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# Linking genes of unknown function with abiotic stress responses by high-throughput phenotype screening

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Over 13% of all genes in the *Arabidopsis thaliana* genome encode for proteins classified as having a completely unknown function, with the function of >30% of the Arabidopsis proteome poorly characterized. Although empirical data in the form of mRNA and proteome profiling experiments suggest that many of these proteins play an important role in different biological processes, their functional characterization remains one of the major challenges in modern biology. To expand the annotation of genes with unknown function involved in the response of Arabidopsis to different environmental stress conditions, we selected 1007 such genes and tested the response of their corresponding homozygous T-DNA insertional mutants to salinity, oxidative, osmotic, heat, cold and hypoxia stresses. Depending on the specific abiotic stresses tested, 12–31% of mutants had an altered stress-response phenotype. Interestingly, 832 out of 1007 mutants showed tolerance or sensitivity to more than one abiotic stress treatment, suggesting that genes of unknown function could play an important role in abiotic stress-response signaling, or general acclimation mechanisms. Further analysis of multiple stress-response phenotypes within different populations of mutants revealed interesting links between acclimation to heat, cold and oxidative stresses, as well as between sensitivity to ABA, osmotic, salinity, oxidative and hypoxia stresses. Our findings provide a significant contribution to the biological characterization of genes with unknown function in Arabidopsis and demonstrate that many of these genes play a key role in the response of plants to abiotic stresses.

## Introduction

Plants are sessile organisms that evolved a large array of adaptive responses to allow them to survive and acclimate to changes in their environment. To date, the function of the proteins encoded by about 13% of the *Arabidopsis thaliana* genome is classified as completely unknown, with the function of >30% of Arabidopsis proteins classified as poorly characterized (Lamesch

*Abbreviations* – ABA, abscisic acid; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species.

et al. 2012, http://www.arabidopsis.org/). mRNA profiling experiments revealed that transcripts encoding many of these proteins are expressed in response to environmental stress in Arabidopsis (Horan et al. 2008). The expression of these genes, which we term 'unknowns', or 'poorly characterized', might be required for novel defense mechanisms or involved in critical signaling pathways (Gollery et al. 2007, Luhua et al. 2008).

Specialized networks of regulatory and defense genes coordinate plant sensing, response and acclimation to environmental conditions such as low water availability, fluctuations in ambient temperature, toxicity of salt and other minerals, limiting oxygen and elevated reactive oxygen species (ROS; Mittler and Blumwald 2010, Reddy et al. 2011, Hauser et al. 2011, Suzuki et al. 2011a, Bailey-Serres et al. 2012). These networks are intertwined with the networks that control plant growth and reproduction (Hirayama and Shinozaki 2010). Despite extensive research efforts in this field, large gaps exist in our understanding of the different regulatory and defense networks that control the response of plants to their dynamic environment. Perhaps the most important gap in our understanding is the large number of unknowns or poorly characterized genes that appear to participate in these networks. Although, we can determine the spatial and temporal expression of these genes during stress, at this point without a dedicated research effort directed at determining their function, we have little structural or functional knowledge that enables us to assign a role for them. Because proteins altered in plants during stress account for >30% of all proteins in Arabidopsis (Horan et al. 2008), it is likely that our current view of the plants' response to abiotic stress would undergo a significant change once the function(s) of the unknowns or poorly characterized genes is revealed. Due to the extreme diversity of their sequences (Gollery et al. 2006, 2007), a tremendous variety of novel molecular and biological functions can be expected in this uncharacterized group of proteins.

To test whether proteins of unknown, or poorly characterized, function play a role in the response of plants to specific abiotic stresses, we chose, in a previous study, 41 different 'unknowns' that respond to oxidative stress in Arabidopsis, and constitutively expressed them in transgenic plants (Luhua et al. 2008). We found that more than 70% of the expressed unknown proteins conferred tolerance to oxidative stress. In contrast, the majority of expressed unknowns (over 90%) did not confer tolerance to other abiotic stresses, such as cold, salinity, heat or osmotic stress, and about 50% of the expressed unknown proteins rendered plants more susceptible to osmotic or salinity stress (Luhua et al. 2008). Our findings demonstrated that tolerance to oxidative stress in Arabidopsis involves unique and specific proteins, pathways and mechanisms, which are unknown at present, including some that might even be specific to *A. thaliana*.

To expand our functional characterization of abiotic stress-response proteins with unknown function, we chose 1007 different genes encoding such proteins in *A. thaliana* ecotype Columbia-0 and tested the response of T-DNA insertional mutants deficient in these genes to salinity, oxidative, osmotic, heat, cold and hypoxia stresses, as well as to abscisic acid (ABA). Depending on the specific abiotic stress or treatment tested, 12–31% of mutants deficient in genes with unknown, or poorly characterized, function had an altered phenotype when subjected to stress. Over 83% of the different mutants displayed altered phenotype to more than one abiotic stress/treatment, and an analysis of multiple stress-response phenotypes revealed interesting links between temperature and oxidative stresses, as well as between sensitivity to ABA, and sensitivity to osmotic, salinity, oxidative and hypoxia stresses. Our findings provide a significant contribution to the annotation of genes with unknown, or poorly characterized, function in Arabidopsis, and demonstrate that many of these genes play a key role in the response of plants to abiotic stresses.

## Materials and methods

### Plant growth qPCR analysis and ROS imaging

*Arabidopsis thaliana* cv Columbia-0 plants were grown under controlled conditions: 21°C, 100 μmol $m^{-2}$ $s^{-1}$ (Suzuki et al. 2005), and monitored for growth and flowering time as described by Miller et al. (2007). Confirmed knockout lines were obtained from ABRC (http://abrc.osu.edu/) and bulked together with wild-type seeds under carefully controlled growth conditions as described in Luhua et al. (2008) and Suzuki et al. (2011a). Quantitative polymerase chain reaction (qPCR) analysis was performed as described by Miller et al. (2009) and Suzuki et al. (2011a), using the primer sets shown in Fig. S1. To image hydrogen peroxide accumulation in seedlings grown under controlled conditions, or subjected to oxidative stress, 5-days-old seedlings grown in the presence or absence of paraquat were treated with 0.2 $\mu$M Amplex® Red (Molecular Probes, Inc., Eugene, OR) for 1 h and imaged as described in Miller et al. (2009) and Luhua et al. (2008).

### Stress assays

For the analysis of stress-tolerance seeds of wild type and confirmed knockouts for the different genes selected for

analysis (Table S1) were surface-sterilized with bleach. Seeds were placed in rows on 1% agar plates (0.5× MS medium), containing different concentrations of paraquat (0.01–0.5 μ*M*), NaCl (75–150 m*M*, Sorbitol (50–250 m*M*), PEG-8000 (−1.2 *w*) or ABA (0.1–1 μ*M*) (Sigma-Aldrich, St. Louis, MO), as described by Luhua et al. (2008), Miller et al. (2007) and Verslues et al. (2006). Each row of seeds (25–30 seedlings) placed on a plate was divided into two parts: control wild-type seeds and seeds of confirmed T-DNA insertion lines for the different proteins of unknown function. Thus, the different seeds were placed side-by-side on the same plate. Plates were maintained vertically in a growth chamber (21–22°C, constant light, 100 μmol photons $m^{-2}$ $s^{-1}$) and percent germination and root length were scored 5 days after seed plating. Four- or five-days-old seedlings grown on 0.5× MS 1% agar plates were also subjected to heat (38°C, 24 h) or cold (10°C, 48 h) stresses and scored for percent germination and root length as described by Miller et al. (2007) and Luhua et al. (2008). For hypoxia stress, wild-type and T-DNA insertion line seeds were plated side-by-side on 0.5× MS medium containing 1% (w/v) sucrose, grown vertically in a growth chamber at 23°C with a 16-h day (50 μmol photons $s^{-1}$ $m^{-2}$) and 8-h night cycle for 7 days, deprived of oxygen and carbon dioxide for 8 or 12 h by replacement of air with argon, or mock treated under dim light (0.15 μmol photons $s^{-1}$ $m^{-2}$ light), and then scored for phenotype and survival as described by Mustroph et al. (2010). All experiments were repeated at least three different times, each with at least three different technical repeats each containing at least 45 seeds per line. Results are shown as mean and SE bars. Statistical analysis was performed as described in Suzuki et al. (2008). Significant difference between each confirmed T-DNA insertion mutant line and the wild-type control was assigned only when differences in root growth were Student's *t*-test significant at $P < 0.05$. Database mining for transcript expression of At4g25990, represented in our screens by the SALK_131539C line, and At1g73750, represented in our screens by the SALK_135747C line was performed as described in Suzuki et al. (2011b) using Genevestigator (https://www.genevestigator.com/gv/plant.jsp).

## Results

### High-throughput phenotype screening of T-DNA insertional mutants deficient in genes of unknown, or poorly characterized, function

The application of DNA microarray technology to the study of abiotic stress responses has led to the realization that about 30% of genes with altered mRNA abundance during abiotic stresses encode for proteins of unknown, or poorly characterized, function (Horan et al. 2008). In 2005, we selected 1007 abiotic stress-response genes that had a completely unknown function at the time (Table S1) for our analysis. These genes were identified in microarray experiments as responsive to salinity, heat, cold, oxidative, hypoxia and/or drought stresses (Seki et al. 2001, Kreps et al. 2002, Rizhsky et al. 2004, Branco-Price et al. 2005, 2008). Although most of the genes we chose were annotated as completely unknown at the time, advances in gene annotation and the development of bioinformatics tools, especially those utilizing profile hidden Markov models for sequence similarity searching (Lamesch et al. 2012), have led in the past few years to the assignment of putative function to about 40% of these genes (Table S2; http://www.arabidopsis.org/).

To assign an experimentally determined abiotic stress-response phenotype to the selected genes shown in Table S1, we obtained 1007 confirmed knockout T-DNA insertion lines for these different genes from the SALK project (http://signal.salk.edu; Alonso et al. 2003). Each line was bulked together with a control wild-type Columbia-0 line and the resulting seeds were used to measure root growth in seedlings grown on agar plates in the presence or absence of different abiotic stresses or ABA (Verslues et al. 2006, Miller et al. 2007, Luhua et al. 2008, Mustroph et al. 2010).

As shown in Fig. 1, 12–31% of the T-DNA mutants in genes with unknown, or poorly characterized, function had an altered phenotype when subjected to a specific abiotic stress condition. Osmotic and oxidative stresses had the most extensive effect on the different mutants (31–32% of mutants showing a phenotype), with heat, cold and salinity moderately (24–27%), and ABA and hypoxia (15–17%) infrequently perturbing growth under the conditions of our assays. In general most mutants showed a reduced root growth in response to the different stresses, with oxidative, salinity and hypoxia showing the highest ratio of reduced survival vs tolerance in their phenotypic effects (Fig. 1). Interestingly, with heat stress more mutants showed tolerance as opposed to sensitivity.

When the list of 1007 mutants was divided into completely unknown or poorly characterized genes (Fig. 2), it was found that genes with a completely unknown function showed a phenotype in 15–32% of the cases, whereas genes with poorly characterized function showed a phenotype in 18–36% of the cases (depending on the different stress tested). These comparable phenotype frequencies indicate that both groups contain similar proportions of genes important for abiotic stress-response pathways in plants.

| | Heat | Cold | Oxidative | Osmotic | NaCl | ABA | Hypoxia |
|---|---|---|---|---|---|---|---|
| Total | 1007 | 1007 | 1007 | 999 | 1007 | 1015 | 762 |
| No Change | 737 | 757 | 695 | 673 | 767 | 857 | 634 |
| Change | 270 | 250 | 312 | 326 | 240 | 158 | 128 |
| Tolerant | 172 | 124 | 78 | 150 | 60 | 72 | 8 |
| Sensitive | 98 | 126 | 234 | 176 | 180 | 86 | 120 |

**Fig. 1.** Phenotypes of mutants deficient in genes with unknown, or poorly characterized, function in response to specific abiotic stress conditions or high ABA levels. For each abiotic stress/treatment the number of mutants is broken down into mutants with a phenotype, as well as mutants that tolerance or sensitive. Significant of phenotype is at $P < 0.05$.

| Poorly Characterized | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Heat | Cold | Oxidative | Osmotic | NaCl | ABA | Hypoxia |
| Total | 401 | 401 | 401 | 397 | 400 | 405 | 286 |
| No Change | 290 | 294 | 293 | 254 | 297 | 345 | 233 |
| Change | 111 | 107 | 108 | 143 | 103 | 60 | 53 |
| Tolerant | 76 | 51 | 27 | 64 | 25 | 29 | 2 |
| Sensitive | 35 | 56 | 81 | 79 | 78 | 31 | 51 |
| | | | | | | | |
| Unknown Function | | | | | | | |
| | Heat | Cold | Oxidative | Osmotic | NaCl | ABA | Hypoxia |
| Total | 471 | 471 | 471 | 461 | 466 | 470 | 348 |
| No Change | 349 | 363 | 316 | 322 | 358 | 394 | 296 |
| Change | 122 | 108 | 155 | 139 | 108 | 76 | 52 |
| Tolerant | 83 | 57 | 34 | 69 | 31 | 33 | 3 |
| Sensitive | 39 | 51 | 121 | 70 | 77 | 43 | 49 |

**Fig. 2.** Phenotypes of mutants deficient in genes with a poorly characterized (top), or completely unknown (bottom), function in response to specific abiotic stress conditions or high ABA levels. For each abiotic stress/treatment the number of mutants is broken down into mutants with a phenotype, as well as mutants that tolerance or sensitive. Significant of phenotype is at $P < 0.05$.

## Tolerance or sensitivity to more than one abiotic stress

Different genes can have different functions in the response of plants to abiotic stresses. Some genes, for example, could have a function that is specific to only one particular stress, whereas others could be involved in the response of plants to several different environmental stresses (Mittler 2006, Mittler and Blumwald 2010, Reddy et al. 2011). To study the possible involvement of different mutants in one or more abiotic stresses, we examined how many mutants were tolerant or sensitive to more than one stress or treatment. Interestingly, 83% of all mutants displayed an altered phenotype to more than one abiotic stress condition (Fig. 3A) suggesting that they could function relatively high on the abiotic stress-response signal transduction pathway or be involved in multiple or general acclimation mechanisms to stress. As shown in Fig. 3B–E, the majority of mutants that were more sensitive to oxidative stress were also more sensitive to other abiotic stresses. Likewise, tolerance to oxidative stress was associated with tolerance to other abiotic stresses. Interestingly, many mutants that were tolerant to heat stress were also tolerant to cold stress and vice versa. A similar association was also observed between tolerance to osmotic, salinity and ABA. Only one mutant was found to be tolerant to five different treatments (heat, cold, salinity, ABA and osmotic) with a few mutants showing sensitivity to a similar number of treatments (Fig. 3E).

## Cross-stress analysis within each group of mutants

Although the analysis shown in Fig. 3 can reveal general associations between different abiotic stresses, it does not specifically examine how different groups of mutants with tolerance or sensitivity to a particular stress respond when subjected to a different set of stresses. To test for such cross-stress associations within specific groups of mutants, we examined how all mutants that are tolerant or sensitive to a particular abiotic stress respond when subjected to other stresses. Figs 4 and 5 show how

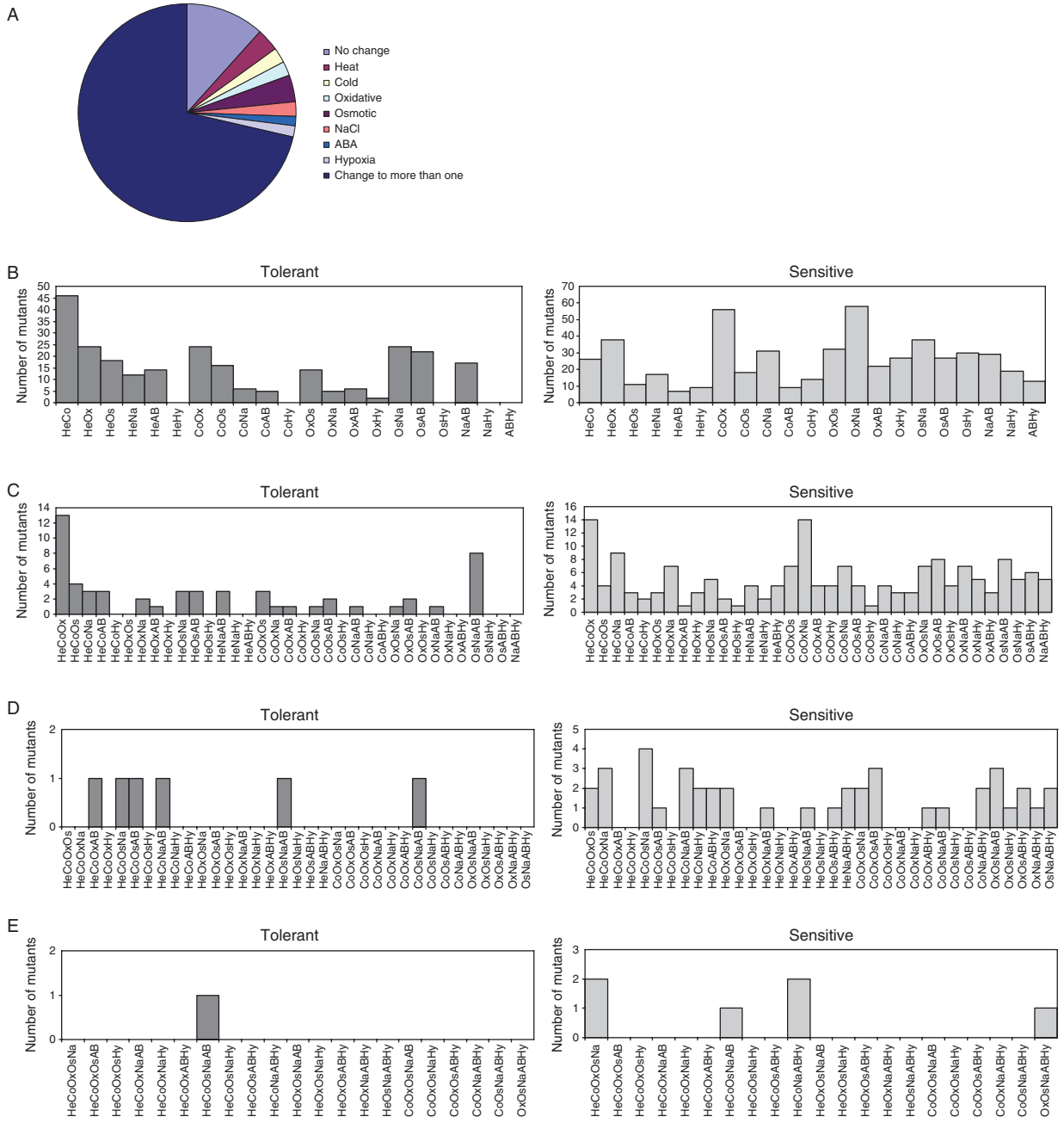


**Fig. 3.** Number of mutants found to be tolerant or sensitive to more than one abiotic stress/treatment tested. (A) Pie chart showing the distribution of mutants with no phenotype at all, a phenotype in a specific abiotic stress/treatment, or phenotypes to more than one abiotic stress/treatment. (B) Tolerance or sensitivity to two different abiotic stresses/treatments. (C) Tolerance or sensitivity to three different abiotic stresses/treatments. (D) Tolerance or sensitivity to four different abiotic stresses/treatments. (E) Tolerance or sensitivity to five different abiotic stresses/treatments. He, Heat; Co, Cold; Ox, Oxidative; Na, Salinity; Hy, hypoxia; Os, Osmotic, AB, ABA.

mutants tolerant or sensitive to a particular stress respond when subjected to other abiotic stresses.

As shown in Fig. 4, some mutants that are tolerant to heat stress are also tolerant to cold stress and vice versa; some mutants that are tolerant to oxidative stress are also tolerant to heat or cold stresses; some mutants that are tolerant to osmotic stress are also tolerant to ABA; some mutants that are tolerant to salinity are also tolerant to osmotic stress and ABA; and some mutants that are tolerant to ABA are also more tolerant to salinity and

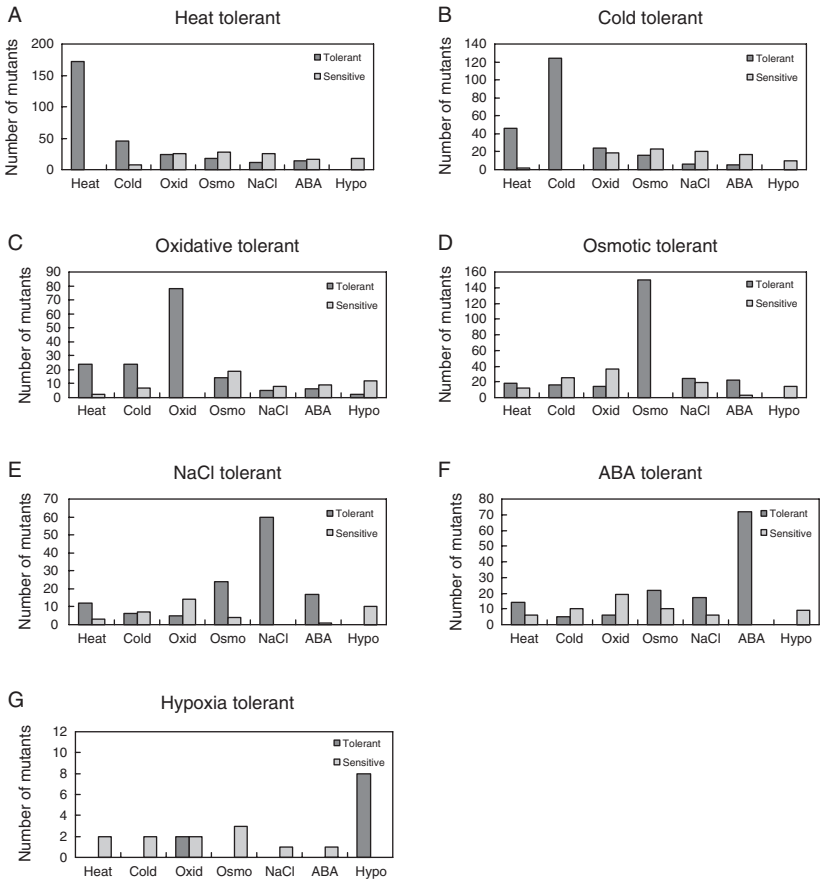


**Fig. 4.** Cross-stress relationships within different groups of mutants tolerant to a specific abiotic stress condition/treatment. (A) Tolerance or sensitivity to different stress conditions in mutants tolerant to heat. (B) Tolerance or sensitivity to different stress conditions in mutants tolerant to cold. (C) Tolerance or sensitivity to different stress conditions in mutants tolerant to oxidative stress. (D) Tolerance or sensitivity to different stress conditions in mutants tolerant to osmotic stress. (E) Tolerance or sensitivity to different stress conditions in mutants tolerant to salinity. (F) Tolerance or sensitivity to different stress conditions in mutants tolerant to ABA. (G) Tolerance or sensitivity to different stress conditions in mutants tolerant to hypoxia.

osmotic stress, but are more sensitive to oxidative stress. These results show an interesting link between heat, cold and oxidative stresses in which tolerance to temperature stress is not necessarily associated with tolerance to oxidative stress, but a reverse association is present. In addition, an interesting link between tolerance to ABA and tolerance to osmotic and salinity stresses is revealed. A reversed effect is also shown between tolerance to ABA and oxidative stress.

As shown in Fig. 5, some mutants that are sensitive to heat or cold are also sensitive to oxidative stress and vice versa (in contrast to the lack of reversed association between tolerance to temperature stress and tolerance to oxidative stress; Fig. 4). We found that some mutants that are sensitive to oxidative stress are also sensitive to salinity stress; some mutants that are sensitive to osmotic stress are also sensitive to salinity, hypoxia and ABA; Some mutants that are more sensitive to salinity are more sensitive to oxidative, cold, osmotic and hypoxia stresses, as well as ABA and vice versa; and some mutants that are more sensitive to hypoxia are more sensitive to osmotic and oxidative stress. These results confirm the links between heat cold and oxidative stress, and reveal and interesting association between sensitivity to ABA and sensitivity to osmotic, salinity, hypoxia and oxidative stress.

## ROS accumulation and transcript expression in two different mutants with altered sensitivity or tolerance to five different abiotic stresses

To test whether our stress screens could reveal new mechanisms involved in the response of plants to abiotic stresses, we selected two genes with an insertion mutant that affected multiple abiotic stress phenotypes and subjected them to further analysis. For this purpose we selected At4g25990, represented in our screens

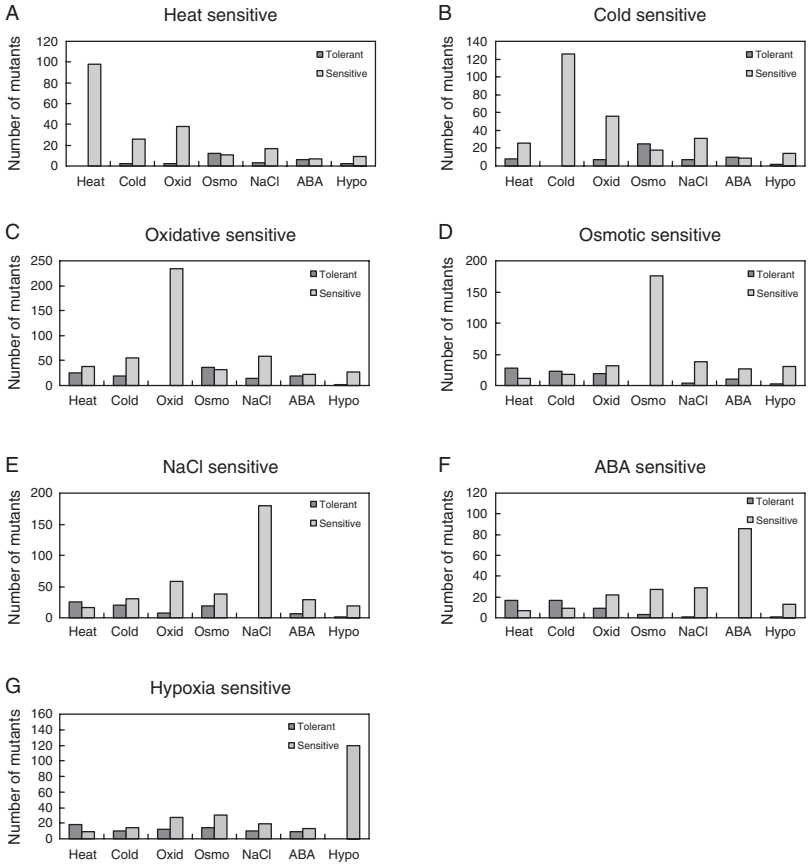


**Fig. 5.** Cross-stress relationships within different groups of mutants sensitive to a specific abiotic stress condition/treatment. (A) Tolerance or sensitivity to different stress conditions in mutants sensitive to heat. (B) Tolerance or sensitivity to different stress conditions in mutants sensitive to cold. (C) Tolerance or sensitivity to different stress conditions in mutants sensitive to oxidative stress. (D) Tolerance or sensitivity to different stress conditions in mutants sensitive to osmotic stress. (E) Tolerance or sensitivity to different stress conditions in mutants sensitive to salinity. (F) Tolerance or sensitivity to different stress conditions in mutants sensitive to ABA. (G) Tolerance or sensitivity to different stress conditions in mutants sensitive to hypoxia.

by the SALK_131539C line that showed sensitivity to four different abiotic stresses, as well as ABA (Fig. 6), and At1g73750, represented in our screens by the SALK_135747C line that showed tolerance to four different abiotic stresses, as well as ABA (Fig. 6). According to TAIR (http://www.arabidopsis.org/), At4g25990 is currently annotated as a CIA2-like protein with biological process unknown, chloroplast, molecular function unknown (CIA2 is a transcription factor which upregulates chloroplast translocon genes). Based on cell-type specific analysis of mRNAs in polyribosome complexes, this protein is primarily synthesized in mesophyll cells of seedling (Mustroph et al. 2009). At1g73750 is currently annotated as an uncharacterized conserved protein UCP031088 containing InterPro domain for $\alpha/\beta$ hydrolase. In contrast to At4g25990, transcripts of At1g73750 are more globally expressed across most cell types of seedlings (Mustroph et al. 2009).

As shown in Fig. 7, both At4g25990 and At1g73750 transcripts are regulated by abiotic stresses. Transcripts of At4g25990 are elevated in response to heat, drought and cold stresses, and those of At1g73750 are elevated in response to heat drought and salt stresses, but are reduced in response to cold. The abundance of both gene transcripts is unchanged during oxidative stress. The results shown in Figs 6 and 7 indicate that while some correlation can be found between change in transcript level during stress and stress tolerance (i.e. salinity for At4g25990), deducing the involvement of a particular gene in a stress response based on change in mRNA abundance might not always be an accurate practice, underscoring the significance of our direct stress tolerance measurements approach for gene function analysis.

Reduced growth, as well as early or late bolting, in plants grown under controlled growth conditions has been previously associated with altered expression

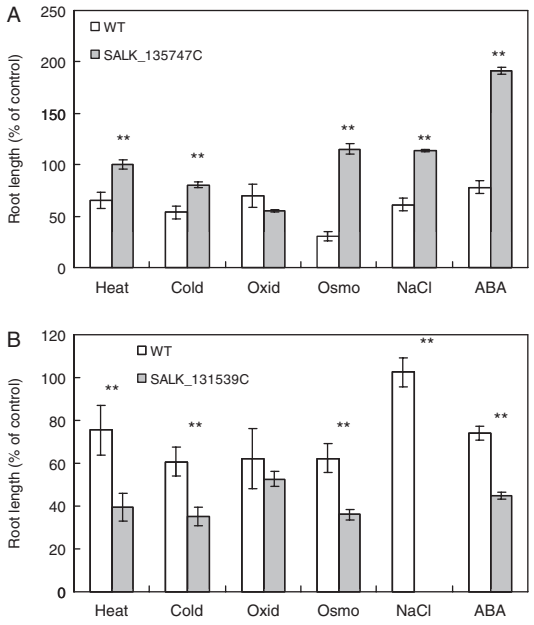


**Fig. 6.** Representative abiotic stress and ABA root growth assays for SALK_135747C (A) and SALK_131539C (B) showing enhanced tolerance of SALK_135747C to different stresses and suppressed tolerance of SALK_131539C to abiotic stresses.

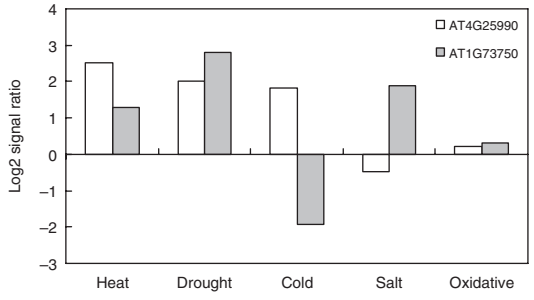


**Fig. 7.** Transcript expression of At4g25990, represented in our screens by the SALK_131539C line, and At1g73750, represented in our screens by the SALK_135747C line, in response to different abiotic stress conditions. Data was obtained from the Genevestigator database as described in section Materials and methods.

of abiotic stress-response genes (Mittler and Blumwald 2010). As shown in Fig. 8, compared to wild-type plants, both SALK lines, deficient in At4g25990 or At1g73750, had reduced growth and delayed bolting, strengthening the link between altered responses to abiotic stresses and plant development and providing additional support to the altered abiotic stress-response phenotype of these mutants.

Accumulation of ROS in plants has been directly linked with responses to abiotic stresses (Suzuki et al. 2012). The accumulated ROS could be a by-product of altered metabolic status during stress or a result of active

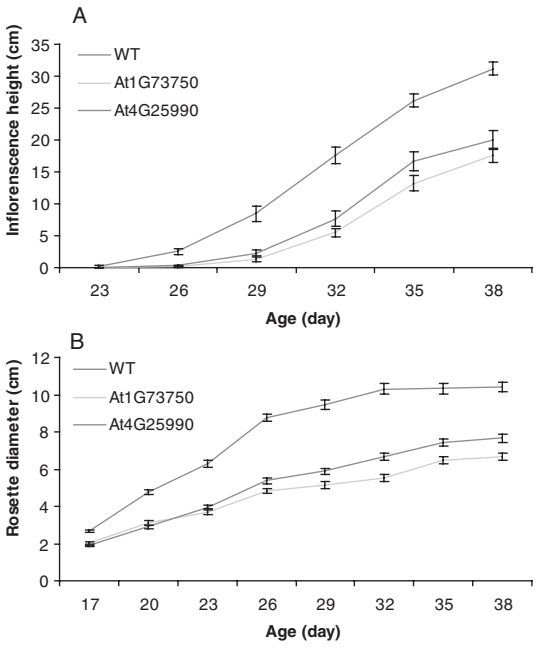


**Fig. 8.** Growth of the SALK_131539C and SALK_135747C lines under controlled growth conditions. Growth of the two lines was assayed by measuring inflorescence height (A) and rosette diameter (B) at different days post germination.

production of ROS as part of the abiotic stress-response signaling pathway (Mittler 2002, Mittler et al. 2004). As shown in Fig. 9, SALK_131539C (At4g25990), which was more sensitive to abiotic stresses than wild type (Fig. 6), had a low basal level of ROS and accumulated ROS to a higher level when treated with paraquat, a superoxide radical producing agent. In contrast, SALK_135747C (At1g73750), which was more tolerant to abiotic stresses (Fig. 6), had a significantly high basal level of ROS and did not accumulate ROS to a level higher than the basal level in the wild type when treated with paraquat. A high basal level of ROS could activate different abiotic stress-response genes and render plants more tolerant to abiotic stresses and could provide a partial explanation to the high abiotic stress tolerance of the SALK_135747C (At1g73750) line.

To test whether altered expression of abiotic stress-response transcripts are associated with the phenotype exhibited by the two mutants, we examined the abundance of six different transcripts in the two mutants and wild-type seedlings grown under controlled growth conditions. For this analysis, we chose the following transcripts: *MBF1c* (AT3G24500) associated with heat and drought (Suzuki et al. 2011a); *ZAT12* (AT5G59820) associated with ROS accumulation and many different abiotic stresses (Miller et al. 2009); *WRKY40* (AT1G80840) and *WRKY70* (AT3G56400) associated

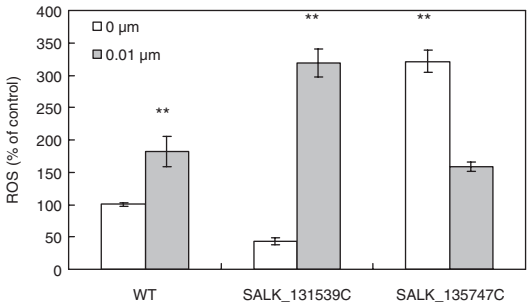


**Fig. 9.** ROS accumulation in seedlings of the SALK_131539C and SALK_135747C lines grown under controlled growth conditions, or treated with 0.01 µ*M* paraquat.

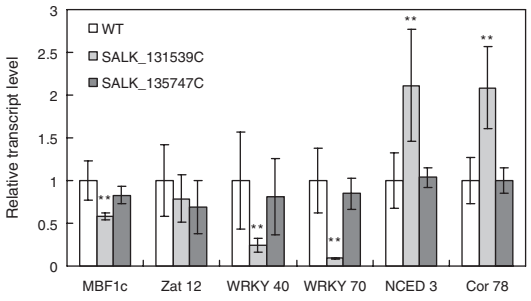


**Fig. 10.** Transcript expression in seedlings of the SALK_131539C and SALK_135747C lines grown under controlled growth conditions. qPCR was used to measure the steady-state transcript level of transcripts encoding MBF1c, associated with heat and drought; Zat12, associated with ROS accumulation and many different abiotic stresses; WRKY40 and WRKY70, associated with ROS levels and responses to biotic and abiotic stresses; NCED3, associated with salinity stress; and Cor78, associated with salinity, cold and drought stresses.

with ROS levels and responses to biotic and abiotic stresses (Davletova et al. 2005); *NCED3* (AT3G14440) associated with salinity stress (Barrero et al. 2006) and *Cor78* (AT5G52310) associated with salinity, cold and drought stresses (Kreps et al. 2002). As shown in Fig. 10, the abundance of *MBF1c*, *WRKY40* and *WRKY70* mRNAs was reduced, and that of *NCED3* and *Cor78* was elevated, in the abiotic stress sensitive SALK_131539C (AT4G25990) line (Fig. 6). In contrast, the abundance of all six transcripts was unaltered under nonstress conditions in the SALK_135747C (AT1G73750) line which was more tolerant to abiotic stresses (Fig. 6) and accumulated high basal levels of ROS (Fig. 9). These findings could provide a partial explanation to the enhanced sensitivity of SALK_131539C (AT4G25990) that appears to be limited in some transcripts associated with stress survival. In contrast, the effects displayed by the SALK_135747C (AT1G73750) line point to a completely unknown mechanism that involves ROS accumulation, but without constitutive elevation of *ZAT12*, *WRKY40* or *WRKY70* mRNAs.

## Discussion

For our abiotic stress analysis we obtained, bulked and analyzed 1007 confirmed knockout lines from the SALK project. Because the mutations in these lines are thought to be 85–90% accurate (Ajjawi et al. 2010), the data reported here should be considered preliminary. Large-scale mutant phenotype screens are very time consuming, and at this stage of this study our results are not supported by a second independent knockout line or complementation experiments. Thus, the false positive rates of phenotypes are likely to be high (~10–15%). Nevertheless, because of the large number of mutants tested (Fig. 1), we believe that our analysis supports many of the findings reported in Figs 3–5 describing cross-associations between different abiotic stresses, as well as provides a valuable resource for determining a putative function and initial annotation for many of the abiotic stress-response proteins with a completely unknown or poorly characterized function. Moreover, our further analysis of two selected mutants (Figs 6–10) demonstrates that the abiotic stress analysis approach we used can identify some very interesting phenotypes.

Among the different abiotic stresses tested, osmotic and oxidative stresses appeared to have the highest phenotypic penetrance (Figs 1 and 2) suggesting that acclimation to these stresses might require the involvement of a larger set of genes compared to the other stresses. Could acclimation to osmotic or oxidative stresses be more complex than acclimation to heat, cold, hypoxic or salinity stresses? More studies are required to address this question; however, it seems likely because osmotic and oxidative stress could affect a larger proportion of cellular functions compared to the other stresses tested. Interestingly, compared to cold, osmotic, oxidative, hypoxic and salinity stresses, in which more mutants were found to be sensitive (as opposed to tolerant) to the stress, more mutants were found to be tolerant than sensitive to heat stress (Figs 1 and 2). This finding could suggest that many of the plant responses to heat stress are regulated by suppressors and that the loss of these (in the knockout lines) causes heat stress tolerance. Alternatively, tolerance to heat stress could be induced by mutations in pathways that are not directly related to heat stress, but cause the triggering of the heat stress response, similar to some of the transgenic lesion mimics or lesion mimic mutants (Mittler and Rizhsky 2000).

Our analysis of tolerance or sensitivity to more than one abiotic stress (Figs 3 and 5) clearly shows that many of the genes disrupted in the different mutants

are involved in responses to more than one abiotic stress (832 out of 1007 mutants showed tolerance or sensitivity to more than one abiotic stress/treatment). These genes could have a general function against stress (e.g. universal stress proteins; Kvint et al. 2003), or function relatively high on the abiotic stress-response signal transduction pathway leading to the activation of multiple acclimation mechanisms (e.g. certain kinases/phosphatases; Reddy et al. 2011). We previously hypothesized, based on bioinformatics analysis of genes with unknown function in different genomes (Gollery et al. 2006, 2007), that genes of unknown function could function in specific signal transduction or networking roles. The findings suggest that the majority of mutants characterized in this study have a phenotype to more than one abiotic stress/treatment (Fig. 3), support our previous hypothesis (Gollery et al. 2006, 2007) and demonstrates that many of the Arabidopsis genes with unknown or poorly characterized function included in this study could be involved in signaling networks regulating the activation of acclimation mechanisms during stress. In contrast to the high proportion of mutants with an altered phenotype to more than one abiotic stress/treatment, only 197 mutants showed specificity to a single stress. These mutants could be related to stress-specific mechanisms that are activated at a downstream point along the signal transduction path. Of six genes with altered insertion mutant survival under hypoxia stress, overexpression lines of all were shown to alter survival of hypoxia and/or prolonged submergence (Mustroph et al. 2010, Lee et al. 2011). Furthermore, only 137 mutants did not show any abiotic stress-related phenotype suggesting that our RNA-profiling-based method for selecting genes of unknown function for further analysis is reliable and that there is a good correlation between transcript expression during abiotic stress and gene function.

At least three major types of abiotic stresses were found to be linked by the analysis shown in Fig. 3 and could use common signaling and/or acclimation pathways. These included heat, cold and oxidative stress (tolerance or sensitivity), salinity, osmotic and ABA (tolerance), and salinity and oxidative stress (sensitivity). A link between salinity and oxidative stress was previously found in our gain-of-function analysis on proteins with unknown function (Luhua et al. 2008), and is now supported by our loss-of-function analysis (Figs 3–5). The relationships between the different abiotic stresses tested were further refined by the analysis shown in Figs 4 and 5. Interestingly, mutants that were tolerant to heat were also tolerant to cold and vice versa, but these mutants were not more tolerant to oxidative stress (Fig. 4). In contrast, mutants that were sensitive to heat were also sensitive to cold as well as oxidative stress and vice versa (Fig. 5). This finding suggests that sensitivity to heat or cold could be mediated by some pathways that are related to oxidative stress. In contrast, tolerance to heat or cold is not. The link between heat and cold stresses (Figs 3–5) is interesting by itself because, discounting the function of some chaperones, many of the known pathways for cold or heat tolerance are not thought to be related and the study of heat or cold signal transduction pathways did not reveal a considerable overlap between these two pathways (Chinnusamy et al. 2007, Mittler et al. 2012). Cold, salinity and osmotic stress are considered to be somewhat related in their effects on plants and the pathways they activate (Kreps et al. 2002). This association was supported by our analysis (Figs 4 and 5). Interestingly, sensitivity to ABA correlated with sensitivity to osmotic, salinity, hypoxia or oxidative stress (Fig. 5). Mutants sensitive to ABA could be deficient in degrading ABA or conjugating it to different compounds. Such deficiency could cause accumulation of ABA to high levels during osmotic or salinity stresses and would explain why these mutants are also sensitive to these stresses (Fig. 5). The finding that mutants sensitive to ABA are also sensitive to hypoxia or oxidative stress suggests an important role for ABA in the response of Arabidopsis to these stresses. A link between sensitivity to oxidative or osmotic stresses and sensitivity to hypoxia was also found (Fig. 5), suggesting that hypoxia stress could involve ROS accumulation and/or alterations in osmotic potentials. Indeed, both ROS and ABA have been shown to be involved hypoxia responses and submergence tolerance in a number of plant species including rice (Bailey-Serres and Voesenek 2008, Fukao et al. 2011).

Our results should be viewed as an initial characterization of genes with unknown, or poorly characterized, function involved in acclimation to different abiotic stresses. We hope that our analysis will contribute to the annotation of these genes and provide a promising ground for new discoveries in the field of abiotic stress responses in plants. Although genes of unknown, or poorly characterized, function are usually not selected by researchers as a subject for further analysis (Gollery et al. 2006), our analysis demonstrates that the function of many of these proteins is important for plant tolerance to abiotic stresses and that these genes should be included in future studies in this field.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1**. Primers used for qPCR analysis shown in Fig. 10.

**Table S1**. Stress screen results of confirmed SALK lines for genes with an unknown or poorly characterized function.

**Table S2**. Gene annotation (October 2011) for the selected genes used in this study.

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