Running title: Transcriptome response to biotic and abiotic stress

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Research area: Signaling and Response
Identification of genes involved in the response of *Arabidopsis thaliana* to simultaneous biotic and abiotic stresses.

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Summary: Arabidopsis responds to simultaneous water stress and nematode infection by activating a unique program of gene expression that is distinct from the response to individual stresses.
Footnotes

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**Abstract**

In field conditions plants may experience numerous environmental stresses at any one time. Research suggests that the plant response to multiple stresses is different from that for individual stresses, producing non-additive effects. In particular, the molecular signalling pathways controlling biotic and abiotic stress responses may interact and antagonise one another.

The transcriptome response of *Arabidopsis thaliana* to concurrent water deficit (abiotic stress) and infection with the plant-parasitic nematode *Heterodera schachtii* (biotic stress) was analysed by microarray. A unique programme of gene expression was activated in response to a combination of water deficit and nematode stress, with 50 specifically multiple-stress regulated genes.

Candidate genes with potential roles in controlling the response to multiple stresses were selected and functionally characterised. *AtRALFL8* (At1g61563) was induced in roots by joint stresses but conferred susceptibility to drought stress and nematode infection when over-expressed. Constitutively expressing plants had stunted root systems and extended root hairs. Plants may produce signal peptides such as *AtRALFL8* to induce cell wall remodelling in response to multiple stresses. The methionine homeostasis gene *AtMGL* (At1g64660) was up-regulated by dual stress in leaves, conferring resistance to nematodes when over-expressed. It may regulate methionine metabolism under conditions of multiple stresses. *AZI1* (At4g12470), involved in defence priming in systemic plant immunity, was down-regulated in leaves by joint stress and conferred drought susceptibility when over-expressed, potentially as part of ABA-induced repression of pathogen response genes. The results highlight the complex nature of multiple stress responses and confirm the importance of studying plant stress factors in combination.
Introduction

Plants are adapted to respond to diverse environmental stress conditions, activating specific molecular and physiological changes in order to minimise damage. In field conditions, plants may be exposed to a variety of concurrent stresses (Mittler and Blumwald, 2010). Despite this, the majority of laboratory studies on plant stress factors have analysed each stress in isolation. There is increasing evidence that the response to multiple environmental stresses is distinct from that for individual stresses, and not merely additive (Atkinson and Urwin, 2012). This has been demonstrated in transcriptome studies on plants subjected to multiple abiotic stresses. In both tobacco and Arabidopsis (*Arabidopsis thaliana*), a combination of drought and heat stress induces a novel program of gene expression, activating transcripts that are not induced by either stress individually (Rizhsky et al., 2002; Rizhsky et al., 2004). Similarly, microarray analysis has revealed that exposure to multiple biotic stresses (two species of herbivorous insect) elicits a transcriptional response that is distinct from each individual response (Voelckel and Baldwin, 2004). It has therefore been proposed that each stress combination should be studied as an entirely new stress (Mittler and Blumwald, 2010). Understanding such mechanisms will be crucial for the future development of broad-spectrum stress-tolerant crops.

The response of plants to simultaneous biotic and abiotic stresses is of particular interest, as the signalling pathways of individual stress responses interact and antagonise one another, a process controlled principally by hormones (Anderson et al., 2004; Asselbergh et al., 2008; Atkinson and Urwin, 2012). Abscisic acid (ABA) is produced primarily in response to abiotic stresses and induces a range of downstream processes resulting in stress tolerance. In contrast, the response to biotic stresses is defined by antagonism between the hormones jasmonic acid, salicylic acid and ethylene. However, ABA can also act as a global regulator of stress responses by dominantly suppressing biotic stress signalling pathways. This may facilitate fine-tuning of plant stress responses to focus on the most severe threat, and can have both positive and negative effects on defence against pathogens (Anderson et al., 2004; Yasuda et al., 2008; Ton et al., 2009). For example, treatment with ABA has been shown to repress systemic acquired resistance to pathogens and prevent the accumulation of defence compounds such as lignins and phenylpropanoids (Mohr and Cahill, 2007; Kusajima et al., 2010). Drought stress or ABA treatment can actually increase the susceptibility of Arabidopsis to an avirulent strain of *Pseudomonas syringae*, whilst ABA treatment in tomato increases susceptibility to the pathogens *Botrytis cinerea* and *Erwinia chrysanthemi* (Audenaert et al., 2002; Mohr and Cahill, 2003; Asselbergh et al., 2008). ABA-
deficient mutants of both Arabidopsis and tomato show increased resistance to pathogens 
(Audenaert et al., 2002; Mohr and Cahill, 2003; Anderson et al., 2004). In contrast, ABA 
signalling is necessary for defence against some fungi and oomycetes, and ABA-induced 
stomatal closure can help prevent microbial invasion (Ton and Mauch-Mani, 2004; Melotto et 
al., 2006; Adie et al., 2007). Downstream regulation of multiple stress signalling pathways is 
achieved by a complex network of interacting components (Atkinson and Urwin, 2012). 
Transcription factors and mitogen-activated protein kinases may be particularly important in 
defining signal specificity, as they frequently control a wide range of downstream events and 
many are induced by more than one stress (Fujita et al., 2006; Zhang et al., 2006).

Water deficit and infection with plant-parasitic nematodes represent two environmental 
stresses with interacting effects under field conditions. Nematode infection can exacerbate 
the effects of water stress on plants as their parasitism of roots severely disrupts plant water 
relations (Bird, 1974; Haverkort et al., 1991; Smit and Vamerali, 1998). The cyst nematode 
_Heterodera sacchari_ increases drought-related losses in upland rice by contributing to 
reduced leaf water potential, stomatal conductance and leaf dry weight (Audebert et al., 
2000). The potato cyst nematode _Globodera pallida_ causes root retardation in potato, which 
in turn has the effect of reducing _Ditylenchus dipsaci_ tolerance (Smit and Vamerali, 1998). Water stress 
and infection with nematodes both negatively affect growth in potatoes, but in combination 
produce non-additive effects (Fasan and Haverkort, 1991; Haverkort et al., 1991).

Transcriptome analysis and functional characterisation of individual genes involved in the 
multiple stress response can provide further opportunities for fully understanding the 
complex interactions that regulate multiple stress responses. In this work the transcriptome 
response of Arabidopsis plants to combined biotic and abiotic stress was analysed using 
Affymetrix ATH1 whole genome arrays. Water stress was imposed through a short 
dehydration treatment, whilst biotic stress comprised infection with the beet cyst nematode 
_Heterodera schachtii_. Candidate genes with potential roles in controlling stress interaction 
were selected and their function characterised through the analysis of over-expression lines 
and loss-of-function mutants under control and stress conditions. The interaction with plant 
hormones was then investigated through the use of hormone signalling mutants.

**Results**

**Transcriptional profiling of Arabidopsis plants subjected to combined water deficit 
and nematode stress.**

Microarrays (Affymetrix, ATH1 chips) were used to determine changes in Arabidopsis gene 
transcript levels as a result of exposure to water deficit, nematode infection or the two 
stresses in combination. Root and leaf tissue were examined separately. Genes were
considered differentially regulated if their expression was significantly different from the control treatment ($p < 0.05$). Each individual or joint stress treatment induced a unique set of differentially expressed genes (Figure 1, Table S1). The number of genes differentially regulated in response to water stress, 996 in roots and 1134 in leaves, was far greater than that induced by nematode stress, 47 in roots and 84 in leaves. Around a quarter of the nematode-induced genes were also regulated by water stress and joint stress, suggesting a more generalised role in stress response. In response to a combination of biotic and abiotic stress, a total of 969 transcripts were found to be significantly different from the control treatment in roots (668 induced and 301 repressed) whilst 1134 were differentially regulated in leaves (624 induced and 509 repressed). The overall pattern of gene induction was highly similar to that observed for water stress, as 96 % of transcripts regulated by joint stress were also regulated by water stress alone. In contrast, only 2 % of joint stress-regulated transcripts were regulated by nematode stress alone. In addition to these overlapping transcript changes, the joint stress treatment induced a set of specific changes that were not differentially regulated by either of the single stresses, revealing that a novel and unique program of gene expression is activated in response to the combined stresses. qRT-PCR verification of expression for 12 genes selected to encompass a range of fold-changes correlated highly ($R^2 = 0.729$) with the results of the microarray (Table S2). All of the genes showed the same direction of fold change using both systems, however almost all genes showed a greater magnitude of fold change when measured by qRT-PCR.

The aim of this study was to identify genes differentially regulated between individual and dual stress treatments. These were genes specifically activated or repressed by the addition of a second stress, and which therefore stood out as potential candidates for orchestrating the multiple stress response. As the response to water deficit and to joint stress was transcriptionally similar, genes with a different pattern of expression under the two conditions were of particular interest. T-tests were used to identify these ‘interaction’ genes that were differentially regulated by joint stress in comparison to individual stress, as opposed to comparison with unstressed plants (Table S3). This list included any genes that were differentially regulated by both individual and joint stress, but to a significantly greater or lesser extent when the stresses occurred together. This system allowed the exclusion of any genes with a very similar pattern of transcription under individual water stress and joint stress, but which only reached the significance threshold in one comparison. Of the interaction genes, several categories of gene function were highly prominent (Table S4). These included genes with both functional and regulatory roles. In roots, 23 up-regulated genes had functions in cell wall modification, including extensins, pectinesterases, polygalacturonases and xyloglucan transferases. Carbohydrate metabolism genes were
abundant amongst the up- and down-regulated interaction genes in roots and leaves, particularly glycosyl and glycoside hydrolases. Sixteen interaction genes with disease resistance annotations, including those with leucine rich repeat (LRR) domains, were down-regulated in leaf tissue, whilst four were down-regulated in roots. Several genes encoding proteins with oxidoreductase functions were repressed in leaves. Signal transduction and regulation genes such as those encoding transcription factors, protein kinases and RALF-like signal molecules were also prevalent amongst the interaction genes. A total of 32 and 27 transcription factors were up-regulated in leaves and roots respectively, whilst 22 and 28 were repressed in those tissues. The largest group of differentially regulated transcription factors belonged to the MYB family, whilst also abundant were those from the no apical meristem (NAM) family, as well as the AP2, the zinc finger (C2H2 type), the basic helix-loop-helix (bHLH) and the Dof-type families. Figure S1 shows the functional gene ontology categories of the interaction genes.

Genes were selected for further analysis from over-represented families of transcription factors, those strongly regulated by hormones (as determined using data from Genevestigator, Figure S2) or those with the largest fold change in expression between individual and joint stress. The role of the candidate genes in stress response mechanisms was then investigated through loss-of-function and constitutive over-expression mutants. The findings obtained from the study of three genes are reported here, namely AtRALFL8, AtMGL and AZI1. These were selected as genes likely to play prominent roles in governing the response to multiple stresses.

Expression of candidate genes during stress treatments.

The genes AtRALFL8 (At1g61563) and AtMGL (At1g64660) were amongst the ‘interaction’ genes identified in the microarray study that were significantly up-regulated due to simultaneous water deficit and nematode stress in root tissue (Table S3). AtRALFL8 encodes a short signalling peptide with similarity to tobacco Rapid Alkalisation Factor (RALF) (Olsen et al., 2002). Three other RALFL genes (AtRALFL23, AtRALFL33 and AtRALFL34) were also induced by the stresses in combination. AtMGL encodes a methionine gamma-lyase involved in cellular methionine homeostasis and isoleucine synthesis (Rebeille et al., 2006). AZI1 (At4g12470) is an example of a gene down-regulated in leaf tissue by the stresses in combination (Table S3). This gene belongs to a family of lipid transfer proteins and is important for defence priming in systemic plant immunity (Jung et al., 2009). Differential regulation of the genes was confirmed by qRT-PCR (Figure 2), and in each case the expression differential was greater than that shown by microarray, a commonly-observed effect likely to arise from the differing sensitivities of the two technologies (Clarke and Zhu, 2006).
Phenotype of loss-of-function and constitutive expression lines under control conditions

Constitutive over-expression lines were created for each gene using the CaMV35S promoter and their relative levels of expression quantified. The most highly over-expressing line for each gene was used for subsequent experiments. The selected 35S::AZI1 line produced 12,500 times the wild type level of AZI1 transcript, the 35S::AtMGL line had 1,590-fold increased expression and the 35S::AtRALFL8 line 21,000-fold. T-DNA insertion lines were obtained for AtMGL and AZI1 although none was available for AtRALFL8. The effect of altered AtRALFL8, AtMGL and AZI1 expression was investigated by studying the growth characteristics of mutants and over-expression lines under control conditions. Figures 3-5 show a range of phenotypic measurements for each mutant in comparison to wild type plants. 35S::AtRALFL8 plants had a severely stunted phenotype, as characterised by a root system that was only 20% of the wild type size (Figure 3). Main root length, number of lateral roots, total root size and lateral root density were significantly reduced. The rosette diameter was smaller than the wild type, whilst biomass accumulation was also greatly reduced, giving plants whose aerial parts weighed only 50 mg (dry weight) after 35 days in comparison to the wild type weight of 500 mg. The inflorescence was significantly shorter than the wild type, even when fully mature, and developed fewer siliques which in turn contained fewer seeds.

The only visible effect of AtMGL gene inactivation was a 15% smaller rosette diameter at 16 days (Figure 4). However, the over-expression of this gene caused several phenotypic differences in development in aerial plant parts. 35S::AtMGL rosettes were slightly reduced in diameter and had a very low rate of biomass accumulation with a dry weight only 44% of the wild type value. Seed yield was also significantly reduced, although the final inflorescence height was slightly greater than the wild type.

Both the azi1 mutant and the 35S::AZI1 constitutive expression line exhibited slow growth in the aerial parts of the plant (Figure 5). The azi1 mutant had a more severe phenotype, with reduced leaf number, rosette diameter, height of 1y inflorescence, silique number and dry weight, as well as fewer lateral roots. The 35S::AZI1 plants had fewer siliques and a lower dry weight than the wild type, although no difference could be found between the root systems and those of control plants. Primary inflorescences in both the mutant and over-expression lines emerged later than the wild type, but seed yield was not affected. To confirm that the observed phenotypes resulted directly from over-expression of the transgene, two additional lines per construct were analysed for their growth characteristics under control conditions, yielding similar results (Figure S3).
Stress tolerance phenotype of loss-of-function and constitutive expression lines

Drought tolerance of Arabidopsis mutant and over-expression lines was assessed by measuring survival rate after a prolonged period without irrigation. 35S::AtRALFL8 plants had a significantly lower survival rate than control plants, with a recovery rate that was only 9% of the wild type value (Figure 6a and 6b). 35S::AZI1 plants also exhibited increased susceptibility to drought stress, showing a recovery rate of 6.7% of the wild type value. The drought tolerance of 35S::AtMGL, atmgl and azi1 plants was not affected by the altered gene expression.

The susceptibility of loss-of-function and over-expression lines to infection with the plant-parasitic nematode *H. schachtii* was assessed. The number of successful parasitism events on each genotype was compared to that on wild type plants. 35S::AtRALFL8 plants supported 2.6 times more nematodes than wild type plants (*p* < 0.05). In contrast, significantly fewer nematodes were observed on 35S::AtMGL plants, which had only 24% the infection rate of wild type plants (*p* < 0.05). The genotypes atmgl, azi1 and 35S::AZI1 showed no altered susceptibility to *H. schachtii* (Figure 7).

Expression of candidate genes in hormone signalling mutants

To investigate the relationship of candidate genes with known hormone signalling pathways, the expression levels of *AtRALFL8, AtMGL* and *AZI1* were analysed in Arabidopsis lines deficient in ethylene, abscisic acid or salicylic acid signalling pathways using qRT-PCR. There were significant differences in expression of each gene in one or more mutants compared to the level in wild type plants (Figure 8). Expression of *AtRALFL8* was significantly higher in ethylene-insensitive *ein3-1* plants, with 2.5 times the level of wild-type transcript (*p* < 0.05) (Figure 8a). Expression of *AtRALFL8* was similar in the constitutive ethylene response mutant *CTR1* and in Col-0 plants. *AtMGL* was expressed 3.8-fold higher in ABA-insensitive *abi2-1* mutant plants, which are deficient in ABA signal transduction, than in the control Ler-0 plants (*p* < 0.01) (Figure 8b). Altered expression of *AZI1* was also observed in hormone signalling mutants (Figure 8c). In the *CTR1* mutant *AZI1* expression was 13 times the wild type value (*p* < 0.01), whilst in the *ein3-1* mutant the expression was lower than, although not significantly different from, the control. *AZI1* expression was increased 6-fold in the *abi2-1* mutant (*p* < 0.01) (Figure 8c). None of the three genes had altered expression in the *abi4-1* mutant or in the NahG salicylic acid signalling mutant.

Investigation of 35S::AtRALFL8 phenotype

In addition to the severely stunted root system of 35S::AtRALFL8 (Figure 3a), roots had longer and more numerous root hairs than the wild type (Figure 9) and a shorter meristem as determined by the number of cortical cells between the quiescent centre and the transition
zone of elongation-differentiation (Figure 10). This phenotype suggests disruption in auxin or
ethylene signalling (Knox et al., 2003; Moubayidin et al., 2010) and was observed in four
independent 35S::AtRALFL8 transgenic lines, thus eliminating the possibility of it being a
secondary effect of the transgene insertion (data not shown). On close observation, the root
epidermal cells were seen to maintain a normal pattern of hair cells and non-hair cells,
although the epidermal cells appeared shorter. To determine whether or not this phenotype
was related to a disruption in auxin signalling, wild type and 35S::AtRALFL8 plants were
grown on media containing the auxin indole-3-acetic acid (IAA), the synthetic auxin 2,4-
dichlorophenoxyacetic acid (2,4-D) and the anti-auxin α-(phenyl ethyl-2-one)-indole-3-acetic
acid (PEO-IAA) and the primary root lengths measured (Figure 11). The 35S::AtRALFL8
plants responded to each compound in a similar manner to wild type plants, producing a
slightly shorter root on IAA, a very short root on 2,4-D and a normal length root on PEO-IAA.
35S::AtRALFL8 homozygous plants were crossed with the auxin-insensitive mutant axr3-1.
This mutant carries a gain-of-function mutation in AXR3 which prevents its auxin-mediated
degradation, thus causing auxin insensitivity and giving a phenotype with no root hairs,
reduced root elongation and agravitropism. The resulting 35S::AtRALFL8/axr3-1
heterozygotes were indistinguishable from the homozygous axr3-1 plants (data not shown),
suggesting that the dominant axr3-1 mutation over-rides the effect of over-expressing
AtRALFL8 from an ectopic promoter. The spatial pattern of auxin response in
35S::AtRALFL8 plants was assessed using the auxin-responsive marker DR5rev::GFP,
however no overall changes were observed in the pattern of DR5::GFP expression (data not
shown). Co-expression analysis using the Arabidopsis Coexpression Data Mining Tool
(arabidopsis.leeds.ac.uk/act) revealed that the expression of AtRALFL8 is extremely highly
correlated with pectin methylesterase genes (r < 0.99). Expression of three pectin
methylesterase genes that are co-expressed with AtRALFL8 (At2g47040, At1g69940,
At3g62170) was analysed in the 35S::AtRALFL8 over-expression line. No difference in
expression was detected between the transgenic line and the wild type plants (data not
shown) suggesting that an increase in AtRALFL8 expression does not affect pectin
methylesterases at the transcriptional level.

Discussion
The effect of two or more concurrent environmental stresses can be far more detrimental to
plants than an individual stress, leading to severe agricultural losses (Craufurd and Peacock,
1993; Savin and Nicolas, 1996; Mittler, 2006). The ability of plants to recognise and respond
to specific stress combinations may be extremely important, particularly when those
individual stresses would elicit conflicting responses. However, the combination of abiotic
and biotic stress factors on the plant whole-genome transcriptome is not well documented. Previous studies have aimed to identify genes important in multiple stress tolerance by comparing lists of genes induced by each stress individually (Seki et al., 2002; Swindell, 2006; Kilian et al., 2007; Kant et al., 2008). With our current knowledge of how stress responses interact, this type of research is no longer considered sufficient for understanding multiple stress responses (Mittler and Blumwald, 2010). Here, the transcriptome response of Arabidopsis following a combination of water deficit and nematode stress treatments displayed a distinct programme of gene expression, supporting the theory that plant responses to stress are highly specialised and unique to the exact set of environmental conditions encountered (Mittler, 2006; Yasuda et al., 2008; Ton et al., 2009). Similar results have been observed in studies of two different abiotic stresses (Rizhsky et al., 2004). Furthermore, a recent study by Rasmussen et al. (2013) has also confirmed that the response of Arabidopsis to dual stress is very different to and cannot be predicted from the individual responses, by comparing single or combined cold, heat, high-light, salt, and flagellin treatments.

Repression of pathogen defence pathways in response to concurrent biotic and abiotic stress

When water deficit and nematode stress were applied to plants in combination, the resulting gene expression profile resembled that of the plant under water deficit alone more closely than under nematode stress alone. The impact of water deficit may be more profound than nematode stress, as water deficit causes rapid physiological changes throughout the plant giving widespread and cellular osmotic imbalance and turgor loss (Chaves et al., 2003). In contrast, plant-parasitic nematodes have evolved mechanisms by which to minimise damage to plant tissues and thus evade standard plant defence systems (Wubben et al., 2008). Therefore, when the stresses occur together the plant response may prioritise the potentially more damaging abiotic stress. Antagonistic crosstalk between biotic and abiotic signalling pathways may also play a role in creating the observed response to the stresses in combination. ABA, although primarily responsible for orchestrating plant response to abiotic stress, also has a prominent role in pathogen and disease resistance (Asselbergh et al., 2008; Yasuda et al., 2008; Ton et al., 2009). There is evidence that ABA produced during abiotic stress suppresses defence pathways including SAR, which is known to be induced by nematode invasion (Wubben et al., 2008; Yasuda et al., 2008). In addition, the down-regulation of genes with disease resistance annotations in the current study, including many containing leucine-rich repeat domains, supports an active suppression of pathogen response pathways as a result of concurrent biotic and abiotic stresses.
AZI1 is a pathogen response gene that was down-regulated when water deficit and nematode stress were imposed in combination. AZI1 is a lipid transfer protein that is locally induced following pathogen infection and important for the establishment of systemic immunity priming in distal tissues. It acts to mobilise lipids as signalling molecules (Parker, 2009) in a process that is dependent on saliclyic acid and the mobile metabolite azelaic acid (Jung et al., 2009). AZI1 also plays a role in abiotic stress response and its over-expression can lead to improved freezing tolerance in Arabidopsis (Xu et al., 2011). Analysis of over-expression and mutant azi1 lines in the current study allowed further characterisation of this gene. AZI1 over-expression conferred drought stress susceptibility. The chemical induction of systemic acquired immunity (SAR) can negatively influence the production of ABA and the activation of ABA-responsive genes (Yasuda et al., 2008). Therefore the observed drought susceptibility may have been due to an over-activation of the SAR priming system, which led to an inhibition of ABA-induced drought response genes. In addition, the expression of AZI1 was 6-fold higher in the ABA insensitive mutant abi2-1 but no different from wild type in abi4-1. Thus ABA may negatively regulate AZI1-mediated systemic immunity priming downstream of the ABA signal transduction gene ABI2 but not dependent on the ABA-responsive AP2 transcription factor ABI4. Ethylene is likely to be a positive regulator of the systemic priming system, however, as shown by the increased expression of AZI1 in CTR1 mutants. The over-expression of AZI1 conferred no resistance to nematode infection. Although a local salicylic acid response is important for resistance to cyst nematodes (Wubben et al., 2008), these results suggest that azelaic acid-associated priming does not influence susceptibility to cyst nematode infection. When biotic and abiotic stresses are encountered simultaneously, AZI1 and the systemic immunity pathway may be specifically repressed in an ABA-responsive manner, in order to focus resources on the abiotic stress response.

**Signal transduction and regulation**

Many of the interaction genes identified in this study were regulatory genes such as transcription factors and small signal molecules. The large number of MYB transcription factors identified amongst the interaction genes contributes to increasing evidence that these factors are central in controlling cross-talk and specificity between different stress signalling pathways (Rizhsky et al., 2004; Mattana et al., 2005; Vannini et al., 2007; AbuQamar et al., 2009; Dubos et al., 2010; Atkinson and Urwin, 2012). MYBs regulate stress-related production of secondary metabolites in the phenylpropanoid pathway such as anthocyanins and lignin, as well as cell wall biosynthesis (Jin et al., 2000; Patzlaff et al., 2003; Wuyts et al., 2006; Dubos et al., 2010), and may make excellent candidates for the improvement of broad-spectrum stress tolerance (Jin et al., 2000; Vannini et al., 2004). In addition, the action
of NAM and AP2 transcription factors in straddling both biotic and abiotic stress signalling pathways is further supported (Nakashima et al., 2007; Xu et al., 2011).

The expression of four small RALF-like signalling molecules was induced by the combination of water deficit and nematode stress (AtRALFL8, AtRALFL23, AtRALFL33 and AtRALFL34). These short signalling peptides show similarity to tobacco Rapid Alkaloinisation Factor (RALF; Pearce et al., 2001). They are growth regulators that can inhibit cell elongation in roots and pollen tubes (Wu et al., 2007; Matos et al., 2008; Srivastava et al., 2009; Covey et al., 2010). RALFLs are expressed in widely differing locations throughout the plant (Figure S4) (Olsen et al., 2002; Hruz et al., 2008), although they have a high degree of sequence similarity. Their identification in the current study suggests a mechanism whereby plants induce such signal molecules in order to reduce growth during severe stress.

**Cell wall modification**

Cell wall modifications can provide an increased physical barrier against potential pathogens, whilst improving tolerance to drought and oxidative stress and maintaining turgor during osmotic stress (Piro et al., 2003; Pelloux et al., 2007; An et al., 2008; Leucci et al., 2008). The observed up-regulation of cell wall modification proteins in response to combined nematode and dehydration stress may therefore be a general defensive mechanism activated to confer broad-spectrum tolerance against multiple stresses. AtRALFL8 may play a role in cell wall modification as it is co-expressed extremely highly with genes encoding cell wall pectinesterases (PMEs). These are crucial for cell wall re-modelling in a variety of growth, reproductive and defence processes and their transcript level varies in response to biotic and abiotic stresses, including nematode infection (An et al., 2008; Pelloux et al., 2007). Different PMEs are specifically active at varying pH levels and their function can be modulated by alkaloinisation (Feijó et al., 1999; Pelloux et al., 2007). AtRALFL8 is myristoylated at its N-terminus and may therefore act as a signalling molecule that binds to the cell membrane and causes alkaloinisation of the cell wall, a similar mechanism to that proposed for SIRALF in tomato (Boisson et al., 2003; Covey et al., 2010). Root surface pH varies along the root tip with distance from the meristem, being lowest at the zone of cell elongation (Staal et al., 2011). Therefore, the constitutive expression of an alkaloinisation factor could directly inhibit the expansion of cells in this zone. Alkaloinisation of the apoplast may have also been responsible for the abnormally long and numerous root hairs of the AtRALFL8 over-expression line, as changes in pH are crucial during rapid cell elongation processes, including the growth of pollen tubes, root epidermal cells and root hair growth (Bibikova et al., 1998; Wu et al., 2007). Changes in cell wall modification proteins may also account for the hyper-susceptibility of the AtRALFL8 over-expression line to drought stress as well as parasitism by the nematode *H. schachtii*.
Root alkanalisation by RALF-like signal molecules may be a point of interaction between the hormones auxin and ethylene during multiple stress responses. There is evidence that auxin acts in concert with ethylene to mediate the alkanalisation of the root surface during growth inhibition (Staal et al., 2011). This, combined with the shortened meristem in the AtRALFL8 over-expression line and the increase in AtRALFL8 expression in the ethylene signalling mutant ein3-1 suggest that AtRALFL8 may be involved in modulating events in the auxin response pathway (Knox et al., 2003; Moubayidin et al., 2010). AtRALFL8 induction during simultaneous biotic and abiotic stress is an illustration of the complex mechanisms by which plants act through different hormone signalling pathways to produce cellular changes that protect them from stress.

Cellular and metabolic responses to multiple stresses
The production and homeostasis of amino acids is intrinsic to many aspects of both biotic and abiotic stress responses. Proline and isoleucine accumulate as osmoprotectants during drought and osmotic stress and can act as scavengers of reactive oxygen species, regulators of pH or as substrates for the synthesis of stress-related proteins (Nambara et al., 1998; Joshi et al., 2010). Isoleucine also has the crucial role of combining with jasmonic acid to make the active defence and pathogen response hormone, JA-Ile (Nambara et al., 1998; Joshi and Jander, 2009). The up-regulation of AtMGL, a methionine homeostasis gene, in joint-stressed plants in the current study suggested that a shift in the metabolism of this amino acid may be important in the response to concurrent biotic and abiotic stresses (Rebeille et al., 2006; Goyer et al., 2007; Joshi and Jander, 2009). Most cellular methionine is converted to S-adenosylmethionine (SAM) for use in essential plant processes such as DNA replication and methylation and the synthesis of ethylene, cell walls, chlorophyll and secondary metabolites. However, AtMGL catabolises methionine in an alternative pathway leading to the synthesis of isoleucine. Previously AtMGL has been induced in response to Pseudomonas syringae and Phytophthora parasitica (Genevestigator) and in response to a combination of heat and drought stress (Rizhsky et al., 2004). Drought-stressed atmgl plants accumulate less isoleucine than wild type plants (Joshi and Jander, 2009). Altering amino acid homeostasis by the channelling of methionine into the isoleucine pathway may be an adaptive strategy providing protection from severe or multiple stresses, albeit with a growth penalty. The increase of AtMGL transcript in the ABA-resistant mutant abi2-1 suggests that this process is negatively regulated by ABA.

The analysis of atmgl mutants revealed a phenotype that was little different from wild type plants under control or drought-stressed conditions, a result also described by Joshi and Jander (2009). These observations are likely to result from redundancy with threonine
deaminase, an alternative isoleucine biosynthesis mechanism (Joshi et al., 2010). The over-expression of *AtMGL*, in contrast, severely affected the growth of aerial parts of the plant under normal conditions. As 80% of methionine is normally directed into SAM, the over-activity of AtMGL would convert excess methionine into the alternative pathway, depleting the pool used as methyl donors for essential plant processes and resulting in the reduced growth phenotype. 35S::*AtMGL* plants were less susceptible to infection with the nematode *H. schachtii*. This may be attributed to a number of factors. Plant-parasitic nematodes are net consumers which depend on amino acids from their hosts. A high sink strength in the feeding cells of *H. schachtii* leads to an enriched methionine concentration as well as an increase in transcription of methionine scavenging genes (Szakasits et al., 2009; Hofmann et al., 2010). The depletion of available methionine by the over-expression of *AtMGL* may therefore inhibit nematode protein synthesis, preventing their development. In addition, the increased production of isoleucine due to *AtMGL* over-expression may boost levels of JA-Ile, creating a heightened defence mechanism which could respond more effectively to the nematode invasion.

**Conclusion**

The study of simultaneous biotic and abiotic stresses highlights the complex mechanisms by which plants tailor their response to precise environmental conditions. A new pattern of transcription was observed that differed from that of either stress individually, which in addition supports the hypothesis that the response to a potentially more damaging abiotic stress can over-ride the response to biotic stress. The importance of various processes such as cell wall re-modelling, methionine homeostasis and immune system priming in the response to multiple stresses has been demonstrated through the functional analysis of individual genes. While the results reported here underline the importance of studying plant stress factors in combination, to fully understand how plants respond to multiple stresses in field conditions further work will be required.

**Materials and methods:**

**Plant and nematode material, growth conditions and stress treatments for microarray experiment.**

Arabidopsis (*Arabidopsis thaliana*) seeds (cv Columbia-0) were sterilised by soaking in 95% ethanol for 2 minutes, 10% bleach for 5 minutes, followed by five washes in sterile distilled water. Seeds were stratified at 4 °C for 48 hours then germinated and grown in square petri dishes on solid media containing half-strength Murashige & Skoog (MS) salts with vitamins.
(Duchefa, Suffolk, UK), 1% sucrose (Sigma, Dorset, UK) and 1% plant agar (Duchefa). Four seeds were sown per 10 cm plate, and plates were held at an angle of approximately 70° in a growth cabinet (Sanyo MLR; Sanyo, Leicestershire, UK); at 20 °C with a light intensity of 140 μmol m⁻² s⁻¹ and under 16 h/8 h light/dark cycles. H. schachtii cysts were extracted from soil then sterilised and hatched as described in Urwin et al. (1997). Overnight hatches of J2s were sterilised in 0.5 mg/ml hexadecyltrimethyl-ammonium bromide and 0.1% chlorhexidine digluconate for 30 minutes and washed twice in sterile tap water. After 18 days of growth plants were divided into four treatment groups: control, water deficit, nematode infection and combined stress. Plants from the nematode-infected and combined stress treatment groups were each challenged with 175 H. schachtii J2s. All other plants were mock-inoculated with sterile tap water. Ten days later dehydration treatment was imposed on the water deficit and combined stress treatment groups. Control and nematode-infected plants were removed from the agar and then immediately replaced and returned to the growth cabinet for 45 minutes. Dehydration-treated plants were removed from the medium and placed in a clean flow of air for 15 minutes, as described by Kilian et al. (2007), during which time plants lost 10% of their fresh weight. Plants were then placed back on the agar and returned to growth cabinets for 30 minutes before harvesting tissue from all four treatment groups. Aerial tissues were separated from roots and both were frozen in liquid nitrogen. Forty plants were used per treatment group. The entire experiment was carried out twice more on different occasions, giving three biological replicates.

RNA isolation, cDNA synthesis and Affymetrix GeneChip analysis

Plant tissue from each complete biological replicate was divided into five pools of eight plants per treatment and RNA isolated from each pool separately using the RNeasy Plant Mini Kit (Qiagen, West Sussex, UK). Equal amounts of RNA were then combined into one sample per treatment group and replicate. A 2100 Expert Bioanalyser (Agilent Technologies, Edinburgh, UK) was used to confirm the quality of all RNA samples before microarray analysis. Twenty-four arrays were used in total, representing four treatments, two tissue types and three replicates. Hybridisation of Biotin-labelled RNA to Affymetrix ATH1 GeneChip arrays and array scanning were performed by the Nottingham Arabidopsis Stock Centre transcriptomics service (Craigon et al., 2004) following the standard Affymetrix protocol. Normalisation and analysis of differential expression was carried out using GeneSpring GX10 (Agilent Technologies). Baseline pre-processing, normalisation and summarisation was carried out using the Robust Multiarray Average summarisation algorithm, as described by Irizarry et al. (2003). Genes were considered differentially regulated if their normalised expression value was significantly different from the control (p < 0.05). One-way ANOVA with Benjamini Hochberg multiple testing correction (false discovery
rate of 0.05) was used to identify genes differentially regulated between treatment groups. Further T-test analyses were used to detect ‘interaction’ genes regulated by joint stress in comparison to individual water deficit stress. From these lists several genes of interest were selected for further study. Data has been deposited in the public repository NASCArrays and is accessible at http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl with the reference number NASCARRAYS-489.

The expression changes in a subset of 12 genes were verified using qRT-PCR. cDNA was generated from 250 ng total RNA per sample using BioScript MMLV reverse transcriptase (Bioline, London, UK), and qRT-PCR carried out on the resulting cDNA using Brilliant II SYBR® Green Master Mix (Agilent Technologies). qRT-PCR conditions consisted of 93°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Fluorescence data was collected at the end of the annealing phase. Differences in transcript level were determined using MxPro software (Stratagene), with ACTIN2 as a normalising gene. All primer pairs had an amplification efficiency of 90-110% and R² correlation coefficients for standard curves ranged between 0.94 and 1.00. (See Table S5 for details of primers used in qRT-PCR analysis).

Generating over-expression lines
Coding regions of the genes of interest were cloned into the plant transformation binary vector pBI121 from which the GUS gene had been removed and replaced by a Kpn I restriction site. The gene coding sequences were placed under the control of a CaMV35S promoter with the nptII gene for kanamycin resistance as the selectable marker. The constructs were introduced into Agrobacterium tumefaciens strain GV3101 which was used to transform Arabidopsis plants using the floral dip method. Transgenic lines were validated by PCR and the increase in target gene expression quantified using qRT-PCR as described earlier. Primer sequences are provided in Table S5.

T-DNA insertion mutants
Insertion mutants were obtained for AtMGL (SALK_074592C) and AZI1 (SALK_085727C) from the Nottingham Arabidopsis Stock Centre. Homozygosity of the lines was confirmed by PCR, using primers annealing either to the left border region of the T-DNA and a 3’ flanking sequence to amplify the insertion, or to 5’ and 3’ flanking regions to amplify the wild type allele.

Phenotypic analysis of transgenic plants
Seeds of each transgenic over-expression and mutant Arabidopsis line together with wild type Col-0 plants were sown onto trays of compost (Sinclair Potting & Growing Medium, East Riding Horticulture) with a depth of 5 cm and grown in a growth chamber (Sanyo) at 20°C.
under 16 h/8 h light/dark cycles (200 μmol m$^{-2}$ s$^{-1}$, 60% humidity). Approximately 14 days after sowing, seedlings were removed from trays and re-potted into 9 cm pots for phenotypic analysis. For over-expression lines, analysis was carried out on the most highly expressing line per construct and two additional independent transgenic lines with lower expression. Phenotypic measurements were recorded at various stages throughout plant development under control conditions, including: Rosette diameter and leaf number 16 days after sowing, timing of primary inflorescence emergence, height of primary inflorescence 35 days after sowing, final height of primary inflorescence, silique number on primary inflorescence 40 days after sowing, seed number per silique (n = 16). Dry weight of aerial plant material was also determined 35 days after sowing (n = 4). For root system analysis plants were grown on half-strength MS medium as described previously. Fourteen days after sowing the plates were scanned using a Hewlett Packard ScanJet 5370C, and root parameters were measured from the digital images using Image-Pro Plus software version 7.0 (MediaCybernetics) (n = 9). Phenotypic differences between plant genotypes were analysed using ANOVA or the Kruskal-Wallis H test.

Stress treatments of transgenic plants

For drought tolerance assays, plants were grown in 25 x 40 x 5 cm trays on compost mixed with sand and loam soil at a ratio of 2:1:1 to facilitate drainage. Plants were transplanted into these trays 2 weeks after germinating. Twelve transgenic plants were alternated with twelve wild type per tray. Trays were then watered to field capacity for 1 week, then irrigation was ceased until the soil moisture level dropped to 3-4 % (2 weeks on average). The plants were then re-watered to field capacity for 1 week and scored for survival. For nematode susceptibility assays, plants were grown in sterile conditions on half-strength MS10 medium as described previously. Eighteen days after sowing each plant was infected with 100 juvenile *H. schachtii* nematodes. Nematodes were allowed to develop for 10 days, the root systems stained in acid fuchsins (Fuller et al., 2007), and the number of established 3rd and 4th stage juvenile nematodes (J3 and J4) per plant counted.

Analysis of gene expression in hormone signalling mutants

The Arabidopsis hormone signalling mutants *abi2-1* (Landsberg erecta; Ler-0), *abi4-1* (Col-0), *ein3-1* (Col-0), *NahG* (Col-0) and *CTR1* (Col-0) together with the corresponding wildtype ecotypes were germinated on half-strength MS10 medium. Abscisic acid (3 μM) was included in the media of *abi2-1* and *abi4-1* plants and the media for *ein3-1* plants contained 1-aminocyclopropane-1-carboxylic-acid (ACC) (1 μM). Fourteen-day old seedlings were planted out into compost/sand/loam. After a further 14 days leaf tissue was harvested from 3 pools of 4 plants for each genotype. RNA was isolated from each pool, and analysed by
qRT-PCR as detailed previously using primers for each of the genes of interest. Differences in gene expression were analysed using ANOVA or the Kruskal-Wallis H test.

**Analysis of 35S::AtRALFL8 root phenotype**

To analyse auxin responses, plants were grown on unsupplemented ATS media (Knox et al., 2003), or that containing 0.1 μM indole-3-acetic acid (IAA), 0.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 1 μM α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA). The plates were held in an upright position and root length measured 7 days post germination. Expression levels of the pectin methylesterase genes At2g47040, At1g69940 and At3g62170 were analysed in the leaves of 14-day old 35S::AtRALFL8 plants by qRT-PCR. Primer sequences are provided in Table S5. Root tips were stained with propidium iodide and confocal microscopy was carried out using an LSM Meta 510 microscope (Zeiss). The spatial pattern of auxin response was visualised by crossing 35S::RALFL8 plants with those containing the *DR5rev::GFP* auxin responsive promoter (Friml et al., 2003).

**Supplemental material**

Table S1: Lists of genes differentially regulated by each individual stress treatment.

Table S2: Expression data for genes used in qRT-PCR validation of microarray experiment.

Table S3: Interaction genes identified by comparison of joint stress with individual stress treatment.

Table S4: Lists of interaction genes in highly represented functional groups.

Table S5: Sequences of all primers used in the study. Specific conditions for qRT-PCR.

Figure S1: Functional Gene Ontology categories of interaction genes.

Figure S2: Figure compiled from Genevestigator, showing the expression pattern of candidate genes in response to various hormone treatments.

Figure S3: Comparative phenotypes of additional A) *AtRALFL8*, B) *AtMGL* and C) *AZI1* over-expression lines under control conditions.

Figure S4: Figure compiled from Genevestigator, showing the expression pattern of *RALFL8* genes in different plant tissues.

**Acknowledgements**
Thanks to Hanma Zhang and Stefan Kepinski (University of Leeds) for providing the Arabidopsis hormone signalling lines used in this study.
Literature Cited


Figure Legends

Figure 1. Venn diagrams showing overlap between genes differentially regulated by water stress, nematode stress, or the two in combination. Genes up- and down-regulated in roots (A and B, respectively) and leaves (C and D, respectively) are shown separately. Genes are shown whose expression levels differed significantly from control arrays where \( p < 0.05 \). Overlapping sections represent genes that were up- or down-regulated by more than one stress treatment. Data shown represent three biological replicates.

Figure 2. Relative expression of candidate genes in leaves of plants under differing stress treatments. The expression levels of A) AtRALFL8, B) AtMGL and C) AZI1 are shown relative to the unstressed samples, as analysed by qRT-PCR. RNA was pooled from 40 plants. Error bars represent standard error of the mean of three technical replicates. ND = not detected.

Figure 3. Phenotype of AtRALFL8 over-expression line under control conditions. 35S::AtRALFL8 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. A) Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** \( p < 0.01 \), * \( p < 0.05 \)). B) Photograph shows wild type and transgenic plants 35 days after sowing.

Figure 4. Phenotype of AtMGL mutant and over-expression lines under control conditions. Mutant atmgl and 35S::AtMGL plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. A) Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** \( p < 0.01 \), * \( p < 0.05 \)). B) Photographs show wild type and mutant/over-expressing plants 35 days after sowing.

Figure 5. Phenotype of AZI1 mutant and over-expression lines under control conditions. Mutant azi1 and 35S::AZI1 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. A) Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** \( p < 0.01 \), * \( p < 0.05 \)). B) Photographs show wild type and mutant/over-expressing plants 35 days after sowing.
Figure 6. Survival rate of mutant and over-expression lines after drought stress. Irrigation was withheld from twelve 21-day old seedlings of each genotype until soil moisture content dropped to < 3% (approximately 2 weeks). Plants were re-watered and scored for survival after a further week. A) Wild type (white bars) and 35S::AtRALFL8 plants (red bars) one week after re-watering. B) Survival rate of each genotype compared to wild type plants (shown as 100%). Asterisks show a significant difference from the wild type (** p < 0.01, * p < 0.05) according to Chi² tests.

Figure 7. Nematode susceptibility assays. Mutant and over-expression lines for each candidate gene were exposed to 100 juvenile H. schachtii nematodes per plant. Nematodes were allowed to develop for 10 days and then roots were stained and the number of established 3rd and 4th stage juvenile nematodes (J3 and J4) counted per plant. The mean number of nematodes per plant for each genotype is expressed as a percentage of the wild type value (100%). Asterisks show a significant difference from the wild type (n=10-12) (* p < 0.05).

Figure 8. Expression levels of candidate genes in hormone signalling mutants. The relative transcript abundance of candidate genes was analysed in Arabidopsis hormone signalling mutants using qRT-PCR. A) AtRALFL8, B) AtMGL, C) AZI1. Values are the average of 3 biological replicates of 4 plants each. Asterisks show significant differences in candidate gene expression level compared to the wild type value (** p < 0.01, * p < 0.05).

Figure 9. Root hair phenotype of 35S::AtRALFL8 plants. Wild type (A, C) and 35S::AtRALFL8 seedlings (B, D) were grown on half strength MS media and photographed after 4 days of growth. Long, dense root hairs are clearly visible in the over-expression line. Bars represent 2.5 mm (A, B) and 500 μm (C, D).

Figure 10. Reduced meristem size in 35S::AtRALFL8 roots. Cortical meristematic cells between the quiescent centre (white arrows) and the transition zone (yellow arrow) were counted in (A) wild type and (B) 35S::AtRALFL8 plants 10 days postgermination. Red indicates cell walls as stained by propidium iodide. (C) The meristem was significantly shorter in the 35S::AtRALFL8 plants (n = 5) (** p < 0.01).

Figure 11. Effect of auxin on root length in 35S::AtRALFL8 over-expression line. Wild type and 35S::AtRALFL8 seedlings were grown on different media and the root length measured after 7 days (n = 8). IAA is a natural auxin, 2,4-D is a synthetic auxin and PEO-IAA is an anti-auxin agent.
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