van de fenotypische variatie voor de ziekte. Slechts een paar QTLs voor meeldauw resistentie overlapten met QTLs voor zouttolerantie. Eén daarvan was gelegen in een klein gebied aan de bovenkant van chromosoom 9, waar ook een aantal receptor-achtige kinases (receptor-like Kinases) zijn gelokaliseerd. De meeste
gedetecteerde QTLs leverden een bijdrage aan ofwel zout tolerantie of gewone meeldauw resistentie, wat erop wijst dat de genetische factoren voor deze eigenschappen in de Lyc4 populatie weinig tot niet met elkaar overlappen. Dit houdt wel de mogelijkheid open van genetisch stapelen van genen voor ziekteresistentie en zouttolerantie om gecombineerde resistentie/tolerantie tegen ziekte en stress te verkrijgen.

De mate van abiotische stress onder natuurlijke omstandigheden kan sterk variëren, van mild tot zeer ernstig. Daarom is in hoofdstuk 4 de respons op gecombineerde gewone meeldauw infectie en zoutstress bestudeerd met verschillende zoutstress niveaus: 50mM, 100mM en 150mM NaCl. De planten die blootgesteld werden aan milde zoutstress (50mM) waren gevoeliger voor gewone meeldauw dan de planten die groeiden zonder stress, en de bladeren verouderden sneller en hadden zelfs afstervingsverschijnselen. Echter, in planten gegroeid bij het hoogste stressniveau van 150mM waren de ziekteverschijnselen juist verminderd. Dit ging gepaard met hogere Na⁺ en Cl⁻ concentraties in het blad. We hebben ook getest in hoeverre de veranderde ziektegevoeligheid onder zoutstress afhankelijk is van het type resistentie en resistentiemechanisme. Verschillende “Near Isogenic Lines (NILs)” elk met andere meeldauw resistentiegenen werden getest (OL-1: geassocieerd bij de langzame overgevoeligheidsreactie (HR); ol-2: een mlo mutant betrokken bij aanmaak van papilla; OL-4 een R-gen betrokken bij een snelle HR reactie), en bleken verschillend te reageren op gecombineerde stress. NIL-OL-1 liet een reactie zien die vergelijkbaar was met de Lyc4 lijnen: verhoogde infectie en snelle veroudering bij milde stress, en juist verminderde symptomen bij 150mM zout. NIL-ol-2 en NIL-OL-4 bleven daarentegen resistent tegen meeldauw bij alle stress niveaus en hadden ook geen verouderingssymptomen, niettegenstaande verminderde callose afzetting van NIL-ol-2. De verhoogde meeldauw infectie, versnelde veroudering en verminderde groei van NIL-OL-1 onder gecombineerde stress ging gepaard met verhoogde ethyleen en jasmonaat biosynthese en verhoogde activiteit van bijbehorende respons routes. Daarnaast was de expressie van het celwand invertase gen LeLIN6 sterk verhoogd, en expressie van genen betrokken bij anti-oxidant activiteit verlaagd. Deze resultaten laten zien dan de effectiviteit van meeldauw resistentie onder zoutstress sterk afhankt van het stressniveau maar ook van het type resistentie, en dat ethyleen een belangrijke rol speelt bij de reactie op en gevoeligheid voor meeldauw onder zilte condities.

In hoofdstuk 4 wordt de rol van hormonen tijdens blootstelling aan gecombineerde stress factoren verder onderzocht in hormoon mutanten van tomaat. De mutanten Notabilis (‘Abscisinezuur, (abscisic acid, ABA) deficiënt), defenseless (jasmijnzuur
(jasmonic acid, JA) deficiënt), en epinastic (Ethyleen (ET) overproductie) zijn gekruist met NIL-OL-1, NIL-ol-2 en NIL-OL-4. De combinatie van NIL-OL-1 met de pleiotrope mutant epinastic verminderde de meeldauw resistentie, en verminderde tegelijkertijd ook de door gecombineerde stress geïnduceerde versnelde veroudering. NIL-ol-2 in combinatie met epinastic reduceerde de afzetting van callose, en de verhoogde ET productie van de mutant verminderde de meeldauw resistentie met name tijdens gecombineerde zoutstress en meeldauw. Het ABA-tekort van notabilis daarentegen verhoogde het resistentieniveau van NIL-OL-1 onder gecombineerde stress, maar dat ging gepaard met sterke groeireductie en sterk verhoogde aanmaak van ROS. De ol-2 x notabilis mutant was vermindert resistent en had minder callose afgezet, maar onder gecombineerde stress was de resistentie tegen meeldauw weer iets verhoogd, en werd ook meer callose afgezet. De jasmijnzuur deficiëntie in de defenseless x NIL-OL-1 en defenseless x NIL-ol-2 lijnen had slechts een gering effect, met een kleine toename in meeldauw gevoeligheid. Deze nam weer af in defenseless x NIL-ol-2 onder zoutstress. defenseless x NIL-OL-4 was net zo resistent als NIL-OL-4 onder al de geteste omstandigheden. Deze resultaten benadrukken de belangrijke rol van de ET en ABA hormonale signaleringsroutes in ziektegevoeligheid en bladveroudering wanneer de plant blootgesteld wordt aan meeldauw en zoutstress tegelijk, en laat tegelijkertijd het belang zien van precieze regulering van de hormoonbalans in het beperken van pleiotrope effecten.

De resultaten worden uitgebreid bediscussieerd met extra aandacht voor de verschillende manieren waarop de abiotische stress respons interacteert met verschillende resistentiemechanismen in de plant, en hoe specifieke onderdelen van de stress respons aangrijpen op deze resistentiemechanismen. Ook wordt bekeken hoe en welke genetische factoren kunnen bijdragen aan verhoogde tolerantie tegen zowel abiotische als biotische tolerantie/resistentie. De mogelijkheden van gebruik van individuele genen worden besproken, maar ook van stapelen van genen die niet negatief op elkaar inwerken, zoals R-genen en genen voor zoutstress tolerantie. Daarnaast wordt onderzocht in hoeverre de inzichten verworven in deze thesis over de interactie tussen de zoutstress respons en gewone meeldauw resistentie kunnen worden vertaald naar andere combinaties van abiotische en biotische stress factoren. Een beter begrip van de mechanismen die ten grondslag liggen aan verbeterde groei van planten die blootgesteld worden aan combinaties van stress factoren kan een grote bijdrage leveren aan de veredeling van gewassen met een duurzaam verhoogde opbrengst onder stressvolle omstandigheden.
Acknowledgements

Reaching the end of this incredible journey I would like to express my deepest gratitude to my supervisors, colleagues and friends that enormously helped in different ways for the culmination of my PhD study.

First and foremost, I thank my promotor Prof. Richard Visser for giving me the opportunity to undertake my PhD in the Plant Breeding department of Wageningen University. Looking for a bigger challenge after finishing my MSc in Greece, Wageningen was the appropriate place to continue for a PhD. And what a challenge that was! The uncharted waters of abiotic and biotic stress combinations! It was a great opportunity to expand my knowledge on the most significant problems crops are facing due to climate change, leading to an opinion review article on the subject. Your comments and improvements on the thesis were always timely and were especially helpful at the final stretch of my studies. I wish you an impactful tenure as the Dean of Research of Wageningen University!

I have to deeply thank my supervisor and co–promotor Dr. Gerard van der Linden. We had a great collaboration throughout my project, giving me enough freedom in the hypothesis generation and experimental part of the thesis. Being a very clever person you could instantly point your considerations, leading to very interesting discussions. I have to apologise though for writing such long manuscripts and you having to go through pages and pages of literature discussions (again and again)! My best wishes for you and your wonderful family!

Many thanks to my second co–promotor Yuling Bai for her insightful suggestions on the disease part of the research and important comments during the writing of the thesis. They helped a lot to organise information, improve readability and passing the important messages to the reader (though it was difficult to convince me to do them!).

I would like to thank Dr. Clemens van de Wiel who kept a close eye on my work throughout my study, giving important advice from his point of view. I greatly appreciate his thorough inspection of all manuscripts for the minutest details which weeded out mistakes escaping my attention and helped improve interpretation of results.

I am especially thankful to my students Rawnaq, Sri, Pedro, Tanjim, Dewi, Mengistu and Yannick who helped me enormously during my PhD. It was an amazing experience to work with you and very fascinating from my point of view to see your skills evolving so quickly. I really enjoyed getting to know your culture and cuisine (especially that!). I wish you all the best for your career and personal life.
It was a great pleasure to work with Hanneke, Fien, Marjan, Anne-Marie, Marga, Jos in the lab and Andre, Bertus, Geurt, Maarten, Casper, Wim and Herman at Unifarm.

I have to express my gratitude to our secretaries, firstly to Annie Marchal who helped me a lot with my enrolment, and Nicole, Janneke and Letty for instantly helping with whatever I requested (abruptly in most occasions!).

Throughout my PhD I was sitting in the same office and I saw many people come and go. I am very grateful to my first officemates Marcel, Bjorn, Antoine and Stathis for helping me to adapt smoothly to the new environment and to PingPing, Giulia, Mario, Cynara, Jarno, Sara L., Alejandro, Sara B. (and Elio!), Naser and Gurnoor for all the nice moments!

In such a long period, bonding with certain people becomes stronger, sharing happy, emotional and weird moments! From waking up very early in the morning due to Myluska’s musical alarm (heard from 20m away!), to sharing a Greek breakfast with Dongli(!), the trip to Italy for Michela’s wedding, Mas’s laughter, the trip to Bordeaux and the weekend dinners and gatherings I had many good memories and I am very grateful to Myluska, Michela, Dongli, Mas, Ernest, Kaile, Atiyeh, Dalia, Anne–Marie and Elly. Best wishes from my heart for your career and for happiness in your life!

I would .like to thank my fellow colleagues Rafael, Long, Anitha, Chang-Lin, Baye, Biructawit, Ali Reza, Marcela, Arwa, Daniela, Peter, Ram, Yusuf, Andres, Xuan, Johan, Gerard, Thijs, Nelson, Giovanni, Anne, Mehdi, Asako, Eva, Shiva, Valentina, Yan, Abe, Carolina, Tim, Jordi, Marine and Jarst for sharing coffee breaks, birthdays, joyful moments and numerous scientific, social and (multi–) cultural discussions.

Many thanks to my Greek friends throughout the years Giannis, Sotiris, Michalis, Olga, Olympia, Eleni, Argyris, Foteini, Nikos, Stelios, Giorgos, Iliana, Manos, Chara and Fokion which made Wageningen feel more like home.

Reaching the end of my PhD I have to express my deep gratitude to my professors from my BSc and MSc in Thessaloniki for providing broad background knowledge on different aspects of plant sciences that greatly helped (and still are helping) to make and approach research questions.

Lastly but most importantly I want to thank my parents for their sacrifices and support through all these years to accomplish my goals. I am indebted to them and now is time to celebrate!
## Education Statement of the Graduate School

**Experimental Plant Sciences**

<table>
<thead>
<tr>
<th>Issued to:</th>
<th>Christos Kissoudis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date:</td>
<td>26 February 2016</td>
</tr>
<tr>
<td>Group:</td>
<td>Laboratory of Plant Breeding</td>
</tr>
<tr>
<td>University:</td>
<td>Wageningen University &amp; Research Centre</td>
</tr>
</tbody>
</table>

### 1) Start-up phase

<table>
<thead>
<tr>
<th><strong>First presentation of your project</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanisms and crosstalk underlying resistance to combined abiotic and biotic stress in tomato</td>
</tr>
<tr>
<td>Feb 22, 2011</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Writing or rewriting a project proposal</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic analysis of abiotic and biotic stress interaction in tomato</td>
</tr>
<tr>
<td>2011</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Writing a review or book chapter</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kissoudis C, van de Wiel C, Visser RGF, Van Der Linden G (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk.</td>
</tr>
<tr>
<td>May 19, 2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>MSc courses</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5 credits</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Laboratory use of isotopes</strong></th>
</tr>
</thead>
</table>

| **Subtotal Start-up Phase** | 13.5 credits |

### 2) Scientific Exposure

<table>
<thead>
<tr>
<th><strong>EPS PhD student days</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS PhD student day, Wageningen University</td>
</tr>
<tr>
<td>May 20, 2011</td>
</tr>
<tr>
<td>EPS PhD student day, University of Amsterdam</td>
</tr>
<tr>
<td>Nov 30, 2012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>EPS theme symposia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS theme 3 'Metabolism and Adaptation', Wageningen University</td>
</tr>
<tr>
<td>Feb 10, 2011</td>
</tr>
<tr>
<td>EPS Theme 4 Symposium 'Genome Biology', Wageningen University</td>
</tr>
<tr>
<td>Dec 09, 2011</td>
</tr>
<tr>
<td>EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, Wageningen University</td>
</tr>
<tr>
<td>Feb 10, 2012</td>
</tr>
<tr>
<td>EPS Theme 3 Symposium 'Metabolism and Adaptation', Utrecht University</td>
</tr>
<tr>
<td>Apr 26, 2012</td>
</tr>
<tr>
<td>EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, Utrecht University</td>
</tr>
<tr>
<td>Jan 24, 2013</td>
</tr>
<tr>
<td>EPS Theme 3 Symposium 'Metabolism and Adaptation', Wageningen University</td>
</tr>
<tr>
<td>Mar 12, 2014</td>
</tr>
<tr>
<td>EPS Theme 3 Symposium 'Metabolism and Adaptation', University of Amsterdam</td>
</tr>
<tr>
<td>Feb 10, 2015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>NWO Lunteren days and other National Platforms</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL</td>
</tr>
<tr>
<td>Apr 04-05, 2011</td>
</tr>
<tr>
<td>NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL</td>
</tr>
<tr>
<td>Apr 02-03, 2012</td>
</tr>
<tr>
<td>NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL</td>
</tr>
<tr>
<td>Apr 22-23, 2013</td>
</tr>
<tr>
<td>NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL</td>
</tr>
<tr>
<td>Apr 14-15, 2014</td>
</tr>
<tr>
<td>NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL</td>
</tr>
<tr>
<td>Apr 13-14, 2015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Seminars (series), workshops and symposia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd EPS Cellular Signaling Symposium, Amsterdam</td>
</tr>
<tr>
<td>Nov 04, 2010</td>
</tr>
<tr>
<td>Seminar Dr. Ian Henderson</td>
</tr>
<tr>
<td>Dec 13, 2010</td>
</tr>
<tr>
<td>Seminar Dr. Javier Palatnik, ‘Biogenesis and function of plant microRNAs’</td>
</tr>
<tr>
<td>Aug 25, 2011</td>
</tr>
<tr>
<td>Plant Sciences Seminar: Virtues and vices of plant modelling</td>
</tr>
<tr>
<td>Oct 11, 2011</td>
</tr>
<tr>
<td>EPS Mini-symposium ‘Plant Breeding in the genomics era’</td>
</tr>
<tr>
<td>Nov 25, 2011</td>
</tr>
<tr>
<td>Seminar Prof. G. Seymour The Tomato Genome: From Genes To QTL and Networks”</td>
</tr>
<tr>
<td>Jan 24, 2012</td>
</tr>
<tr>
<td>Plant Breeding Research Day 2012 &quot;Next generation sequencing – What’s in it for me&quot;</td>
</tr>
<tr>
<td>Feb 28, 2012</td>
</tr>
<tr>
<td>Seminar Prof. Sir D.C.Baulcombe ‘Plant versus virus: defense, counter defense and counter counter defense’</td>
</tr>
<tr>
<td>Oct 10, 2012</td>
</tr>
<tr>
<td>Seminar Prof.dr. Mark Tester</td>
</tr>
<tr>
<td>Oct 24, 2012</td>
</tr>
<tr>
<td>EPS Flying Seminar Dr. Detlef Weigel</td>
</tr>
<tr>
<td>Feb 27, 2013</td>
</tr>
<tr>
<td>Plant Sciences Seminar on Bioinformatics</td>
</tr>
<tr>
<td>Mar 12, 2013</td>
</tr>
<tr>
<td>TRANSPLANT workshop</td>
</tr>
<tr>
<td>Oct 13-14, 2014</td>
</tr>
<tr>
<td>Symposium All-inclusive Breeding: Integrating high-throughput science</td>
</tr>
<tr>
<td>Oct 16, 2014</td>
</tr>
<tr>
<td>EPS Flying Seminar Prof.dr. George Coupland</td>
</tr>
<tr>
<td>Jan 19, 2015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>International symposia and congresses</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>GRC Salt &amp; Water Stress in Plants, Hong Kong</td>
</tr>
<tr>
<td>Jun 24-29, 2012</td>
</tr>
<tr>
<td>Next Generation Plant Breeding conference</td>
</tr>
<tr>
<td>Nov 11-14, 2013</td>
</tr>
<tr>
<td>7th EPSO Conference, Greece</td>
</tr>
<tr>
<td>Sep 01-04, 2013</td>
</tr>
<tr>
<td>12th Solanaceae Conference, Bordeaux</td>
</tr>
<tr>
<td>Oct 25-29, 2015</td>
</tr>
<tr>
<td>Presentations (highly recommended)</td>
</tr>
<tr>
<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren (poster)</td>
</tr>
<tr>
<td>Apr 04-05, 2011</td>
</tr>
<tr>
<td>GRC Salt &amp; Water Stress in Plants, Hong Kong (poster)</td>
</tr>
<tr>
<td>Jun 24-29, 2012</td>
</tr>
<tr>
<td>Next Generation Plant Breeding conference (oral)</td>
</tr>
<tr>
<td>Nov 11-14, 2012</td>
</tr>
</tbody>
</table>

### Seminar plus

<table>
<thead>
<tr>
<th><strong>GRC Salt &amp; Water Stress in Plants, Hong Kong</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun 24-29, 2012</td>
</tr>
<tr>
<td>Next Generation Plant Breeding conference</td>
</tr>
<tr>
<td>Nov 11-14, 2012</td>
</tr>
<tr>
<td>7th EPSO Conference, Greece</td>
</tr>
<tr>
<td>Sep 01-04, 2013</td>
</tr>
<tr>
<td>12th Solanaceae Conference, Bordeaux</td>
</tr>
<tr>
<td>Oct 25-29, 2015</td>
</tr>
<tr>
<td>Presentations (highly recommended)</td>
</tr>
<tr>
<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren (poster)</td>
</tr>
<tr>
<td>Apr 04-05, 2011</td>
</tr>
<tr>
<td>GRC Salt &amp; Water Stress in Plants, Hong Kong (poster)</td>
</tr>
<tr>
<td>Jun 24-29, 2012</td>
</tr>
<tr>
<td>Next Generation Plant Breeding conference (oral)</td>
</tr>
<tr>
<td>Nov 11-14, 2012</td>
</tr>
<tr>
<td>Event Description</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ALW meeting 'Experimental Plant Sciences', Lunteren (oral)</td>
</tr>
<tr>
<td>7th EPSO Conference, Greece (poster)</td>
</tr>
<tr>
<td>ALW meeting 'Experimental Plant Sciences', Lunteren (oral)</td>
</tr>
<tr>
<td>IAB interview</td>
</tr>
<tr>
<td>Excursions</td>
</tr>
</tbody>
</table>

**Subtotal Scientific Exposure** 21.8 credits*

3) **In-Depth Studies**

- EPS courses or other PhD courses
  - 6th Utrecht EPS-PhD Summerschool on Environmental Signaling       Aug 22-24, 2011
  - Introduction to R for statistical Analysis                        Jun 11-12, 2012
  - PhD Summer School 'Natural Variation of Plants'                   Aug 21-24, 2012
  - The Power of RNA-seq                                              Dec 09-14, 2013
- Journal club
  - Participation in a literature discussion group at Plant Breeding  2011-2014
- Individual research training

**Subtotal In-Depth Studies** 8.1 credits*

4) **Personal development**

- Skill training courses
  - Course: Project and Time Management
  - Course: Writing Grant Proposals
- Organisation of PhD students day, course or conference
- Membership of Board, Committee or PhD council

**Subtotal Personal Development** 3.5 credits*

**TOTAL NUMBER OF CREDIT POINTS** 46.9

* A credit represents a normative study load of 28 hours of study.
This thesis was financially supported by “Alexander S. Onassis” Public Benefit Foundation scholarship to the author.

Cover design and thesis layout by the author

Printed by Proefschriftmaken.nl || Uitgeverij BOXPress
INVITATION

You are kindly invited to attend the defence of my PhD thesis

Genetics and regulation of combined abiotic and biotic stress tolerance in tomato

Friday, February 26th 2016
At 16.00 in the Aula of Wageningen University, General Foulkesweg 1a, Wageningen

You are kindly invited to the reception after the defence at the same address

Christos Kissoudis
christos.kissoudis@wur.nl

PARANYMPS

Cynara Romero
cynara.romero@wur.nl

Mas Muniroh
mas.muniroh@wur.nl

Genetics and regulation of combined abiotic and biotic stress tolerance in tomato

Christos Kissoudis

Genetics and regulation of combined abiotic and biotic stress tolerance in tomato
C. Kissoudis 2016

Christos Kissoudis