Molecular analysis of menadione-induced resistance against biotic stress in Arabidopsis

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Abstract

Menadione sodium bisulphite (MSB) is a water-soluble derivative of Vitamin K_3, or menadione, and has been previously demonstrated to function as a plant defence activator against several pathogens in several plant species. However, there are no reports of the role of this vitamin in the induction of resistance in the plant model *Arabidopsis thaliana*. In the current study, we demonstrate that MSB induces resistance by priming in Arabidopsis against the virulent strain *Pseudomonas syringae pv. tomato DC3000 (Pto)* without inducing necrosis or visible damage. Changes in gene expression in response to 0.2 mM MSB were analysed in Arabidopsis at 3, 6 and 24 hours post-treatment (hpt) using microarray technology. In general, the treatment with MSB does not correlate with other publicly available data, thus MSB produces a unique molecular footprint. We observed 158 differentially regulated genes among all the possible trends. More up-regulated genes are included in categories such as "Response to stress" than the background, and the behaviour of these genes in different treatments confirms their role in response to biotic and abiotic stress. In addition, there is an over-representation of the G-box in their promoters. Some

interesting functions are represented among the individual up-regulated genes, such as glutathione S-transferases, transcription factors (including putative regulators of the G-box) and cytochrome P450s. This work provides a wide insight into the molecular cues underlying the effect of MSB as a plant resistance inducer.

Keywords

Arabidopsis, menadione sodium bisulphite, induced resistance, priming, Pseudomonas

Introduction

Menadione sodium bisulphite (MSB) is a water-soluble addition compound of vitamin K_3, or pro-vitamin K. In addition, menadione is a redox-active compound that is often used in the study of oxidant stress in plant (Sun *et al.,* 1999), mammal (Shi *et al.,*, 1996), fungal (Emri et al. 1999) and bacterial (Mongkolsuk *et al.,* 1998) cells, and it is a reactive oxygen species (ROS) generator, readily undergoing cell-mediated one-electron reduction, producing superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Hassan and Fridovich 1979).

Vitamin K-like compounds are widely distributed in plants, but their role and function are still partially unknown. The most studied of such compounds, vitamin K_1 or phylloquinone, is largely present in thylakoid membranes as an electron carrier within the photosystem I redox chain. Several studies suggest the involvement of vitamin K in the transport chain transferring electrons across the plasma membranes, and the possibility that this molecule contributes to the maintenance of a proper oxidation state of some important proteins embedded in the cell membrane. Phylloquinone is a metabolite of the shikimate pathway. This pathway is widely used by plants and bacteria but not by animals, which, for this very reason, must obtain some compounds including vitamin K through their diet. The physiological function of vitamin K in plants is directly linked to its redox properties deriving from the presence of a double quinonic function on the naphthalenic ring. In fact, similarly to many other quinones and naphthoquinones, vitamin K can be reduced and reoxidised in a cyclical manner by several substances and enzymatic pools.

MSB was first studied as a plant growth regulator (Rama-Rao *et al.,* 1985). The application of MSB induced higher levels, about 3 to 4-fold, of free indole-3-acetic acid

(IAA) in tomato, cucumber, capsicum and maize, and the detected increase in tomato fruit yield was correlated with the observed higher level of free IAA (Rama-Rao *et al.,* 1985). MSB has also been shown to induce resistance against Panama disease, a vascular wilt disease of banana, caused by *Fusarium oxysporum* f.sp. cubense (Borges et al. 2003a; Borges *et al.,* 2004) and to induce resistance, both locally and systemically, in oilseed rape plants (Borges *et al.,* 2003b; Liu *et al.,* 2006; ShengYi *et al.,* 2007), to infection by A-type *Leptosphaeria maculans*, a necrotrophic fungal pathogen causing phoma stem canker (West *et al.,* 2001). Another study showed MSB-induced resistance against downy mildew in pearl millet (Pushpalatha *et al.,* 2007). Currently, practical applications of MSB in agriculture have been patented (Borges-Pérez and Fernández-Falcón, 1995; Borges-Pérez and Fernández-Falcón, 1996) and several MSB-based formulations have been commercially developed as plant defence elicitors.

On the other hand, using a differential display approach in olive leaves, menadione was found to induce the expression of several genes that were also induced by H_2O_2 (Benitez *et al.,* 2005), some of which were also induced by salicylic acid (SA). In many instances, the initial defensive response in a plant–pathogen interaction activates a signalling process that renders the plant not only locally, but also systemically, more resistant to subsequent infections by a broad spectrum of pathogens. This response is known as systemic acquired resistance (SAR), which is associated with the activation of many plant genes. It has been reported that SA is a key modulator of SAR. Although the specific role of SA as a systemic signal remains unclear, its accumulation in local and systemic (uninoculated) tissues is correlated with the coordinated expression of a specific subset of defence genes (Dong 2001). Finally, menadione was also found to induce tolerance to chilling stress in maize seedlings, suggesting that exogenous application of

menadione and H_2O_2 to the seedlings might induce a mild oxidative stress leading to chilling tolerance (Prasad *et al.,* 1994). Furthermore, two recent works have used menadione as an oxidant inducer to study the metabolic response of heterotrophic Arabidopsis cells (Baxter et al., 2007) and roots (Lehmann et al., 2008) to oxidative stress.

In order to study the effect of MSB in *Arabidopsis thaliana* against biotic stress we performed bacterial growth curves using the hemibiotrophic pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pto*) at different MSB doses and times for treatment prior to inoculation. Several MSB concentrations and times prior to inoculation were assayed, the optimal conditions for induced resistance being obtained with 0.2 mM MSB 24 h prior to inoculation.

Molecular and physiological changes induced by MSB in plants are still unknown. The analysis of such molecular changes in plants is necessary to understand their effect on metabolism and physiological functions. Herein we report on a molecular analysis of the transcriptional changes in Arabidopsis in response to MSB using the whole genome microarray. Furthermore, we demonstrate that MSB acts by inducing a primed state of the plant making it more resistant to *Pto*. The results are discussed in the context of the diverse biological effects under MSB treatment.

Results

MSB-induced defence response in Arabidopsis

MSB has been reported to function as a plant growth regulator and as a plant defence activator. However, the molecular mechanisms underlying the effects of MSB on plants remain largely unknown. The choice model for unravelling molecular mechanisms in plants

is *Arabidopsis thaliana* (Arabidopsis). Arabidopsis has several major advantages over other plant species for genetic and molecular studies. It is a small, rapid cycling, self-fertilizing member of the Brassicaceae family. Most significantly, it has a small genome (130 Mb) which has been completely sequenced (The Arabidopsis Genome Initiative 2000). This plant also serves as a good genetic and molecular model for the study of plant-pathogen interactions (Gonzalez *et al.,* 2006).

Experiments performed in Arabidopsis to show whether MSB was capable of inducing resistance led to Arabidopsis as a good system for the unveiling of molecular mechanisms underpinning MSB effects. As a pathogen, we used another model, the Gram-negative bacterium *Pseudomonas syringae pv. tomato* DC3000 (*Pto*). There is an extensive literature on the relationship between these two organisms (Katagiri *et al.,* 2002). We assayed eight MSB concentrations from 20 mM to 0.1 mM in Arabidopsis (ecotype Col-0) and found that the concentration which provides protection against *Pto* by foliar spraying application is 0.2 mM (Figure 1A), a MSB dose that has worked well inducing resistance against other pathogens such as *L. maculans* (Borges *et al*., 2003b) and *F. oxysporum* (Borges *et al*., 2004). We also optimized the best time for MSB treatment prior to inoculation (Figure 1B). Clearly, 24 hours before inoculation is the most appropriate time to obtain the desired effects. Moreover, from the treatments subsequently performed, we inferred that MSB has no direct effect against *Pto*. To further confirm this result, we also assayed a possible direct effect of MSB *in vitro* by adding it to LB medium ranging from 0.2 to 1 mM and found that MSB is not toxic to *Pto* at these concentrations (data not shown). Hence, we concluded that Arabidopsis is a good model for the study of the molecular mechanisms underlying the effect of MSB, and that this chemical acts by inducing resistance in Arabidopsis.

Even if MSB is not toxic to the bacteria *per se*, it could trigger a known defence mechanism in the plant. MSB-treated Arabidopsis plants were 3,3'-diaminobenzidine (DAB) stained in order to study ROS induced by treatment and/or pathogen challenge. As shown in Figure 2A, MSB does not induce ROS *per se*. However, after challenge with *Pto,* MSB-treated plants appeared to show ROS spreading, whereas water-treated (mock) plants only showed localized ROS. Another mechanism that could explain the resistance induced by MSB is SAR (reviewed by Durrant *et al.,* 2004). However, two characteristics of SAR do not fit with the resistance induced by MSB. First, SAR requires at least 48 hours between the first and the second challenge in Arabidopsis to be deployed (Cameron *et al*., 1994). In the case of MSB, 24 hours is the best timing (Figure 1B). Second, SAR requires cell death to be triggered. Arabidopsis MSB-treated plants were also trypan blue stained in order to study cell death induced by treatment pre or post-*Pto* inoculation. MSB does not appear to induce cell death prior to inoculation that could act as a trigger of SAR. On the contrary, after bacterial inoculation, cell death in mock-inoculated leaves is more intense than in MSB-inoculated leaves (Figure 2B). Therefore, the effect does not fit into the generation of ROS or SAR *in planta*.

MSB induces resistance by priming

Upon different treatments, many plants acquire an enhanced capacity for activating defence responses to biotic and abiotic stress, a process called priming (Beckers and Conrath, 2007). Therefore, a plausible hypothesis is that MSB acts by inducing a primed state of the plant. To test this possibility we performed a western blot analysis of the known SA signalling pathway marker PR1 (Dong, 2001) and found that MSB does not induce PR1 in the absence of bacterial challenge. However, three days after inoculation MSB pretreated

plants produce an increment in PR1 protein of over two fold as compared to mock plants (Figure 2C). This result demonstrates that MSB is inducing resistance against *Pto* by priming.

Microarray analysis: MSB produces a unique molecular footprint

Given the oxidant nature of menadione effects, more information can be obtained performing large-scale gene expression studies in order to understand overlapping functions and signalling networks. We used the ATH1 Genome Array and microarray hybridization for direct quantitative measurements of changes in several thousand transcripts simultaneously. The ATH1 array includes 22,500 probe sets representing approximately 24,000 Arabidopsis gene sequences, and thus can provide genome-scale transcript changes in response to MSB treatment.

To assess the effect of MSB on cellular transcriptional response, Arabidopsis seedlings were treated with 0.2 mM MSB or mock treated, and time-course microarray hybridizations were performed at 3, 6 and 24 hours post-treatment (hpt). Total RNA from the aerial part of the control and treated plants was labelled, and hybridized to Arabidopsis ATH1 microarrays. Based on the hybridization data, MSB up-regulated and MSB down-regulated transcripts were identified as described in 'Experimental procedures'. While ATH1 arrays are widely used for their reliability, it is standard among the scientific community to validate the most relevant genes by real-time RT-PCR. The results of such validation from three independent biological replicates are shown in Figure 3. MSB-induced genes exhibited approximately 1.4-6.7-fold-changes in expression in their expression levels using the microarray analysis (Table 1) whereas the fold-change in expression levels quantified using real-time PCR for selected genes were much higher than

for the microarray data (Figure 3). Hence, it can be concluded that the information provided by the microarray is correct. Table 1 lists selected MSB-upregulated genes with corresponding annotations. On the other hand, the greater part of the menadione-induced genes we found were also up-regulated after menadione treatment in other publicly microarray data in Arabidopsis despite the fact that these were performed in response to heterotrophic Arabidopsis cells or in root tissues (Baxter et al., 2007; Lehmann et al., 2008, respectively).

As an initial approach, we sought to determine whether the effect of the MSB was similar to that of other chemicals already used in Arabidopsis, or in other words, determine whether the information obtained from the microarray analysis could be explained as the combination of known treatments (e.g. SA, ethylene, jasmonic acid (JA), etc). For this purpose, the average value of expression of each time-point vs. treatment was calculated. Then, this information was uploaded in the program "SampleAngler" (www.bar.utoronto.ca) which compares the molecular profile of one treatment against the profiles present in the public databases. There are no treatments in this database showing correlation with those produced by MSB treatment. The highest correlation found was similar to the one obtained with the mock control (data not shown). Therefore, from the point of view of its molecular profile, treatment with MSB produces a new molecular footprint.

Gene ontology: group of MSB- regulated genes

158 differentially expressed genes were identified including up-regulated, down-regulated or up- and down-regulated genes in response to MSB along the different time-points assayed. Since a mere list does not convey all of the potential information, a customized

cluster of these differentially expressed genes is shown in Figure 4. In addition, this figure shows the trend line for each cluster. The complete information is provided in Table 1 and as Supplementary Material in Table S2. Among the 158 differentially expressed genes, 4 genes were up-regulated only at 3 hpt and 6 genes only at 6 hpt, 56 genes were up-regulated at 3 and 6 hpt, and 39 genes were up-regulated at the three time-points assayed. In addition, only 3 genes were down-regulated only at 6 htp, 6 genes only at 6 hpt, 11 genes only at 24 hpt and 20 genes were up- and down-regulated.

Next, we generated a list of up-and down-regulated genes at each time point. The possibility that the number of genes could be insufficient for some analyses (such as ontology of motifs in *cis*, see below) prompted the creation of two additional lists: genes up-regulated at any time point, and down-regulated at any time point. The analysis of these lists provides a robust frame, since they are not dependent on any given gene, but on the group.

The first analysis of the lists was carried out to check the ontology, revealing whether any category is over-represented in the list of genes provided. An overview of the functional classification of the genes up-regulated (Figure 5A) or down-regulated (Figure 5B) by MSB at any time point was performed using the program "Classification SuperViewer" (see Experimental procedures). A class score for normalization was calculated based on the data of the up- or down-regulated genes versus the number of genes in each class present on the chip. These values are represented in the X axis. The input sets were bootstrapped one hundred times and the standard deviation from the bootstrap scores generated is displayed along with the normalized class score (Provart and Zhu, 2003). It is immediately clear that in the list of up-regulated genes there is an over-abundance of genes in the categories of "Response to abiotic or biotic stimuli" and "Response to stress". These

two categories do not only correspond to the role of MSB as an inducer of defence shown in Figure 1, but to published role of protection by menadione against chilling-induced oxidative stress in maize seedlings (Prasad *et al.,* 1994). The remaining categories are similarly represented in both gene lists.

Biclustering analysis

While the MSB treatments as a whole do not correlate with any known chemical treatment as mentioned above, the up-regulated genes have a strong component of response to stimuli or stress. Therefore, these genes should behave as such in other treatments or situations. To analyze this point, a biclustering was performed with the lists of genes. The biclustering tool groups genes that show similar responses to specific conditions. One of the most important ideas implied by this type of clustering is that a shared regulatory element (or elements) may be acting on a subset of genes. Figure 6 shows the most informative part of the analysis of the up-regulated genes at 3 hpt. In this graph, every gene is considered to be up-regulated (light red) or down-regulated (light blue) based on its own microarray data for each specific condition; the columns represent the genes and the rows are the different conditions or treatments. It is clear that the conditions in which most of these genes are co-regulated are biotic (pathogens and molecules from pathogens) and abiotic (salt, ROS elicitors, $AgNO_3$, etc.) stress. The complete figures for each time point can be found as Supplementary material figures S1A, S1B and S1C. Results from genes listed as down-regulated were considered poor (only 2 genes were biclustered).

Promoter analysis of cis-elements

The genes selected in the above-mentioned lists are co-regulated, at least in their response to MSB. It is thus likely that they share common regulatory controls and hence common sets of *cis*-elements in their promoters (e.g. Maleck *et al.,* 2000). Therefore, the next analysis carried out with these gene lists was to look for those motifs. To do so, we obtained the up-stream regions of the genes represented on the ATH1 GeneChip (TAIR), and then looked for motifs that were over-represented in each list. We first identified the plant *cis*-elements already described. However, since the motifs that regulate the response to MSB could be different from those previously published, we used two programs to identify these potential motifs. The "BioProspector" program searches for motifs of a given size, starting from the background of all the genes present in the microarray (see Experimental procedures). On the other hand, "AlignACE" is a program that does not require the background, and the motifs proposed are of a variety of sizes. These two programs were used in the lists of genes generated (Supplementary material Table S4) and, together with the plant *cis*-elements database, produced a total of 225 elements. We then re-analyzed the output by bootstrap analysis (see below) to select the most strikingly enriched promoter motifs (see Experimental procedures). Table 2 shows the results of this search, listing the motifs found with a probability $p<1x10^{-6}$ of being randomly enriched. Since the G-box (Menkens *et al.,* 1995) enriched in the up-regulated genes was immediately clear, the motifs in Table 2 which contain the core of the G-box are underlined.

The bootstrap analysis was performed for each motif in each list to ensure thoroughness and, at the same time, to detect over- and under-representation. In short, if the list of genes up-regulated by MSB has 89 genes, we created a hundred thousand different bootstrap-generated clusters from those 89 genes and then counted the number of times that

a particular motif was found. We also produced a hundred thousand clusters generated from the same number of genes picked randomly from the background of the whole microarray (see Experimental procedures). We then compared the values derived from the list with the values derived from the background using a Student's statistical distribution. To illustrate the distribution of motifs following bootstrapping, we plotted the results for two selected putative *cis*-elements, the G-box (Supplementary material Figure S2a) and the ABRE-like binding site (Simpson *et al.,* 2003; Supplementary material Figure S2b).

The number of times that a motif was found per bootstrap-generated cluster is plotted on the X-axis, while the Y-axis represents the number of bootstrap-generated clusters having a given occurrence of that motif (see Experimental procedures). This method greatly simplified the visualisation of the differences in frequency for a list vs. background for any putative *cis*-element.

As the motifs in Table 2 show, we not only found the core of the G-box, but several variations thereof. It is possible that the genes up-regulated by MSB that contain the G-box have additional information surrounding the core. To resolve the issue, all the G-boxes in the promoters of the genes in the chip were compiled (3574, see Experimental procedures) and the program “WebLogo” was used to represent the context around the core of the box. The result (Supplementary Material Figure S2c) revealed a small amount of information at positions -1, 6 and 8 with respect to the core. When the same analysis was performed in the list of genes up-regulated by MSB (37, Supplementary Material Figure S2d), the relative importance of nucleotides -1 and 8 increased, nucleotide 6 was found to be irrelevant, and positions 7 and 9 afforded some information. While the differences are small, the consensus presented is a starting point for further experiments.

The last gene list analysis was performed to search for the physical clustering of co-regulated genes or RIDGE (Regions of Increased Gene Expression; Caron *et al.*,, 2001), though none were found (data not shown).

Individual genes affected by MSB

Once we extracted all possible information from the gene pool making up the gene lists, we focused on the next level of information, that is, the individual genes that were up- or down-regulated by MSB treatment. In this work and using whole genome Arabidopsis ATH1 arrays, MSB treatment was observed to increase the transcript level of several defence-related genes. The identification of these and other genes (see below) supports the role of MSB in plant defence response. Among them, we selected several genes that play important roles as resistance genes. The most highly induced gene in our work (At3g28740), encoded for a cytochrome P450 (Table 1), which belongs to a superfamily of genes involved in the responses to abiotic and biotic stresses in Arabidopsis (Narusaka *et al.,* 2004). MSB strongly up-regulated several of these genes, mainly *CYP81D11* (At3g28740; Figure 3) and *CYP81D8* (At4g37370), which are also induced by the necrotrophic pathogen *Alternaria brassicicola*, SA, JA, abscisic acid (ABA) and by abiotic stress such as high salinity (Narusaka *et al.,* 2004). MSB also increased the transcript level of several glucosyltransferase genes involved in resistance to the virulent strain of the bacterial pathogen *Pto* (Langlois-Meurinne *et al.,* 2005). Members of group D of this large multigenic family displayed distinct induction profiles, indicating potential roles in stress or defence responses notably for *UGT73B3* (At4g34131) and *UGT73B5* (At2g15480) the latter being strongly induced by MSB in the present study (Figure 3).

MSB also up-regulated a gene (At1g28480; Figure 3) encoding for the GRX480 protein, a member of the glutaredoxin family that regulates protein redox state. GRX480 interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Besides, GRX480 transcription is SA-inducible and requires *NPR1* and may be involved in SA/JA cross-talk (Ndamukong *et al.,* 2007). Thus, MSB treatment was observed to increase the transcript level of several pathogen-induced genes, including two resistance (R) genes (At1g72900; see Figure 3 and At1g66090) with nucleotide-binding site leucine-rich repeats (NBS-LRRs) and toll-related immune receptors (TIR).

As already mentioned, MSB is a redox-active compound that is often used in the study of oxidative stress in plants. We identified several cellular detoxification genes involved in oxidative stress. MSB induced several glutathione-S-transferases (GSTs) and ABC transporters that may scavenge toxic compounds generated during oxidative stress. Indeed, one of the most highly induced genes in the present study (At1g17170; Table 1 and Figure 3) encoded for a GST and was also highly induced by H_2O_2 (Vanderauwera *et al*., 2005) and nitric oxide (NO) treatments (Parani *et al.,* 2004).

MSB up-regulated several TFs that moderate plant defence responses. Of particular interest are several genes which encode C2H2 and C3HC4-type zinc finger proteins. C2H2-type TFs have been shown to be involved in dehydration and high-salt response such as the DREB2A (At5g05410; Table 1) transcriptional activator (Sakuma *et al.,* 2006) as well as a JA-inducible transcription factor (Wang *et al.,* 2008). Among the C3HC4-type genes, we found an increased transcript level for one (At5g59820; Figure 3) encoding for a zinc finger protein of special interest named Zat12, which plays a central role in abiotic stress such as high-intensity light, cold acclimation, and in response to oxidative stress and hyperosmotic salinity through a negative regulatory circuit of the CBF pathway (Davletova *et al.,* 2005).

Other genes of particular importance which moderate plant defence responses are WRKY TFs (Xu *et al.,* 2006), which bind to the W-box present in the promoters of many plant defence genes. In our microarray we observed a 2.5-fold induction in the expression of AtWRKY40 in response to MSB treatment at 3 hpt, with this transcription level gradually decreasing at 6 and 24 hpt (Table 1). AtWRKY18 is known to be a positive regulator of basal defence and systemic acquired resistance (SAR) operating downstream from *NPR1*. However, recent protein-protein studies suggest that a complex interplay between AtWRKY18 and two structurally-related family members, AtWRKY40 and AtWRKY60, modulates their function (Xu *et al.,* 2006). The other class of defence-related TFs induced by MSB was ethylene-responsive element-binding proteins (EREBPs). McGrath *et al.* (2005) studied several members of this family of TFs and among them, *TDR1*(At3g23230) and *ATERF-1*(At4g17500), whose transcript levels in real-time PCR analysis were increased after MeJA application and *Alternaria brassicicola* inoculation. These genes were also MSB-induced in our microarray.

MSB treatment also induced transcripts coding for calcium ion binding proteins such as calmodulin and calcium-transport ATPase. Calcium signalling is one of the best documented pathways in plants; it has been demonstrated to be operative in a series of biological processes from cell division to plant responses to a wide range of stimuli including hormones, light, pathogen elicitors and abiotic stresses (Reddy 2001). MSB treatment increased the transcript level at 6 hpt 3.2-fold (Table 1 and Figure 3) over untreated control plants of one of these calcium-transport ATPase (At3g01830), a P-type Ca^{2+} pump (ACA12), which is induced in response to salt stress (Maathuis, 2006).

Finally, MSB enhanced the transcript level of several defence-related TFs that bind specifically to the *cis*-element CACGTG of the G-box. Among the TFs worth mentioning

are the genes which encode C2H2-type zinc finger proteins, AP2 domain-containing TFs such as *F26K10_20,* which encodes a member of the DREB A-6 subfamily of ERF/AP2 transcription factor family, *TDR1* (At3g23230) another ethylene responsive factor or *ZAT11* (At3g53600) involved in abiotic stress, among others (Table 1). Therefore, we have found several candidates that bind to the G-box, and they are present at the same time and place as the induction of the target genes. Further work is needed to pinpoint the gene or genes responsible for this response.

Discussion

The present work clearly shows that MSB modulates the expression of an important number of genes at a transcriptional level. While treatment with MSB does not resemble other treatments available in the database, the genes induced clearly establish a link to treatments in response to stress.

MSB-induced resistance against the virulent strain of the hemibiotrophic pathogen *Pto* under the experimental conditions assayed is modest. Indeed, we found a bell dose-response curve in all the experiments. We hypothesize that this peculiar behaviour reflects the pro-oxidant nature of MSB that at low dose induces a plant response that is weak or insufficient to induce a defence response against *Pto* but at higher although non-lethal concentrations is capable of inducing resistance by priming without inducing necrosis or visible damage. The fact that the same dose of MSB (0.2 mM) had been used to induce resistance against other pathogens (Borges et al. 2003b; Borges *et al.,* 2004; Liu *et al.,* 2006; ShengYi *et al.,* 2007) appears to support this hypothesis.

Another interesting finding is the mode of action of MSB. In plants, biotic and abiotic stresses can trigger the generation of ROS, such as superoxide and hydrogen

peroxide. As previously mentioned, MSB is a ROS generator (Hassan and Fridovich, 1979), which may be a trivial explanation of the mechanism. Previous studies in oilseed rape plants (*Brassica napus* cv Bristol) treated with MSB 24 h prior to inoculation with *L. maculans* showed that, after staining with aniline blue in lactophenol, rings of necrotic mesophyll cells surrounded the invasive hyphae of *L. maculans*, whilst at infection sites in water pre-treated control plants, unhindered *L. maculans* hyphal growth was observed, with no visible host reaction (Liu *et al.,* 2007). In the present study staining assays in Arabidopsis did not fit into the generation of ROS or SAR *in planta* (Figure 2A, 2B). Another possibility is that MSB is inducing resistance by priming. The primed state can be induced by the colonization of plant roots by beneficial micro-organisms or by treatment of plants with various natural and synthetic compounds (Beckers and Conrath, 2007). It has been suggested that sustained alterations in levels of key signalling metabolites or transcription factors (TFs) may provide an explanation of plant metabolism altered by exposure to various stresses leading to priming. Alternatively, epigenetic changes could play a role by enabling long-term changes in gene expression (Bruce *et al.*,, 2007). The result of the PR1 western blot clearly demonstrates that MSB acts by inducing a primed state of the plant rendering it more resistant to the pathogen (Figure 2C). The large number of defence-related TFs induced by MSB in our microarray (see Table 1) supports the accumulation of these gene transcriptional activators as a possible mechanism to explain this type of induced resistance.

Although the molecular footprint of the MSB does not resemble other stresses, the genes up-regulated in the MSB treated plants have a close connection with other stresses. Plant growth is greatly affected by a combination of environmental stresses such as extreme temperatures, drought, or high salinity. From an agricultural point of view, such stresses are

among the most significant factors responsible for substantial and unpredictable losses in crop production. In addition, due to global climate change, drought and salinity are increasing problems for agriculture and ecosystems. The resulting abiotic stress is the primary cause of crop loss worldwide and reduces average yields for most crop plants by more than 50% (Bray *et al.,* 2000). Interestingly, MSB increases the transcripts of several abiotic stress-related genes (Table 1).

Interestingly, the promoter analysis of *cis*-elements clearly reveals that most of the genes up-regulated by the MSB contain the G-box in their promoter regions (Table 2). Strikingly, there is an important role for the G-box in the response and the candidates which bind to these G-boxes are also identified. The G-box is a ubiquitous element, and it has been proposed that it functions in concert with neighbouring *cis*-elements in regulating gene expression related to different functions, including pathogen attack (Kim *et al.,* 1992; Menkens *et al.,* 1995). The G-box element was first identified as a highly conserved protein binding site upstream of many genes, encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) (Giuliano *et al.,* 1988). The CACGTG motif (G-box) is a highly conserved DNA sequence that has been identified in the 5' upstream region of plant genes exhibiting regulation by a variety of environmental signals and physiological cues (Williams *et al.,* 1992). Recently, two evolutionarily conserved Arabidopsis protein kinases have been found, KIN10/At3g01090 and KIN11/At3g29160, with pivotal roles in linking stress, sugar and developmental signals to globally regulate plant metabolism, energy balance, growth and survival (Baena-González *et al.,* 2007). To identify critical DNA sequences involved in stress and KIN10-mediated responses, the authors performed systematic mutagenesis of predicted *cis*-regulatory elements and found that a specific mutation of the G-box (CACGTG) proximal to the TATA box abolished most of the stress

responses activated by this gene such as hypoxia, darkness or herbicide. They concluded that a common *cis*-element mediates convergence of diverse signals, most probably through KIN10 (Baena-González *et al.,* 2007). Similarly, Pozo *et al.,* (2008) studied the molecular mechanisms of the rhizobacteria-induced priming response using a whole-genome transcript profiling approach in Arabidopsis. Promoter analysis of induced systemic resistance (ISR)-primed, methyljasmonate (MeJA)-responsive genes and ISR-primed, *Pto*-responsive genes revealed the over-representation of another G-box-like motif, CACATG. This motif is a binding site for the transcription factor MYC2, which plays a central role in JA- and abscisic acid-regulated signalling. In general, the proteins that bind to the G-box are GBF factors from the bZIP superfamily. These TFs have been characterized in tobacco, soybean, and Arabidopsis (Singh *et al.,* 2002).

Here we show a molecule, MSB, which protects plants through priming of existing defence mechanisms avoiding unnecessary allocation and energy costs and manipulation of the genome. Primed plants do not require costly defence investments (Heil, 2002). Collected data will provide a better understanding of the defence pathways and other physiological and metabolic effects underlying MSB treatment. Current research is under way to unveil the role of some of the genes shown here in MSB-treated plants, as well as their transcriptional regulation.

Experimental procedures

Inoculation and plant treatment

Arabidopsis thaliana (ecotype Col-0) plants were grown under a short-day regime (8 hours of light at 21 °C, 16 hours of darkness at 19 °C). A stock of 6000 ppm (20 mM), light-

sensitive MSB (M5750, Sigma-Aldrich-Aldrich, St. Louis, MO, USA) was prepared in water for each treatment and kept in the dark. A dilution in distilled water was applied one day before the bacterial inoculation at 60 ppm (0.2 mM) unless otherwise stated. When indicated, a mock inoculation of distilled water was performed. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) containing pVSP61 (empty vector) were maintained essentially as described (Ritter and Dangl 1996). The bacteria were grown, inoculated and measured as described (Tornero and Dangl, 2001) with minor changes. For both the growth curves and RNA extraction, three independent treatments were performed (three independent sets of plants sowed and treated on different dates). The MSB or mock treatments were applied on the 17th day after transferring the plants to the growing chamber, and the bacteria on the 18th day. The treatments started 30 minutes after the initiation of the artificial day to ensure reproducibility. Samples (c. 1 g of fresh weight) were taken at the indicated times after the MSB or mock treatments and the tissue was frozen in liquid nitrogen and stored at –80 °C. The staining was performed as described (Tornero *et al.,* 2002).

RNA isolation and microarray hybridization

Total RNA was isolated with "Trizol" (Invitrogen, San Diego, CA, USA) following the manufacturer's recommendation. Following extraction, an additional step of column purification with "RNeasy Mini Kit" (Qiagen, Valencia, CA, USA) was added to ensure the purity of the RNA. Array hybridization to an Arabidopsis GeneChip ATH1 (Affymetrix, Santa Clara, CA, USA) was performed following the manufacturer's recommendation. The hybridization was carried out in the "Sección de Chips de DNA-S.C.S.I.E.", University of Valencia (Valencia, Spain).

Western blot analysis

Proteins from Arabidopsis were extracted from grinded 18 days-old plants using TBS buffer (10mM Tris pH 7.4, 150 mM NaCl,) plus 1X protease inhibitor cocktail (P9599, SIGMA, St. Louis, MO, USA). Crude extract was centrifuged and protein content of the supernatant was quantified (Bradford 1976). Equal amount of protein was electrophoresed on a 12% SDS-PAGE. Proteins were then transferred to a PVDF membrane (GE Healthcare, Little Chalfont, UK). Equal amount of transferred protein was verified by Ponceau-S staining. Immunoblot detection was carried out using Amersham ECL Plus Western Blotting Detection Reagents (GE HealthCare, Little Chalfont, UK) with a 1:5000 dilution of polyclonal antibodies against PR1 (Wang *et al.,* 2005) and a 1:25000 dilution of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, USA). Chemiluminescent signal was detected using a LA-3000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, CT, USA).

Reverse transcriptase polymerase chain reaction

Gene-specific primers were synthesized (Invitrogen, San Diego, CA, USA) for 9 selected probe sets plus 2 internal control genes which were used for normalization and RT-PCR was carried out to verify the microarray results. One microgram of purified total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) following the manufacturer's recommendation.

Real-Time RT-PCR

Amplification primers (Supplementary material Table S2) were designed using PRIMER3 software (Rozen and Skaletsky, 2006). In order to control for genomic DNA

contamination, when possible, the primers for real-time PCR were targeted to different exons (Supplementary material Table S2). Real-time amplification reactions were performed using SYBR Green detection chemistry and run in triplicate on 96-well plates with the iCycler iQ5 thermocycler (Bio-Rad, CA, USA). Reactions were prepared in a total volume of 20 µl containing: 4 µl template, 3 µl of each amplification primer (working concentration, 0.4 µM) and 10 µl iQ SYBR Green Supermix (Bio-Rad, CA, USA). Blank controls were run for each master mix. A RT-minus amplification reaction was included for each cDNA sample. The cycling conditions were set as follows: initial denaturation step of 95 ºC for 10 min to activate the Taq DNA polymerase, followed by 45 cycles of denaturation at 95 ºC for 15 s, annealing at 60 ºC for 50 s and extension at 72 ºC for 20 s. The amplification process was followed by a melting curve analysis, ranging from 60 °C to 90 °C, with temperature increasing steps of 0.2°C every 10 s. Baseline and crossing points (Cp) were automatically determined using the iQ5 Software 2.0. Threshold levels were set at 200 standard deviations (background fluorescence) above baseline. Expression data were processed following an efficiency-corrected model for relative quantification (Pfaffl, 2001) and normalization with multiple internal controls (Vandesompele *et al.,* 2002) as implemented in the qBase software (Hellemans *et al.,* 2007). The PCR efficiency was determined for each primer pair with the DART-PCR workbook (Peirson *et al.,* 2003), which uses fluorescence data captured during the exponential phase of each amplification reaction. Three genes which showed high expression stability in the microarray expression analyses were tested as potential reference genes using the qBASE software normalization tools. Two of the mentioned three internal control genes were finally selected. A relative expression software tool (REST 2008) (www.genequantification.com) was used to obtain

the corresponding significance level ($P < 0.05$) to each individual gene expression (Pfaffl *et al.*, 2002).

Microarray data analysis

The original hybridization signals were normalized using the web-based resource for microarrays, Gene Expression Profile Analysis Suite (GEPAS, www.gepas.org). Relative expression values for three independent MSB-treated samples were calculated using control treatments as calibrators. Since one of the MSB treatments produced values that differ from those of the other two, it was not taken in account. We later confirmed by real-time RT-PCR that the difference was not due to the microarray but to the RNA itself (data not shown). Normalized relative quantities from two replicas were averaged and the estimation of biological variance was made using a coefficient of variance (CV= standard deviation divided by the mean). Finally, gene expression values were arranged according to the mentioned CV values. Genes were selected on the basis of the upper limit for biological variance compatible with a statistical power higher than 0.95 in the detection of gene expression changes with two biological replicates (see above), using a two-tailed t-distribution as provided by http://www.univie.ac.at/medstat.Genes that fulfilled these requirements, for at least one of the time-points assayed were included in the set of candidate MSB-responsive genes.

From these data, we created lists of up- and down-regulated genes at each time point, and also a list of genes up-or down-regulated at any of the time points. For the subsequent analysis, only non-redundant and non-ambiguous genes were used. In the cases where the genome was taken as a background, we used all the non-redundant and non-ambiguous genes in the microarray.

The original hybridization data files were submitted to the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository and the Accession number E-MEXP-1934 was assigned to this experiment.

Gene ontology and biclustering.

The program "Classification SuperViewer" was used for the gene ontology analysis and the program "Genevestigator V3" was used for the biclustering. Default settings were used, except with the biclustering, where a renderization factor of 2 was used due to computer limitations.

Motif analysis

Putative *cis*-acting motifs present in the promoters were studied as follows. 1 kb of promoter sequence of each gene was downloaded from TAIR (Version 8, www.arabidopsis.org), along with the list of genes present in the microarray. The gene lists were then analyzed with three different programs with their default settings. First, the program "Athena" (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) was used to identify known motifs present in the gene lists. Second, the program "BioProspector" (http://ai.stanford.edu/~xsliu/BioProspector/) defined motifs of a given size (10 nucleotides in the present analysis) that were overrepresented in each list. For this search, the whole genome was previously computed as a background. Third, the program "AlignACE" (http://atlas.med.harvard.edu/cgi-bin/alignace.pl) defined motifs of several sizes found to be overrepresented. Thus, we constructed a dataset of 225 motifs.

This dataset became the input for a bootstrap analysis where all the motifs were evaluated in all the lists. For this evaluation, we generated $1x10^5$ clusters from each list and

$1x10^5$ control sets of equal size randomly chosen from the chip. In each bootstrap, we counted the number of any particular motif found in the cluster and in the control set. We performed a t-test analysis with this information, and kept only the events that occurred with a probability $p< 1x10^{-6}$ of occurring by chance (74 events).

In the case of the G-box, the program "WebLogo" (http://weblogo.berkeley.edu/logo.cgi) was used to represent the context around the core of the box. We compiled all the G-boxes (along with four nucleotides before and four after the core) in 1 kb upstream of each gene in the microarray and in the list of genes. The flow of information to and from BioProspector and AlignACE, and the bootstrapping analysis were accomplished using custom Perl scripts, available upon request.

Supplementary material

The complete lists of up-regulated, down-regulated and up- and-down-regulated genes in response to MSB treatment at different time-points are available as Supplementary material tables and figures:

Table S1 Ranking of gene expression level.

Table S2 List of oligonucleotides used as primers in the quantification of selected genes by real-time RT-PCR.

Table S3 List of co-regulated genes.

Figure S1A Complete biclustering of the up-regulated genes at 3 hpt.

Figure S1B Complete biclustering of the up-regulated genes at 6 hpt.

Figure S1C Complete biclustering of the up-regulated genes at 24 hpt.

Figure S2 Examples of the motif distribution and the context of the G-box.

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Table 1 Microarray analysis. Selected list of genes differentially expressed over control in response to MSB at 0.2 mM.

	3 h		6 h		24 h			
Affymetrix	**Fold**	**CV**	**Fold**	**CV**	**Fold**	**CV**	**AGI code**	**Annotation**
256589_at	**4.75**	0.01	**4.00**	0.12	**3.06**	0.20	At3g28740	Cytochrome P450 CYP81D11; crosstalk in the response to abiotic and biotic stresses
262518_at	**6.68**	0.16	**4.29**	0.20	**2.45**	0.01	At1g17170	Glutathione-S-transferase 24
262911_s_at	**3.03**	0.7	**3.13**	0.15	**2.67**	0.11	At1g59860	Putative Heat shock hsp20 protein
266290_at	**3.22**	0.14	**2.76**	0.12	**1.96**	0.22	At2g29490	Glutathione-S-transferase 19
253268_s_at	**3.51**	0.02	**3.73**	0.34	**1.46**	0.07	At4g34135	Glucosyltransferase -related protein UGT73B2
262118_at	**2.30**	0.03	**3.18**	0.29	**1.71**	0.27	At1g02850	β-Glucosidase; Pseudomonas fluorescents FPT9601-T5a induced more than 2-fold
265499_at	**3.85**	0.01	**2.84**	0.35	**1.46**	0.15	At2g15480	UDP-glucuronosyl/UDP-glucosyl transferase family protein ;UGT73B5
260706_at	**1.29**	0.17	**2.63**	0.83	**0.58**	0.23	At2g29460	Glutathione-S-transferase 22
266267_at	**1.96**	0.4	**3.34**	0.22	**2.04**	0.11	At1g17180	Glutathione-S-transferase 28
262517_at	**2.18**	0.32	**3.06**	0.15	**1.10**	0.16	At5g16980	Quinone oxidoreductase-like protein
256245_at	**1.38**	0.18	**2.70**	0.21	**2.39**	0.03	At4g01870	Unknown protein; NOT induced
255543_at	**2.77**	0.16	**2.21**	0.05	**1.40**	0.09	At1g77450	GRAB1-like protein
246464_at	**3.52**	0.09	**2.08**	0.32	**1.23**	0.26	At3g12580	Response to heat, protein folding, response to biotic and abiotic treatments; cytosol localized
259705_at	**2.76**	0.02	**2.35**	0.00	**1.22**	0.09	At3g46230	Arabidopsis thaliana heat shock protein 17.4
252515_at	**1.50**	0.29	**3.22**	0.55	**2.96**	0.26	AtCg00870	Hypothetical protein
244990_s_at	**1.77**	0.11	**2.29**	0.00	**0.58**	0.06	At1g05575	Expressed protein
264202_at	**4.70**	0.02	**2.32**	0.42	**0.90**	0.19	At5g17000	Quinone oxidoreductase-like protein
246465_at	**2.57**	0.3	**1.61**	0.21	**1.16**	0.03	At4g22530	Embryo-abundant protein
254318_at	**2.56**	0.27	**1.78**	0.14	**1.22**	0.19	At5g12030	Heat shock protein 17.6A
250351_at	**2.59**	0.59	**3.70**	0.52	**3.87**	0.31	At3g23550	Unknown protein
252084_at	**1.22**	0.48	**1.41**	0.21	**0.50**	0.16	At2g17500	Auxin efflux carrier family protein, contains auxin efflux
250054_at	**2.05**	0.23	**2.19**	0.04	**1.30**	0.30	At5g17860	Cation exchanger, putative (CAX7). Calcium: sodium activity
263073_at	**2.05**	0.18	**2.29**	0.14	**1.24**	0.17	At1g78440	Similar to F-box family protein
258100_at	**2.17**	0.13	**2.56**	0.47	**1.28**	0.15	At3g51970	Encodes for a novel sterol O-acyltransferase; wax synthase-like protein
260773_at	**0.52**	0.05	**0.67**	0.15	**1.11**	0.12	At2g19790	Clathrin adaptor complex small chain family protein
266294_at	**2.45**	0.62	**2.64**	0.24	**2.06**	0.22	At5g45380	Sodium: solute symporter family protein
258252_at	**0.74**	0.24	**0.58**	0.26	**0.80**	0.04	At2g46660	CYP78A6 (cytochrome P450, family)
266321_at	**1.16**	0.13	**1.44**	0.17	**0.54**	0.03	At3g15720	Polygalacturonase activity; plant-type cell wall anchored to membrane
253799_at	**0.73**	0.08	**2.08**	0.10	**1.18**	0.18	At4g28140	AP2 domain-containing transcription factor, putative
248970_at	**1.62**	0.03	**2.01**	0.27	**1.77**	0.30	At1g72900	Disease resistance protein (TIR-NBS class), putative
262381_at	**2.41**	0.16	**1.62**	0.27	**1.13**	0.17	At3g26690	Nudix family protein; located in mitochondrion; hydrolase activity
257830_at	**2.07**	0.12	**1.41**	0.10	**1.58**	0.05	At2g37760	Aldo/keto reductase family protein

245277_at	**1.59**	0.1	**2.12**	0.13	**1.17**	0.02	At5g64230	Unknown protein
267181_at	**1.74**	0.05	**2.07**	0.19	**1.29**	0.09	At1g05575	Unknown protein
251950_at	**1.63**	0.28	**1.67**	0.50	**0.57**	0.13	At1g15520	ABC transporter family involved in resistance to lead. Localizes to plasma membrane
261658_at	**1.09**	0.28	**1.15**	0.27	**0.55**	0.14	At5g48430	Dermal glycoprotein precursor, extracellular-like
245369_at	**2.00**	0.25	**1.24**	0.29	**1.37**	0.05	At4g15975	Zinc finger (C3HC4-type RING finger) family protein; transcription factors activity
261763_at	**2.28**	0.18	**3.28**	0.66	**0.97**	0.11	At3g53600	Zinc finger (C2H2 type) family protein; transcription factor activity
259964_at	**1.23**	0.17	**0.95**	0.21	**0.58**	0.27	At1g32350	AOX1D (Alternative oxidase 1D)
248703_at	**2.24**	0.13	**1.47**	0.25	**1.27**	0.04	At1g53680	Glutathione-S-transferase (Class TAU) 28
264518_at	**0.80**	0.12	**0.59**	0.11	**1.36**	0.23	At1g50040	Unknown protein
263182_at	**3.20**	0.08	**2.23**	0.66	**1.03**	0.04	At1g01250	AP2 domain-containing transcription factor, putative; transcription factor TINY-like protein
261059_at	**0.83**	0.04	**1.15**	0.06	**0.56**	0.03	At1g09990	Unknown protein
263273_x_at	**0.59**	0.04	**1.12**	0.24	**0.80**	0.23	At2g38840	Guanylate-binding family protein; GTPase activity
256576_at	**1.63**	0.29	**2.83**	0.68	**2.23**	0.12	At2g23680	Stress-responsive protein; similar to cold acclimation protein WCOR413 [T. aestivum]
267288_at	**2.02**	0.26	**1.52**	0.14	**1.07**	0.03	At3g28210	Zinc ion binding; zinc finger protein; ABF3
266316_at	**1.29**	0.03	**1.64**	0.01	**0.58**	0.36	At2g27080	Harpin-induced protein-related / HIN1-related
248332_at	**2.01**	0.55	**2.53**	0.36	**2.03**	0.23	At2g22880	Unknown molecular functions
266800_at	**2.73**	0.34	**2.96**	0.60	**1.30**	0.28	At5g41080	Glycerophosphoryl diester phosphodiesterase family protein
260178_at	**0.95**	0.3	**0.85**	0.11	**0.57**	0.23	At3g62150	ABC transporter related; ATPase activity
251248_at	**2.20**	0.28	**1.14**	0.30	**1.10**	0.05	At5g05410	ERF/AP2 transcription factor family (DREB2A)
249337_at	**2.32**	0.23	**1.34**	0.23	**1.01**	0.25	At5g10830	Embryonic abundant protein -related embryonic abundant
252938_at	**0.89**	0.23	**0.59**	0.00	**1.03**	0.02	At5g52640	Encodes a cytosolic heat shock protein AtHSP90.1
250955_at	**0.72**	0.21	**0.59**	0.18	**0.98**	0.42	At2g45080	CYCP3;1 (cyclin p3;1); cyclin-dependent protein kinase
254629_at	**1.01**	0.21	**0.72**	0.24	**0.57**	0.31	At2g46915	Unknown protein
255538_at	**0.78**	0.2	**1.08**	0.11	**0.58**	0.07	At4g18425	Unknown protein
250781_at	**2.16**	0.2	**1.65**	0.38	**1.67**	0.09	At4g00160	Similar to F-box family protein; similar to Cyclin-like F-box
266759_at	**1.03**	0.15	**1.16**	0.04	**2.00**	0.30	At1g70720	Invertase/pectin methylesterase inhibitor family protein
266124_at	**1.04**	0.03	**1.06**	0.12	**0.52**	0.21	At4g39190	Unknown protein
250449_at	**2.16**	0.02	**1.46**	0.12	**1.07**	0.13	At4g01680	Encodes a putative transcription factor (MYB55).
255705_at	**0.97**	0	**0.59**	0.11	**1.05**	0.09	At5g03190	Unknown protein
263210_at	**1.53**	0.47	**2.55**	0.49	**1.24**	0.04	At1g05680	UDP-glucuronosyl/UDP-glucosyl transferase family protein
267168_at	**1.74**	0.34	**2.24**	0.22	**1.36**	0.27	At1g68620	Expressed protein similar to PrMC3 [Pinus radiata]
262229_at	**1.86**	0.28	**2.42**	0.24	**1.40**	0.49	At3g11840	U-box domain-containing protein; involved in protein ubiquitination
258787_at	**1.76**	0.28	**2.13**	0.50	**1.38**	0.03	At2g37770	Similar to aldo/keto reductase family protein; oxidoreductase activity
248434_at	**1.70**	0.26	**2.41**	0.39	**1.34**	0.03	At5g51440	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)
263231_at	**4.15**	0.07	**1.91**	0.60	**0.63**	0.33	At1g10585	Transcription factor
265674_at	**2.63**	0.78	**3.21**	0.83	**1.34**	0.11	At5g39550	Zinc finger (C3HC4-type RING finger) family protein

249459_at	**3.24**	0.63	**2.59**	0.48	**1.03**	0.42	At4g37370	Cytochrome P450, CYP81D8; crosstalk in the response to abiotic and biotic stresses
249449_at	**1.31**	0.43	**0.75**	0.03	**0.60**	0.07	At4g00430	Member of the plasma membrane intrinsic protein subfamily PIP1; response to drought
258815_at	**1.48**	0.35	**2.16**	0.17	**1.25**	0.25	At1g79710	Integral membrane transporter family protein
249494_at	**2.06**	0.27	**1.41**	0.23	**1.25**	0.42	At3g01830	Calmodulin-related protein, putative; similar to CML38, calcium ion binding
261344_at	**2.44**	0.1	**1.38**	0.10	**1.24**	0.34	At5g39050	Transferase family protein
258947_at	**2.23**	0.08	**2.55**	1.07	**0.94**	0.03	At3g04000	Short-chain dehydrogenase/reductase (SDR) family protein; oxidoreductase activity
256356_s_at	**1.12**	0.06	**2.00**	0.40	**1.33**	0.25	At5g39430	Unknown protein.
253046_at	**2.65**	0.04	**2.05**	0.69	**0.94**	0.04	At1g66500	Zinc finger (C2H2-type) family protein; similar to S-locus protein-related
253859_at	**1.80**	0.43	**2.21**	0.15	**1.00**	0.13	At1g28480	Encodes GRX480. Maybe involved in SA/JA cross-talk
252882_at	**1.00**	0.32	**2.29**	0.10	**1.81**	0.03	At4g27657	Unknown protein
261443_at	**2.22**	0.23	**1.87**	0.37	**0.99**	0.08	At4g39675	Senescence-associated protein-related
263403_at	**2.66**	0.48	**2.44**	0.67	**1.16**	0.28	At2g04040	AtDTX1 has been identified as a detoxifying efflux carrier for plant-derived antibiotics
265501_at	**2.07**	0.41	**2.13**	0.41	**1.07**	0.03	At5g18470	Curculin-like (mannose-binding) lectin family protein
247717_at	**0.82**	0.32	**0.59**	0.25	**0.99**	0.11	At2g15490	UDP-glucuronosyl/UDP-glucosyl transferase family
259439_at	**1.57**	0.3	**1.59**	0.66	**0.58**	0.10	At1g01480	1-aminocyclopropane-1-carboxylate synthase 2 / ACC synthase 2 (ACS2)
249174_at	**0.93**	0.2	**1.05**	0.05	**0.57**	0.46	At1g66160	U-box domain-containing protein; involved in protein ubiquitation
266754_at	**0.60**	0.18	**0.83**	0.23	**0.84**	0.29	At3g09730	Unknown protein
258702_at	**0.96**	0.13	**0.60**	0.17	**0.93**	0.06	At5g42900	Unknown protein
249983_at	**2.24**	0.12	**2.21**	0.62	**1.47**	0.11	At3g49310	Unknown protein
256522_at	**1.46**	0.01	**2.06**	0.31	**0.95**	0.11	At5g59320	Lipid transfer protein 3, LTP3, MNC17.10, MNC17_10 ; involved in response to ABA
247814_at	**0.82**	0.01	**0.61**	0.24	**0.90**	0.17	At5g58310	Hydrolase, alpha/beta fold family protein; esterase/lipase/thioesterase family protein
252308_at	**0.87**	0.01	**0.89**	0.16	**0.52**	0.49	At2g46980	Unknown protein
253796_at	**1.40**	0.24	**1.88**	0.89	**0.55**	0.15	At3g10930	Unknown protein
260040_at	**1.52**	0.07	**1.40**	0.72	**0.58**	0.02	At2g37430	Zinc finger (C2H2 type) family protein
256442_at	**2.52**	0.04	**1.78**	0.83	**0.97**	0.09	At1g68765	Inflorescence deficient in abscission
266010_at	**2.34**	0.01	**1.56**	0.76	**0.68**	0.23	At4g28710	Myosin heavy chain - like protein (fragment).
245082_at	**1.84**	0.48	**1.83**	1.02	**0.47**	0.47	At5g59820	Encodes a zinc finger protein involved in high light and cold acclimation
251176_at	**1.29**	0.46	**3.18**	0.74	**1.00**	0.22	At2g32030	GCN5-related N-acetyltransferase (GNAT) family protein
259143_at	**1.07**	0.42	**1.15**	0.22	**0.58**	0.38	At2g23270	Unknown protein
266841_at	**1.37**	0.39	**2.36**	0.38	**1.29**	0.07	At2g26150	Heat shock transcription factor A2 ; response to heat, high light intensity, oxidative stress
255577_at	**1.09**	0.18	**1.34**	0.48	**0.55**	0.48	At3g63380	Calcium-transporting ATPase, putative / Ca(2+)-ATPase (ACA12)
266372_at	**0.81**	0.17	**0.61**	0.34	**1.26**	0.19	At4g01410	Harpin-induced family protein / HIN1 family protein / harpin-responsive family
267614_at	**0.77**	0.11	**1.06**	0.23	**0.59**	0.58	At3g10190	Calmodulin putative
247655_at	**2.45**	0.08	**2.15**	0.65	**0.93**	0.05	At2g41310	Encodes an A-type response Regulator that is primarily expressed in the root
265725_at	**2.07**	0.03	**2.23**	0.96	**0.99**	0.03	At2g26710	Cytochrome p450 family ; involved in brassinosteroid metabolism process
253946_at	**0.54**	0.03	**0.70**	0.08	**1.06**	0.67	At4g27030	Small conjugating protein ligase; regulation of protein metabolic process

260978_at	**1.74**	0.43	**2.85**	0.32	**2.44**	0.51	At5g14730	Unknown protein
245252_at	**2.05**	0.32	**1.80**	0.42	**1.01**	0.09	At1g80840	WRKY40 transcription factor ; hydrogen peroxide induced
246584_at	**3.48**	0.24	**1.90**	0.74	**0.75**	0.70	At3g23230	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2
257918_at	**2.08**	0.2	**1.84**	0.61	**1.16**	0.24	At4g17500	Encodes a member of the ERF (ethylene response factor, ATERF-1)
266071_at	**1.89**	0.18	**2.48**	0.65	**1.19**	0.29	At2g18680	Unknown protein
261892_at	**2.50**	0.15	**1.96**	0.81	**1.12**	0.31	At1g53540	17.6 kDa class I small heat shock protein
247704_at	**3.46**	0.76	**2.09**	0.60	**1.09**	0.30	At5g59510	DVL18/RTFL5 (rotundifolia like 5); involved in shoot development
263569_at	**0.84**	0.37	**0.55**	0.23	**0.98**	0.35	At2g33710	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2
256252_at	**1.42**	0.26	**2.32**	0.64	**1.33**	0.27	At3g11340	UDP-glucuronosyl/UDP-glucosyl transferase family protein; similar to UGT76C2
261216_at	**1.28**	0.22	**2.04**	0.67	**1.27**	0.29	At1g26420	FAD-binding domain-containing protein; similar to FAD-binding domain-containing protein
258277_at	**1.29**	0.18	**2.37**	0.60	**1.22**	0.25	At3g26830	CYP71B15, MDJ14.12, PAD3, phytoalexin deficient 3
261005_at	**1.39**	0.14	**2.11**	0.72	**1.33**	0.15	At1g33030	O-methyltransferase family 2 protein; similar to ATOMT1 (O-methyltransferase 1)
260668_at	**1.23**	0.14	**2.07**	0.54	**1.38**	0.16	At3g25655	Unknown protein
267451_at	**2.04**	0.09	**2.01**	0.51	**1.32**	0.38	At1g19530	Unknown protein
256762_at	**1.24**	0.04	**1.00**	0.57	**0.56**	0.25	At2g27170	Cohesin; involved in chromosome segregation
253832_at	**2.37**	0.54	**1.80**	0.12	**0.80**	0.03	At2g35710	Glycogenin glucosyltransferase; involved in carbohydrate biosynthetic process
253181_at	**2.25**	0.46	**1.65**	0.60	**0.77**	0.06	At4g27654	Unknown protein
266743_at	**1.75**	0.38	**2.57**	0.22	**0.95**	0.63	At2g44840	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2
265841_at	**2.60**	0.38	**1.75**	0.77	**1.02**	0.10	At1g35210	Unknown protein
252487_at	**1.72**	0.36	**2.05**	0.41	**1.13**	0.33	At4g35180	LHT7 (Lys/His transporter 7);amino acid transmembranetransporter
245755_at	**2.29**	0.31	**1.70**	0.78	**0.77**	0.03	At1g25400	Unknown protein
258606_at	**1.92**	0.29	**2.03**	0.72	**0.91**	0.23	At5g27420	Zinc finger (C3HC4-type) family protein; involved in response to abscisic acid stimulus
248164_at	**2.13**	0.17	**1.95**	0.75	**0.93**	0.19	At5g54400	Unknown protein
266821_at	**2.33**	0.13	**1.82**	0.96	**0.92**	0.26	At1g66090	Disease resistance protein (TIR-NBS class)
265670_s_at	**1.90**	0.12	**2.13**	0.56	**0.95**	0.13	At3g02840	Immediate-early fungal elicitor family protein
255733_at	**2.19**	0.12	**1.77**	0.51	**0.99**	0.13	At2g32210	Unknown protein
246777_at	**2.15**	0.03	**1.95**	0.60	**1.03**	0.05	At2g02990	Member of the ribonuclease T2 family, responds to inorganic phosphate starvation
256526_at	**2.08**	0.03	**1.90**	0.87	**1.06**	0.07	At3g46660	UDP-glucuronosyl/UDP-glucosyl transferase family protein
250296_at	**1.47**	0.59	**2.62**	0.23	**2.33**	0.57	At4g39670	Glycolipid binding / glycolipid transporter; similar to ACD11 (accel. cell death 11)
257536_at	**2.33**	0.5	**2.02**	0.72	**1.00**	0.25	At3g02800	Phosphoprotein phosphatase; similar to tyrosine specific protein phosphatase family
250493_at	**2.17**	0.49	**1.48**	0.44	**1.42**	0.50	At4g37290	Unknown protein
253044_at	**2.27**	0.46	**2.13**	0.75	1.07	0.14	At5g09800	U-box domain-containing protein; involved in protein ubiquitination
263402_at	**1.40**	0.42	**2.28**	0.85	**0.74**	0.25	At2g36790	Encodes a UDP-glucose:flavonol-3-O-glycoside-7-O-glucosyltransferase (UGT73C6)
252908_at	**2.40**	0.35	**2.21**	0.87	**0.83**	0.02	At5g12020	HSP17.6II (17.6 KDA Class II heat shock protein)
252334_at	**1.43**	0.14	**2.29**	0.53	**1.01**	0.14	At3g48850	Mitochondrial phosphate transporter
265200_s_at	**2.07**	0.04	**1.37**	0.64	**1.04**	0.29	At2g04050	Similar to ATDTX1, antiporter/ multidrug efflux pump/ multidrug transporter/ transporter

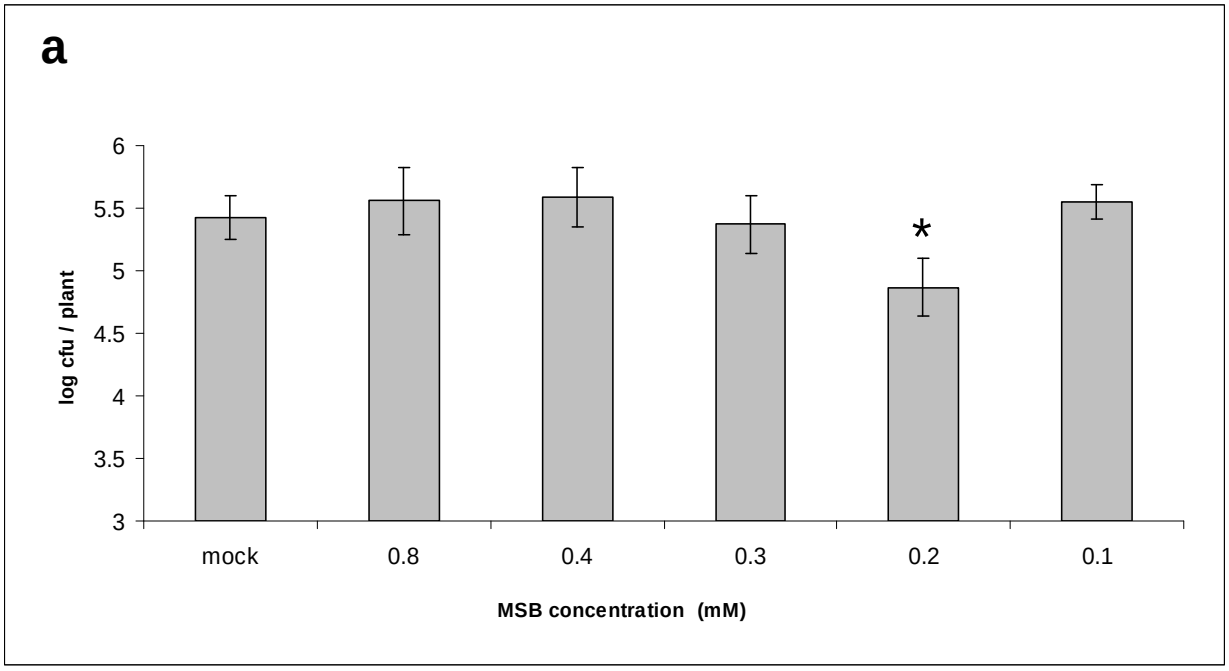
252934_at	**0.88**	0.03	**0.87**	0.02	**0.58**	0.56	At4g39120	Inositol monophosphatase family protein; involved in sulfur metabolic process
266995_at	**1.73**	0.72	**2.79**	0.38	**1.18**	0.34	At3g50260	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family
252214_at	**2.08**	0.62	**1.53**	0.09	**1.26**	0.42	At4g21680	Proton-dependent oligopeptide transport (POT) family protein
260248_at	**1.51**	0.54	**2.38**	0.50	**1.26**	0.13	At1g15010	Unknown protein
254396_at	**2.04**	0.45	**1.77**	0.57	**1.18**	0.06	At2g39510	Nodulin MtN21 family protein
260744_at	**2.00**	0.39	**1.33**	0.37	**0.63**	0.36	At1g74310	Encodes ClpB1, which belongs to the Casein lytic proteinase/heat shock protein 100
245136_at	**2.33**	0.33	**1.45**	0.05	**1.29**	0.63	At2g45210	Auxin-responsive protein-related; involved in response to auxin stimulus
257061_at	**1.20**	0.27	**2.00**	0.35	**1.35**	0.61	At3g28550	Proline-rich extensin-like family protein; involved in plant-type cell wall organization
263376_at	**1.05**	0.07	**0.82**	0.02	**2.00**	0.57	At2g20520	Fasciclin-like arabinogalactan-protein 6 (Fla6).
246927_s_at	**2.20**	0.5	**1.63**	0.68	**0.97**	0.25	At5g25260	Unknown protein
253827_at	**2.03**	0.41	**1.88**	0.67	**0.96**	0.24	At1g27730	Salt tolerance zinc finger protein; Related to Cys2/His2-type zinc-finger proteins
261648_at	**2.09**	0.37	**1.83**	0.63	**1.04**	0.02	At4g28085	Unknown protein
246405_at	**1.74**	0.31	**2.12**	0.62	**1.09**	0.25	At1g57630	Disease resistance protein (TIR class)
253378_at	**1.46**	0.33	**1.07**	0.09	**2.31**	0.87	At4g33310	Unknown protein
254006_at	**0.60**	0.2	**1.10**	0.37	**1.37**	0.79	At4g26340	F-box family protein; Identical to F-box/FBD/LRR-repeat protein
245209_at	**2.85**	0.7	**1.83**	0.76	**1.02**	0.19	At5g62480	Glutathione-S-transferase (Class TAU) 9
247435_at	**2.24**	0.55	**1.77**	1.10	**0.65**	0.12	At5g12340	Unknown protein
247026_at	**2.32**	0.78	**2.24**	0.79	**0.87**	0.02	At5g67080	Mitogen-activated protein kinase kinase kinase 19
263475_at	**1.89**	0.76	**2.06**	0.69	**1.58**	0.04	At2g31945	Unknown protein

Table 2 DNA sequence motifs over-represented in the promoters of the lists. The gene promoters grouped by the lists were analyzed as described in Experimental procedures to determine the frequency of the motifs. We display the motifs with a probability less than $1x10^{-6}$ of being overrepresented by chance, and more than two-fold abundance. The motifs that contain the G-box are underlined.

Name	Seq	Fold	List
Up3h_AA8	CRTMAGCAATGAS	87.6	Upall
Upall_AA19	AAATTGYAGA	6.6	Upall
Upall_AA18	GAGATTTSTT	5.5	Upall
Up24h_AA20	TGAMMCRTGA	5.1	Upall
Up6h_AA12	GWCACGTGKS	4.3	Upall
Upall_AA12	TTTGAAAART	4.2	Upall
Up3h_0	ACACGTKKMR	3.8	Upall
Up6h_AA11	KTTGAAAART	3.6	Upall
Upall_AA5	TGMCACGTGKM	3.6	Upall
Upall_AA11	AAAGTTTSMAA	3.2	Upall
Up6h_3	TKMCACGTRW	3.2	Upall
Up3h_1	ACACGTKKMW	3.1	Upall
Upall_4	CAYGTGBSWY	3.0	Upall
Up6h_0	KMCACGYRYS	2.9	Upall
GBF1/2/3 BS in ADH1	CCACGTGG	2.9	Upall
Up3h_4	GBAMACGYRW	2.9	Upall
Up6h_AA9	TYWATATWTWYAWAWA	2.9	Upall
Upall_0	ACACGTGKMM	2.9	Upall
Up24h_3	TARSTCWRGY	2.9	Upall
Up3h_AA5	AWAAWATTWATW	2.8	Upall
Up6h_2	SWCACGTGYM	2.7	Upall
Up6h_4	KWCACGTKKM	2.3	Upall
Upall_3	CACGTGKMMH	2.3	Upall
Up3h_AA3	WTTTTRWWAAAWAAA	2.2	Upall
Up3h_AA4	WTTTTAAWAWARA	2.2	Upall
Down24h_0	YACACACWYA	2.2	Upall
Down3h_AA8	TATMTATATAAA	2.1	Upall
Upall_1	MCACGTGWMM	2.1	Upall
CACGTGMotif	CACGTG	2.1	Upall
Down6h_4	ATTTTRWATG	2.0	Upall
Down24h_AA2	AWWWTATYAWATWT	2.0	Upall
Down24h_AA3	CACACACAYA	2.0	Upall
GADOWNAT	ACGTGTC	1.9	Upall
Up24h_AA15	AAAAWHRYKKGAWY	1.9	Upall
Up3h_2	WKMMACGTGT	1.9	Upall
Downall_0	YACAMACAYA	1.8	Upall
Down6h_0	ATTTTRAAKG	1.7	Upall
Down24h_2	CACACWCAYA	1.7	Upall
Up24h_AA2	AAAARWYWRWAWWWWT	1.7	Upall
Up24h_AA12	WWRWKAMYAAMTT	1.6	Upall
ABRE-like binding site motif	BACGTGKM	1.6	Upall
Up24h_0	WWATGGGCCT	1.6	Upall
Upall_AA8	KTTGACMAAA	1.5	Upall
Upall_AA16	AAAWAAAAAT	1.5	Upall
Downall_AA3	TTKTGTMTTGAACA	212.0	Downall

Down3h_AA3	GAACATCACTGTKKCTTTKTGTMTTG	186.7	Downall
Down3h_0	TCACTGTGGC	131.5	Downall
Down6h_AA3	ATCGGDKTWGGT	93.0	Downall
Down3h_AA7	ACWGTGRYKTTS	42.2	Downall
Down3h_AA12	TAWGTGGTYWK	20.1	Downall
Down6h_1	TCATATRAWT	7.1	Downall
Down24h_AA2	AWWWTATYAWATWT	7.0	Downall
Downall_AA6	ATWTATATAMAM	5.4	Downall
Down6h_2	CATWYAAAAT	4.7	Downall
Down24h_1	TRTGTKTGTR	4.7	Downall
Down24h_AA3	CACACACAYA	4.2	Downall
Down24h_0	YACACACWYA	3.8	Downall
Down24h_2	CACACWCAYA	3.5	Downall
Down24h_AA1	WWMAAAAWAAAAWWWRA	3.4	Downall
Downall_0	YACAMACAYA	3.3	Downall
Downall_AA7	AAAHHTTGAA	3.3	Downall
Upall_AA2	AWWWWRMAAAAWRWAAA	2.9	Downall
Downall_1	TRTGTRTRTR	2.7	Downall
Downall_4	AYAYAYAYAC	2.7	Downall
Downall_AA5	WTWWTTMAAAA	2.6	Downall
Up6h_AA2	AWWWWWAWARARAWAWAWW	2.5	Downall
Upall_AA3	AWANAWARAWANAWA	2.3	Downall
Downall_2	CAMAMACAYA	2.2	Downall
Downall_3	GTRTRTRTRT	2.2	Downall
Downall_AA1	WRAARAAAARAAAA	2.0	Downall
Down3h_3	YYWGRCCCRK	1.9	Downall
Up3h_AA1	MAAAMAAAAWWA	1.7	Downall
Up24h_AA16	WWAAANMMAAANAA	1.7	Downall
GADOWNAT	ACGTGTC	1.7	Downall

Figure 1


a
6
5.5
5
4.5
4
3.5
3
log cfu / plant
*
mock
0.8
0.4
0.3
0.2
0.1
MSB concentration (mM)

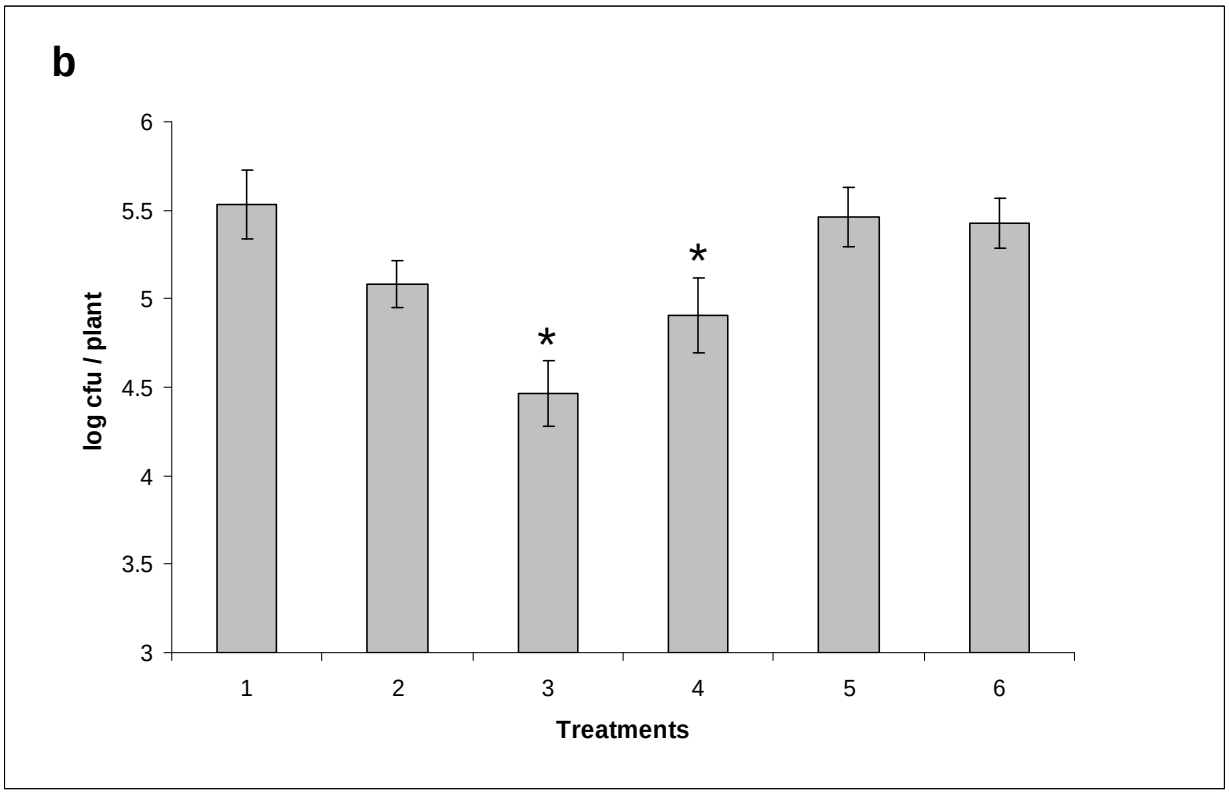
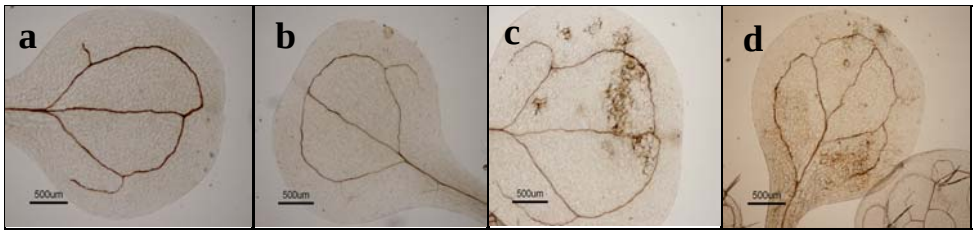

b
6
5.5
5
4.5
4
3.5
3
log cfu / plant
*
*
1
2
3
4
5
6
Treatments

Figure Legends

Figure 1 a MSB induces resistance. *Arabidopsis thaliana* (Arabidopsis) plants were treated with different concentrations (mM) of MSB and 24 hours later inoculated with *Pseudomonas syringae pv. tomato* DC3000 (*Pto*). The bars show the average growth of the bacteria three days later (±SD) in a logarithmic scale of colony forming units per plant (Y-axis). The concentrations are expressed in mM of MSB (X-axis). Asterisk indicates data significantly different from mock ($P < 0.06$; Tukey test).This experiment was repeated three times with similar results. **b** Different MSB treatments prior to inoculation were performed in order to optimize the timing prior to inoculation for inducing resistance. Treatments: mock (1); MSB 48 h (2); MSB 24 h (3); SA 24 h (4); MSB 12 h (5); MSB 12 h post-inoculation (6). Asterisks indicate data significantly different from mock ($P < 0.05$; Tukey test). This experiment was repeated three times with similar results.

Figure 2

a



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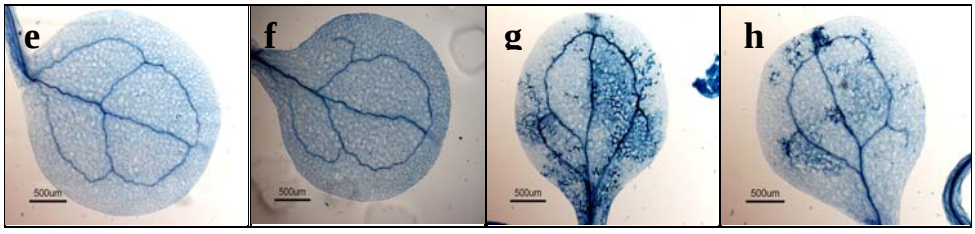


c

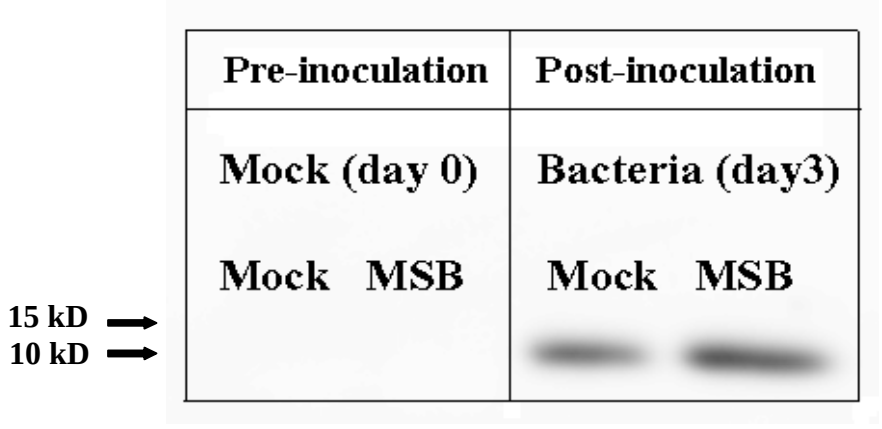


Figure 2 a Staining for ROS and cell death. Plants were either mock (**a** and **c**) or MSB (**b** and **d**) treated. 24 hours later, an inoculation with *Pto* was performed (**c** and **d**). **a** and **b** were treated as control for the inoculation. Three days later, a DAB staining was performed as described in Experimental procedures, showing the reactive oxygen species. **b** To unveil cell death, plants

were either mock (**e** and **g**) or MSB (**f** and **h**) treated. 24 hours later, an inoculation with *Pto* was performed (**g** and **h**). **e** and **f** were treated as control of the inoculation. Three days later, a trypan blue staining was performed as described in Experimental procedures, showing the dead cells. **c** Western blot of PR1 protein. 18 days-old plants were pre-treated with Mock or MSB prior to inoculation with *Pto*. MSB does not induce PR1 protein *per se* (day 0). In contrast, a considerable effect of the MSB treatment can be seen 3 days post-inoculation, where ample amounts of PR1 protein are observed versus the Mock treatment (Priming effect). These experiments were repeated three times with similar results.

Figure 3

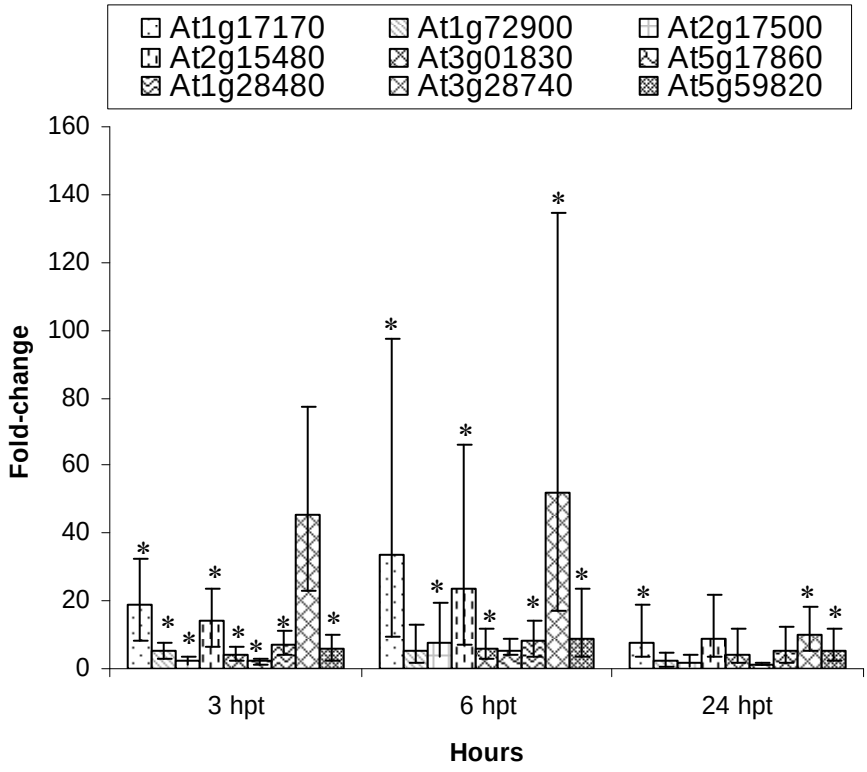


Figure 3 Validation of gene expression by real-time RT-PCR. Changes in gene expression were estimated as fold-change over the mock (distilled water treatment). The genes tested are indicated above bars. The bars represent the average fold-change and confidence intervals in transcript changes estimated from three independent biological replicates. Asterisks indicate data significantly different from mock ($P < 0.05$; REST software, see 'Experimental procedures'). As standard internal controls GAPC-2 (Gliceraldehyde-3-phosphate dehydrogenase2, At1g13440) and NDK1 (Nucleoside diphosphate kinase 1, At4g09320) were used and showed an average M (geNorm) 0.38 and CV (coefficient of variance) 13,3%, respectively.

Figure 4

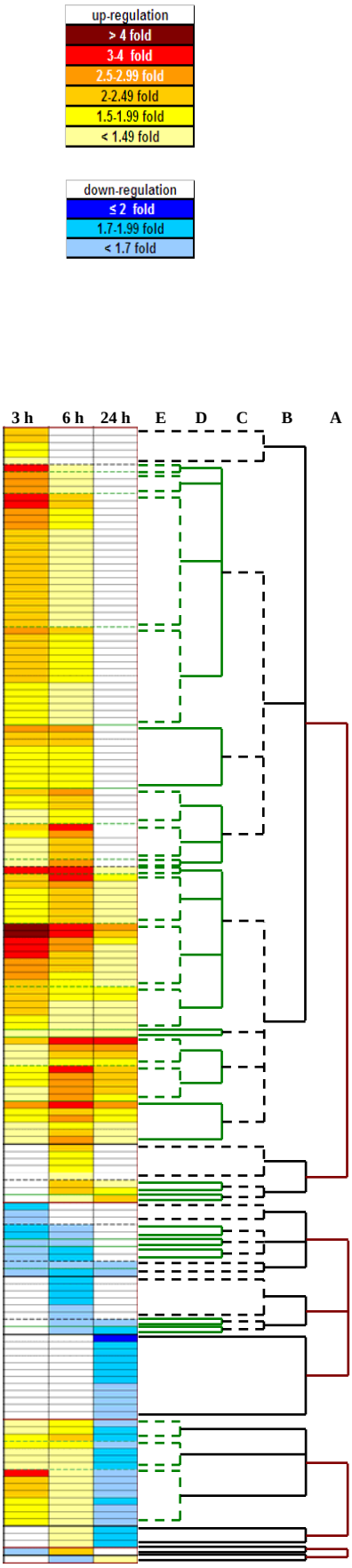
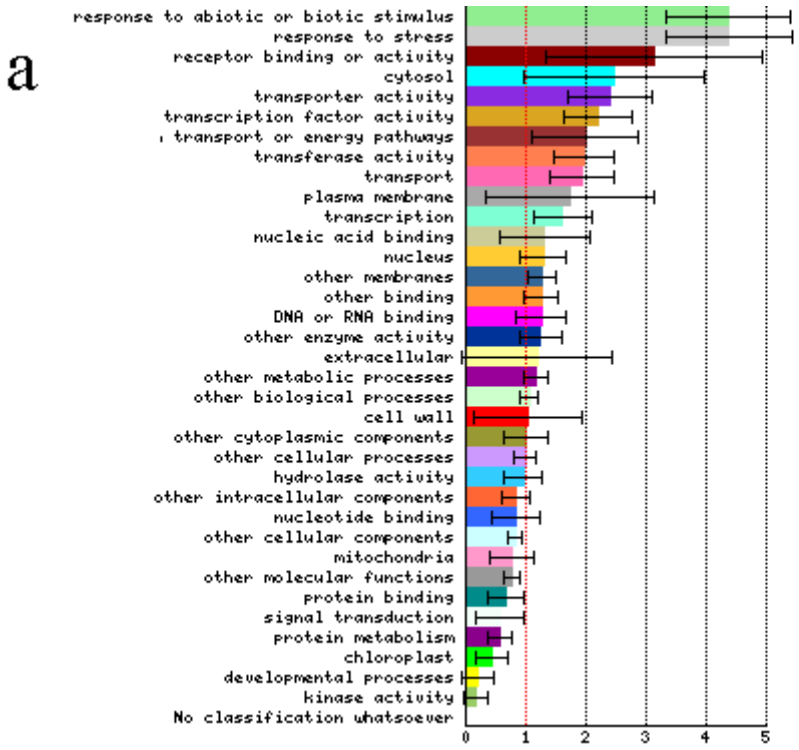

up-regulation
> 4 fold
3-4 fold
2.5-2.99 fold
2-2.49 fold
1.5-1.99 fold
< 1.49 fold
down-regulation
≤ 2 fold
1.7-1.99 fold
< 1.7 fold
3 h
6 h
24 h
E
D
C
B
A

Figure 4 Customized clustering. (**A**) Direction of change expression: up, down or in both directions. (**B**) Response onset: early, medium, late. (**C**) Expression change detected at one, two or three time-points, respectively. (**D**) Trends: increasing, constant, decreasing. (**E**) Trends subtypes.

Figure 5



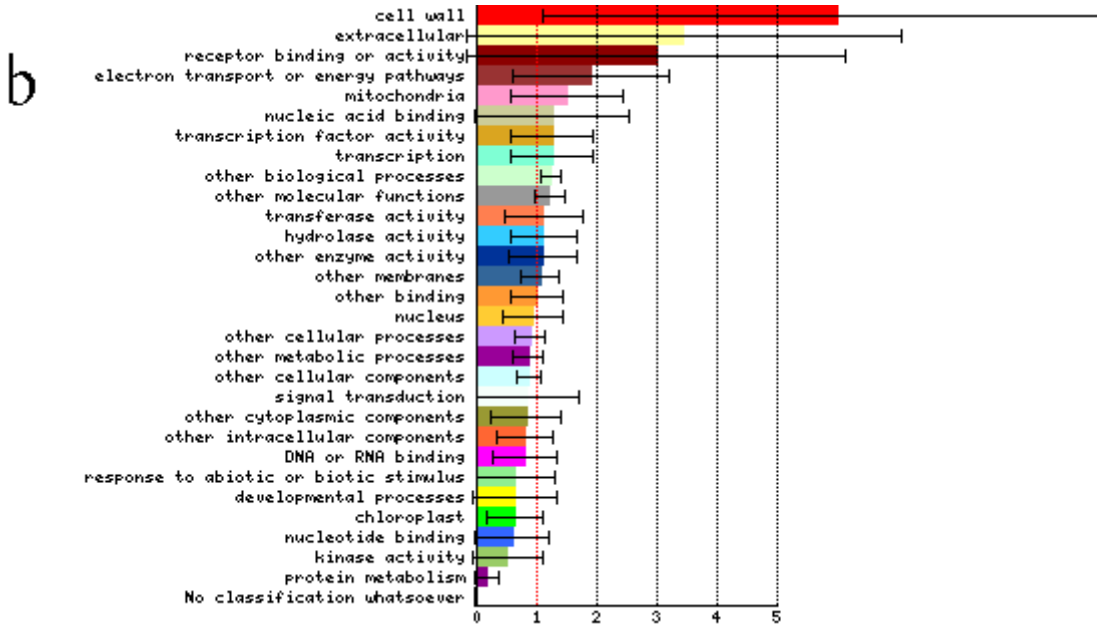


Figure 5 Ontology. The list of up-regulated genes **(a)** and the down-regulated genes **(b)** was uploaded into the program "Classification SuperViewer" (see Experimental procedures). This is an overview of the functional classification based on the available databases.

Figure 6

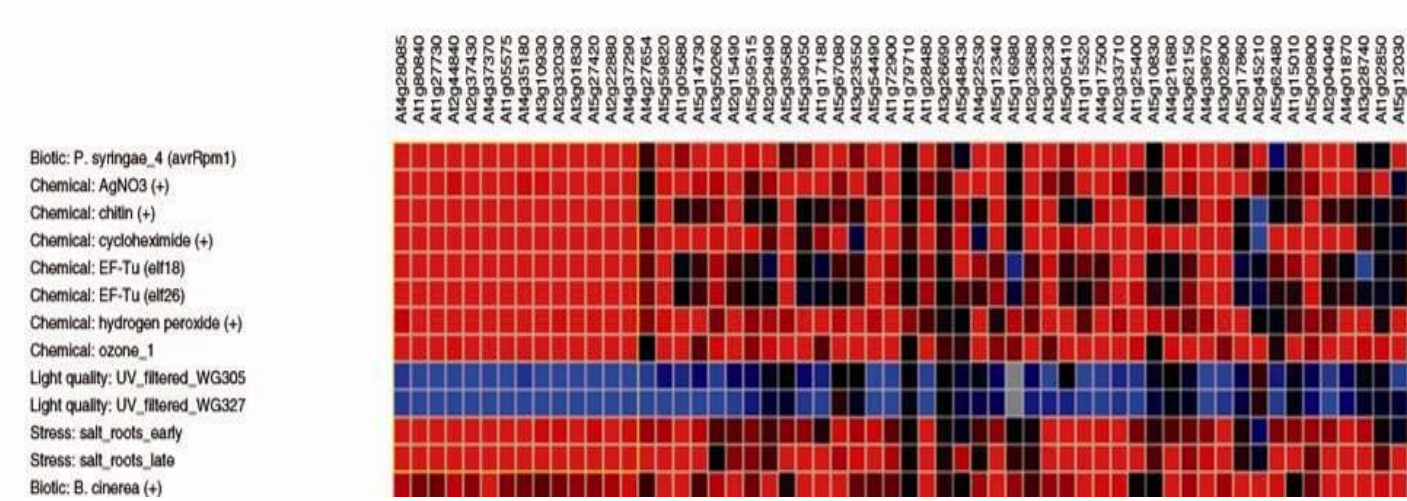


Figure 6 Biclustering. The list of up-regulated genes at 3 hpt was uploaded into the program "Genevestigator" (see Experimental procedures). This tool describes every gene as upregulated (light red) or down-regulated (light blue) based on its own microarray data for each specific condition, a matrix of red and blue squares is obtained where the columns represent the genes and the rows are the different conditions or treatments. Since the complete figure is of considerable size, only the most informative treatments are shown. The whole image is presented as supplemental figures S1A, S1B and S1C.

Supplementary material: table and figure legends

Table S1 Ranking of gene expression level. List of normalized data followed by coefficient of variation values at 3, 6 and 24 hpt.

Table S2 List of oligonucleotides used as primers in the quantification of selected genes by real-time RT-PCR.

Table S3. Lists of genes used in the paper. Genes were selected for their up or down regulation in response to MSB (see Experimental procedures).

Figure S1 Biclustering. The list of up-regulated genes at 3 (**a**), 6 (**b**) or 24 hpt (**c**) was uploaded into the program "Genevestigator" (see Experimental procedures). This tool describes every gene as up-regulated (light red) or down-regulated (light blue) based on its own microarray data for every specific condition, a matrix of red and blue squares is obtained where the columns represent the genes and the rows are the different conditions or treatments. Note that part of panel **a** (the most informative) is presented as Figure 8. The down-regulated genes do not produce any significant information.

Figure S2 Examples of the motif distribution and the context of the G-box. A hundred thousand clusters were generated by bootstrapping, and the frequencies of motifs present counted (see Experimental procedures). In the X-axis is plotted the number of times a particular motif was found, and in the Y-axis the number of times that a cluster generated by bootstrapping produced that number. **(a)** G-box in the up-regulated genes. **(b)** ABRE-like binding site motif in up-regulated genes. In blue rhombi is the distribution of the motif in the genome, in pink squares, the distribution in the list of genes. **(c)** Context of the G-box in the genome **(d)** Context of the G-box in the up-regulated genes.