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Identification of novel stress-responsive biomarkers from gene expression datasets in tomato roots

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Abiotic stresses such as heat, drought or salinity have been widely studied individually. Nevertheless, in the nature and in the field, plants and crops are commonly exposed to a different combination of stresses, which often result in a synergistic response mediated by the activation of several molecular pathways that cannot be inferred from the response to each individual stress. By screening microarray data obtained from different plant species and under different stresses, we identified several conserved stress-responsive genes whose expression was differentially regulated in tomato (*Solanum lycopersicum* L.) roots in response to one or several stresses. We validated 10 of these genes as reliable biomarkers whose expression levels are related to different signalling pathways involved in adaptive stress responses. In addition, the genes identified in this work could be used as general salt-stress biomarkers to rapidly evaluate the response of salt-tolerant cultivars and wild species for which sufficient genetic information is not yet available.

Additional keywords: abiotic stress, gene expression profiling, stress biomarkers, salt-stress responsive genes.

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Stress-responsive biomarkers in tomato roots

In nature and in the field, plants and crops are commonly exposed to different stresses. By screening public gene expression data, we identified several conserved stress-responsive genes in tomato roots that could be used as biomarkers to rapidly evaluate the response of salt-tolerant cultivars and wild species for which sufficient genetic information is not yet available.

Introduction

Several lines of evidence indicate that the increase in atmospheric CO_2 level is driving global climate changes that will likely result in a higher frequency of heatwaves, episodes of prolonged drought and flooding (Bates et al. 2008). Since plants are not able to physically escape these stresses, adverse environmental conditions represent a serious challenge for agricultural production (Mittler and

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Blumwald 2010). This fact, together with the increasing demand for food caused by a rising worldwide population make necessary an increasing understanding of how plants and crops respond to a combination of stresses. Abiotic stresses such as heat, drought or salinity have been widely studied individually, but in ecosystems and in the field, plants and crops are commonly exposed to a combination of different stresses such as heat and drought, or salinity and drought, for example (Mittler 2006). Further, the combination of different abiotic stresses often results in a synergistic response mediated by the activation of several molecular pathways that cannot be inferred from the response to each individual stress alone (Pnueli et al. 2002; Rizhsky et al. 2002, 2004; Mittler 2006). In turn, the adaptive responses to different abiotic stresses also imply the activation of specific biotic-response pathways that could positively or negatively affect plant survival. For instance, it has been reported that *Arabidopsis* plants showed an enhanced susceptibility to *Pseudomonas syringae* and *Hyaloperonospera arabidopsidis* in drought conditions (Mohr and Cahill 2003). Furthermore, the increase of tolerance to drought, salt and osmotic stress in tomato and barley fosters the resistance to different pathogens (Wiese et al. 2004; Achuo et al. 2006).

These and other results indicate that the response to diverse stresses involves a complex and coordinated crosstalk between different signalling pathways (Zhu 2002). The level at which these pathways interact includes different signalling complexes; calcium and/or reactive oxygen species (ROS) signalling; mitogen-activated protein kinase (MAPK) cascades; and stress hormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (Bowler and Fluhr 2000; Cardinale et al. 2002; Zhu 2002; Xiong and Yang 2003; Mittler 2006). Among stress hormones, ABA is one of the most important components of the abiotic-stress transduction pathways. However, diverse studies have determined the existence of a complex interplay between ABA, SA, JA and ethylene in response to different abiotic stresses (Grant and Jones 2009; Pieterse et al. 2009).

The existence of multiple transcriptome datasets for different abiotic stresses and in different species provides the opportunity to identify and characterise key conserved genes downstream of shared stress responses. Some of these genes might be eventually used as biomarkers for the evaluation of stress responses in crop breeding programs; they could also expand our knowledge about the genetic mechanisms underlying the responses to combined stresses in non-model crops. In tomato (*Solanum lycopersicum* L.), for instance, salt-specific responsive genes have been identified using a combination of suppression subtractive hybridisation and microarray analyses (Ouyang et al. 2007; Sun et al. 2010).

Because of its agronomic interest, we are using tomato as a crop model system. Given that a large number of microarray datasets are publicly available and with the aim to contribute to a better knowledge of the complex molecular mechanisms of plant abiotic responses in this species, we performed a data mining approach to identify conserved salt stress-responsive genes and to search for tomato genes whose expression in roots was significantly affected by more than one abiotic stress

conditions. Next, we experimentally validated whether the expression of some of the genes selected was affected by several stresses, in order to be used as biomarkers for the quantification of molecular adaptive responses to single or combined abiotic stresses in this and other species.

Materials and methods

Data mining for the identification of putative stress-responsive genes

To find genes whose expression was altered in response to salt stress, we searched available literature for comparative microarray analyses between salt-tolerant and salt-susceptible cultivars in different plant species (Rensink et al. 2005; Cotsaftis et al. 2011; Fujita et al. 2011; Iyer-Pascuzzi et al. 2011; Yao et al. 2011; Huang et al. 2012). We selected for further analysis some of the functionally validated genes whose expression was significantly altered upon salt stress (see Table S1, available as Supplementary Material to this paper). To identify putative tomato (*Solanum lycopersicum* L.) homologues of the genes selected, their protein sequences were searched for homology against all annotated tomato proteins (ITAG release 2.30) using the BLAST tool in the Sol Genomics Network database (https://solgenomics.net/). The expression in roots of these tomato orthologues upon salt and drought stress was confirmed by the analysis of available microarray data (GSE16401; (Sun et al. 2010) using the Anatomy Tool in Genevestigator 4.

In addition, four tomato microarray datasets from experiments related to abiotic stresses: salinity (GSE16401; (Sun et al. 2010), nitrogen availability (GSE21020; (Ruzicka et al. 2010), and drought and heat stresses (GSE22304), were screened for genes with differential expression by using the GEO2R tool. Additional information about the datasets considered could be found at Gene Expression Omnibus (Barrett et al. 2013); http://www.ncbi.nlm.nih.gov/geo/) The Affymetrix microarrays used in these studies contained probes for 7405 tomato unigenes, which only represent 39% of the 18 051 genes identified in the tomato genome (The Tomato Genome Consortium 2012). Differentially expressed genes were defined by probes with a Benjamini-Hochberg adjusted $P \leq 0.05$ (Benjamini and Hochberg 1995) and a fold change >1.75. Only unambiguous probes hybridising with unique tomato genes were kept for further analyses. Relative expression data from the selected genes was processed using the pheatmap package of R Development Core Team (R Foundation for Statistical Computing, Vienna, Austria); http://www.r-project.org/). Euclidean distance matrixes between genes (rows) and between fold-change expression in different experiments (columns) were calculated to build the dendrograms. Gene ontology (GO) terms analysis in the set of differentially expressed genes and pathways that were affected by the stress-treatment was performed using the Plant MetGenMAP (Joung et al. 2009) and statistically significantly-enriched GO categories assigned based on a Benjamini-Hochberg adjusted $P \leq 0.05$

Plant material and growth conditions

Seeds of Ailsa Craig (AC), a wild-type tomato cultivar, and *flacca* (*flc*), an ABA deficient mutant affected in a molybdenum cofactor sulfurase required for ABA biosynthesis (Sagi et al. 2002) were kindly provided by Ian Dodd (University of Lancaster, UK). Seeds were washed in 70% (v/v) ethanol for 30 s, surface-sterilised in 2% (w/v) sodium hypochlorite for 12 min, and rinsed thoroughly with sterile distilled water (five times). On the first experiment (early response), 3–5 seeds of each genotype were sown on a square Petri dish containing 100 mL of half-Murashige and Skoog (Murashige and Skoog 1962) basal salt medium (Duchefa Biochemie, BH Haarlen, The Netherlands), 20 g l^{-1} sucrose (Duchefa Biochemie), 8 g l^{-1} plant agar (Duchefa Biochemie), and 0.5 g l^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES; Duchefa Biochemie), pH 5.8. Six plates were sown per genotype. After a 4 day stratification period at 4°C in the dark, seeds were left to germinate in near-vertical positions in a growth chamber at 24 ± 1°C, 60% RH and a 16 h day (PPFD at 150 µmol m^{-2} s^{-1}) and 8 h night cycle. Seedlings of both genotypes were grown as described until the first lateral roots emerged (at ~7 days), when plants were transferred to new Petri dishes supplemented with 75 mM NaCl, 1 µM 2-*cis*, 4-*trans*-abscisic acid (ABA; Duchefa Biochemie) or mock (DMSO) treatment. Three randomly collected roots from each plate were harvested at 6 h after the transfer to the supplemented media, frozen in liquid nitrogen and stored at −65°C until analysis. Three biological replicates were collected per each genotype, treatment and time-point.

For the long-term experiments on salt, seeds of AC were germinated in vermiculite at 26–28°C, 80–90% RH and darkness. Following germination, growth chamber conditions were set at 16 h day (PPFD at 245 µmoles m^{-2} s^{-1}) and 8 h night cycle and 40–60% RH. The air temperature ranged from 25 to 28°C during the day and 17 to 18°C at night. Fourty days after sowing, the plants were transferred to a hydroponic culture by using 20 L plastic trays containing half-strength Hoagland's nutrient solution. After 1 week of acclimatisation, plants were exposed to 0 (control) or 100 mM NaCl added to the nutrient solution for 11 days. The roots of each plant were harvested and immediately frozen with liquid nitrogen and stored at –80°C until analysis. Three biological replicates per each treatment were chosen for RNA isolation and subsequent real-time quantitative PCR quantitative (RT-qPCR) analysis.

For microarray experiments, a recombinant inbred line known as A4 from the cross *S. lycopersicum* var. *cerasiforme* E9 × *Solanum pimpinellifolium* L. line L5 (Monforte et al. 1997) was used. This RIL was a control line in a study to fine map a quantitative trait loci (QTL) that affected fruit weight under salinity (AAJ Thompson, Z Kevei, unpubl. data). The rootstock of A4 was grafted to scions of *S. lycopersicum* cv. Boludo in 20 mL modules of peat-based compost, and then 3-week-old grafted plants were transplanted into pots containing medium grade perlite (Sinclair, LBS Horticulture, Colne, Lancashire, UK). For the control treatment, plants were irrigated daily to pot capacity with Hoagland solution containing 1 mM H_3PO_4 (control treatment with excess phosphorus). For the drought and low

phosphorus multi-stress treatment, plants were initially irrigated to pot capacity with Hoagland solution containing no added H_3PO_4 for a period of 19 days after transplanting (to deplete phosphorus in the transplanted peat block); then, for the subsequent 10 days, the pots were irrigated to 70% of pot capacity with Hoagland solution containing a low H_3PO_4 concentration (10 μM) to provide the drought and low phosphorus treatment. For microarray analysis ~0.5 g of root tissue was sampled midway between the base of the plant and the base of the pot by removing a handful of root/perlite material during the time period 1000 and 1100 hours Greenwich mean time (GMT) (dawn was at 0548 hours). Perlite was washed from the roots with tap water (sampling process taking ~90 s) and then the roots were frozen in liquid nitrogen.

RNA isolation and first-strand cDNA synthesis for real-time quantitative PCR assays

Total RNA from frozen tomato roots (~150 mg) was extracted using Tri-Reagent (Sigma-Aldrich, St Louis, MO, USA). Contaminating genomic DNA was removed by 20 min incubation at 37°C with 4 units of DNase I (Thermo Fisher Scientific, Waltham, MA, USA). After DNase I inactivation at 70°C for 15 min, RNA was ethanol-precipitated and resuspended in 30 μL of diethylpyrocarbonate (DEPC)-treated water. The first strand cDNA was synthesised with 1 μg of purified RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). The resulting cDNA was diluted by adding 40 μL of sterile distilled water.

Gene expression analysis by real-time quantitative PCR

For primer design, small amplicons (92 to 151 bp) were chosen within the first third of the cDNA sequences. To avoid amplifying genomic DNA, forward and reverse primers (20–27 nt) were designed to bind to different exons and the reverse primer was designed to hybridise across consecutive exons. Primer sequences were confirmed to hybridise to unique tomato genes using the BLAST tool (Madden 2013).

Reaction mixes (10 μL) were prepared with 5 μL of the SsoAdvanced SYBR Green Supermix (Bio-Rad), 1 μM of specific primer pairs (Tables 1 and 2) and 0.8 μL of cDNA. PCR amplifications were carried out in 96 well optical reaction plates on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). At least two independent RNA samples and three technical replicates were used per genotype, treatment and time-point. The thermal cycling program started with a polymerase activation step of 30 s at 95°C, followed by 40 cycles (5 s at 95°C, 10 s at 55°C and 20 s at 72°C), and a melt curve (from 65 to 95°C, with increments of 1°C every 5 s). Dissociation kinetic analyses of each amplified product and agarose gel loading and sequencing of the PCR product confirmed its specificity.

To quickly identify the most appropriate housekeeping gene for RT-qPCR validation, we initially selected four known tomato reference genes from a previous study: *ACTIN*, *GAPDH*, *TIP41* and *UBIQUITIN* (Dekkers et al. 2012). Based on the probe expression data from former microarray

experiments (Fei et al. 2011), *SlACTIN7* (*Solyc11 g005330*), encoding the closest homologue of *Arabidopsis ACTIN2*, was selected as the most stable reference gene under different stress treatments.

Relative quantification of gene expression levels was performed using the comparative C_t method (Schmittgen and Livak 2008). Data were represented as the relative gene expression normalised to the C_t value for the tomato housekeeping gene *SlACTIN7* (F: CCAAGCAGCATGAAAATTAAGG; R: CCTTTGAAATCCACATCTGCTG) according to the following calculation: fold-change = $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t$ (target gene) − C_t (*SlACTIN7*) and $\Delta\Delta C_t = \Delta C_t$ (treatment) − ΔC_t (control). Mean of fold-change values were used for graphic representation. ΔC_T values were analysed using SPSS 21.0.0 (SPSS Inc., Cary, NC, USA) by applying the Mann–Whitney *U* test for statistical differences between treated and control samples ($P \leq 0.05$).

Microarray hybridisation and data analysis

Total RNA samples of A4 RIL line roots, using three biological replicates per treatment, were extracted using the SpectrumPlant Total RNA Kit (Sigma-Aldrich) including on–column DNase digestion: 200 ng of RNA was used for cDNA synthesis and Cy3-labelling using the Low Input Quick Amp Labelling Kit for One-Colour Microarray-Based Gene Expression Agilent analysis (Agilent). 1.65 µg of linearly amplified and labelled cDNA was hybridised for 17 h at 65°C on 4 ×180 k format 60-mer oligonucleotide probes designed against the *S. lycopersicum* cv. Heinz 1706 build 2.4 (annotation 2.5) genome (Agilent design ID = 069672; GEO GSE79307). Each array contained ~5 probes for 34 619 transcripts. Arrays were imaged using an MS200 microarray scanner using only the 480 nm laser using the autogain feature of the NimbleScan software. Image (tiff) files were imported into the Agilent Feature Extraction software for quality control assessment, grid alignment and expression value extraction at the probe and transcript level the RMA algorithm (Irizarry et al. 2003) used to carry out background subtraction, quantile normalisation and summarisation via median polish, and output $\log_2$ normalised gene expression levels (GEO GSE79307). Linear Models for Microarray Data (package *limma* in R) was then used to fit linear models to pairs of samples, identifying genes that contrasted the most between the experimental pairs (Smyth 2004). Transcripts were termed to be differentially expressed if they showed a Benjamini-Hochberg adjusted *P*-value ≤ 0.05 in the comparison between treatment and control.

Results

Bibliographic review to identify conserved salt stress-responsive genes

From existing literature, we searched microarray experiments designed to identify salt-stress regulated genes in plant species other than tomato (see 'Materials and methods'). A subset of 133 functionally validated salt-stress responsive genes was selected from these experiments and their putative tomato orthologues were identified by protein sequence similarity. This generated a list of

107 genes, 85 of them with expression data available (Table S1). GO term analyses were performed on this dataset and genes belonging to 'response to osmotic stress' (15 genes), most of which are involved in hormone responses, mainly ABA and JA, were found significantly enriched ($P < 0.001$). We then evaluated the stress-regulated expression of the selected tomato homologues by studying available microarray data (See 'Materials and methods'), and clustered them to eight groups (I to VIII) determined according to their differential expression upon salt stress and drought stress, respectively (Fig. 1a; Table S1). We found that ~85% of the tomato genes selected from the literature ($n = 73$) were found differentially expressed in response to salt and/or drought stresses in these microarray experiments (Fig. 1a). Finally, a subset of 13 genes representing most of the expression clusters identified (Table S1) were chosen for functional validation by RT-qPCR as tomato salt stress-responsive biomarkers (see 'Materials and methods'; Table 1). In a first experiment, relative gene expression was assayed in young AC tomato roots after a short pulse (6 h) of mild salt-stress (75 mM NaCl) or control treatment (see 'Materials and methods'). Nine of the genes studied (□70%) displayed a slight but significant differential expression upon salt-stress in these conditions (Fig. 1b). Three of these genes (Solyc04 g078840, Solyc09 g015770 and Solyc12 g013620) displayed an increase or decrease of >1.75-fold after the salt treatment, suggesting that their expression was highly sensitive to the salt signal.

Next we asked whether these early salt-responsive genes identified were also influenced by ABA (see 'Materials and methods'). The *Solyc04 g078840*, *Solyc08 g043170*, *Solyc11 g011050*, *Solyc11 g069030* and *Solyc12 g013620* genes also showed altered expression upon ABA treatment (Fig. 1c), which suggests that their salt-responsiveness might be directly regulated through the ABA signalling pathway. On the other hand, the expression of the *Solyc09 g015770* gene, encoding a homologue of the *Arabidopsis* WRKY70 (Li et al. 2004), was not significantly affected by the ABA treatment despite its higher downregulation by salt. We noted that the expression of all the other genes that were not affected by the salt-pulse in the wild-type accession, was significantly altered in the roots of the ABA-deficient *flacca* (*flc*) mutant (Sagi et al. 2002) (Fig. 1d), suggesting that their salt-induced regulation occurred in later stages.

Data mining public microarray data to identify abiotic stress-responsive genes

To identify a general set of abiotic stress biomarkers in tomato, we selected four experiments from the BioProject database (Pruitt et al. 2011) that compared microarray profiling of stressed vs non-stressed samples of different tomato cultivars with contrasting tolerance to a given abiotic stress (salt, drought, heat, and nitrogen availability; see 'Materials and methods'). Pairwise comparisons for probe gene expression among the entire dataset indicate moderate but significant correlations between salt stress and drought stress (Fig. 2a; Table S2). Hereafter, we selected 2028 genes (27.4% of the genes represented in the array) showing differential expression ($P \leq 0.05$) upon salt stress in either susceptible or tolerant cultivars (5 h with 200 mM NaCl) for further analysis. On the one hand, ~45%

of salt-upregulated genes and 34% of salt-downregulated genes were shared between susceptible and tolerant tomato cultivars where the most significantly-enriched GO categories for shared upregulated genes were ‘ion binding’ (GO:0043167) and ‘response to stimulus’ (GO:0050896), whereas ‘tetrapyrrole binding’ (GO:0046906) was significantly enriched among shared downregulated genes (Fig. 2b). On the other hand, several genes were found to have altered expression in both susceptible (Table S2) and tolerant (Fig. 2c) tomato species in response to several individual stresses.

To reveal the shared molecular mechanisms of different stress responses in tomato, we further classified the selected genes according to their differential expression ($P \leq 0.05$) after salt stress, drought stress and heat stress either in susceptible or tolerant subtypes respectively (Fig. 2d; Table S3). We noted that the expression of genes belonging to the GO category ‘response to stimulus’ (GO:0050896), most of which were involved in ABA, auxin and chitin responses, were found significantly altered by one or more of these stresses (Fig. 2d; Table S3). Genes encoding integral proteins of the chloroplast envelope and thylakoids (GO:0009579) were significantly enriched among the set of genes whose expression changed by salt stress or drought stress in susceptible cultivars. Gene functions involved in tetrapyrrole binding (GO:00046906) were significantly enriched only by the salt stress (Fig. 2d; Table S3), which might be related to the alteration of the photosynthetic function through tetrapyrrole-mediated retrograde signalling (Brzezowski et al. 2015) by salt stress, similarly as it has been described for high light stress (Estavillo et al. 2011). These results suggest (i) that the quantitative regulation of the expression in a subset of these genes might account for the adaptive responses observed in tolerant species, and (ii) that multiple genes are similarly responding to different stress signals.

We then selected 501 genes whose expression was significantly changed ($P \leq 0.05$) upon salt stress in both susceptible and tolerant tomato cultivars and that also showed moderate differential expression ($P \leq 0.10$) upon drought stress and heat stress in susceptible cultivars (Table S4). Some of the most significantly-enriched GO categories within this dataset were ‘response to stress’ (GO:0006950; 77 genes; $P = 3.8e\text{-}04$) and ‘response to hormone stimulus’ (GO:0009725; 41 genes; $P = 2.41e\text{-}05$). A heatmap representation of the relative gene expression levels enabled us to group them into 22 clusters, ranging in size from five (cluster 4) to 40 (cluster 11) genes (Fig. 3; Table S4). Genes in clusters 1 to 11 were clearly downregulated in response to several abiotic stresses, mainly salt and drought, and the GO categories ‘regulation of G2/M transition of mitotic cell cycle’ (GO:0010389; seven genes; P value = 2.1e-04) and ‘chlorophyll biosynthetic process’ (GO:0015995; four genes; $P = 3.4e\text{-}04$) were found over-represented in these clusters. Conversely, genes in clusters 12 to 22 were upregulated by stress and some of the most significantly-enriched GO categories were ‘response to stress’ (47 genes; $P = 1.1e\text{-}04$) and ‘cellular amino acid catabolic process’ (GO:0009063; eight genes; $P = 1.9e\text{-}06$). Other genes belonging to the categories ‘ethylene-activated signalling pathway’ (GO:0009873; seven genes; $P = 7.4e\text{-}04$), ‘response to chitin’ (GO:0010200; eight genes; P = 2.4e-

04), and ‘response to ABA stimulus’ (GO:0009737; 13 genes; $P = 1.3e\text{-}04$) were also found commonly upregulated by salt and drought stress.

Fourteen genes across a range of the expression clusters shown in Fig. 3 were selected based on the following criteria: (1) their putative encoding proteins belonged to some of the most GO-enriched categories found, and (2) they were expressed in roots in previous microarray experiments (see Materials and methods; Table 2). Their relative gene expression levels were tested in young roots of the AC background after a pulse of salt-stress (6 h, 75 mM NaCl) and compared with that of control-treated plants (see ‘Materials and methods’). The expression of 11 of the studied genes (~80%) was found significantly differentially regulated by the salt treatment (Fig. 4A). Six genes (*Solyc01 g107730*, *Solyc01 g111660*, *Solyc02 g069490*, *Solyc03 g096460*, *Solyc05 g007180* and *Solyc07 g043130*) were downregulated, whereas the other five (*Solyc01 g079200*, *Solyc02 g084850*, *Solyc03 g006880*, *Solyc03 g095510* and *Solyc07 g049530*) were upregulated after the salt pulse. Our qPCR results were mostly in agreement with the results from previous microarray data and confirmed that the selected genes might be used as early-responsive, root-specific and salt stress-regulated biomarkers. In the other three genes tested in our study, the differences in relative expression levels between the two treatments were not statistically significant (Fig. 4a), although two of them showed a significant downregulation in the ABA-deficient *flacca* mutant (Fig. 4b). Next, we tested whether the expression of these 11 salt stress-regulated genes was also dependent on the ABA signal (Fig. 4c). Only four of these genes (*Solyc02 g084850*, *Solyc03 g006880*, *Solyc03 g095510* and *Solyc07 g049530*) displayed differential expression upon 1 μM ABA treatment. Among those, *Solyc07 g049530* encodes a putative ACC oxidase involved in ethylene biosynthesis and whose *Arabidopsis* orthologue, *At1 g05010*, was upregulated under drought stress (Winter et al. 2007). Another gene strongly upregulated by salt and ABA was *Solyc02 g084850*, encoding the Tas.14 dehydrin (Godoy et al. 1994).

Verification of selected genes as multiple abiotic stress-responsive biomarkers

We next asked whether the 28 stress-responsive genes selected in previous sections for RT-qPCR validation (Tables 1, 2) could also be used to quantify the responses of the root system to other stresses, such as drought or nutrient deficiency. To this end, we gathered microarray data obtained from a multi-stress experiment using RIL A4 derived from *S. lycopersicum* var. *cerasiforme* line ‘E9’ and *S. pimpinellifolium* line ‘L5’ cross (Estañ et al. 2009). This line was grown in either combined low phosphorus (low P) and drought conditions or optimal conditions (see ‘Materials and methods’). Fourteen of the stress-responsive genes selected were found to be significantly ($P < 0.05$) differentially expressed in low P and drought conditions compared with control plants (Table S5). Only 18% of the genes on the array were found differentially expressed by the combined stress treatment applied, whereas 50% of the selected genes (14 genes out of 28) were responsive to the combined stress treatment, hence, we reasoned that the genes selected were significantly enriched

among the microarray dataset, confirming their value as root-specific multistress biomarkers. Additionally, we collected roots from 10-week-old plants (AC) that were grown in soil during 11 days in salt (100 mM NaCl) or control conditions (Fig. 5a) and studied the expression of several of the stress-responsive genes validated previously (Fig. 5b). The majority (seven) of genes were similarly upregulated by single (salt) or combined (low P and drought) stresses: *GA2ox-3*, *GH3.3*, *Tas.14*, *KIN2*, *AREB1*, *WRKY70*, and *JA2*. We noted that three of the genes selected based on their differential expression after a pulse of salt treatment (*CYCD3*;*2*, *DIM* and *PLAT*) were not significantly affected by the long-term stress conditions assayed here, suggesting that these genes might be involved in the early responses to stress. Several the selected genes whose expression was differentially regulated by several stresses were also affected by a pulse of ABA, while others did not respond to our ABA treatment (Figs 1c, 4c, 5c).

Discussion

In our first approach, we found that the expression of nine putative salt-responsive genes in tomato, identified from expression data gathered from other species, was significantly altered upon a pulse of salt stress (~70% of the genes assayed). These results validate our literature-mining strategy to identify salt-responsive genes in tomato roots through comparative transcriptomic profiling in different plant species challenged by high salt conditions. Several early salt-responsive genes identified in this way were also influenced by ABA. Indeed, *Solyc04 g078840* encodes AREB1, a transcription factor induced by drought and salinity through a canonical ABA-responsive pathway, and whose overexpression confers increased tolerance to salt and water stress in tomato (Orellana et al. 2010). Notably, *Solyc12 g013620* encodes the JA2 transcription factor of the NAC family, which has been recently shown to be activated by ABA and that mediates stomatal closure in rice (Du et al. 2014). We have shown here for the first time that the tomato *JA2* gene is an early salt-stress and ABA-responsive gene in roots, which suggests that the hormonal crosstalk between ABA and JA might also regulate specific adaptive responses under salt stress in tomato. Recent studies have implicated some WRKYs in the negative regulation of the oxidative stress responses (Chen et al. 2010). Indeed, *wrky54 wrky70* double mutants exhibited enhanced tolerance to osmotic stress in *Arabidopsis*, which was likely caused by higher stomatal closure in these mutants (Sun et al. 2010). Although the rapid downregulation found in roots for the *WRKY70* homologue (*Solyc09 g015770*) after the salt treatment might indirectly contribute to osmotic stress adaptation through the regulation of stomatal conductance, additional experiments are required to assess whether the WRKY protein encoded by this gene plays a similar role to that proposed for its *Arabidopsis* counterpart (Li et al. 2013). The expression of other salt-induced genes was significantly altered only at later stages. An example is *Solyc01 g067710*, which encodes a Na^+/H^+ exchanger that has been recently proposed to play a role in long-term Na^+ accumulation in the roots of salt-treated tomato accessions (Almeida et al. 2014). Our results suggest that an evolutionary conserved set of salt-stress responsive genes might control a

similar range of physiological, metabolic and cellular processes in different species. Indeed, salinity stress involves similar changes in different plant species involving various physiological and metabolic processes, such as ion salt homeostasis, biosynthesis of osmoprotectants, antioxidant regulation and hormone modulation (Gupta and Huang 2014). Through appropriate primer design, the genes identified in this work could be used as general salt-stress biomarkers to rapidly evaluate the response of salt-tolerant cultivars and wild species for which not sufficient genetic information is available, such as *Asparagus officinalis* and *Spergularia marina* among others.

Recent work in *Arabidopsis* and rice found that a subset of downstream responses was shared between biotic and abiotic stresses (Sham et al. 2014; Zhang et al. 2015). Consistent with the hypothesis of shared signalling components between biotic and abiotic stresses, chitinase-defective mutants of *Arabidopsis* are also hypersensitive to salt, drought and heat stress due to cell wall alterations that indirectly impair membrane integrity (Kwon et al. 2007). By comparing the responses of *Arabidopsis* to a variety of abiotic and biotic stresses, a common 'universal stress response transcriptome' was previously identified and that contained conserved pathways, such as those related to MAPK cascades, vesicle transport, mitochondrial function and transcription machinery (Ma and Bohnert 2007). In addition, several ABA- and JA-regulated genes were found specifically upregulated in roots in response to several stresses in this species (Ma and Bohnert 2007). We found that a large proportion of differentially expressed genes in tomato were specifically regulated upon salt stress or drought stress. These results suggest that, mirroring findings in *Arabidopsis*, the different abiotic stresses activated similar signal transduction pathways and metabolic responses in tomato roots (Fujita et al. 2006). Additional experiments will be needed to assess whether some signalling components between biotic and abiotic stresses are also shared in tomato.

We have experimentally verified by RT-qPCR that 20 of the genes selected from tomato microarray data displayed differential expression in tomato roots after a pulse of mild salt stress, suggesting their utility as root-autonomous salt stress-responsive biomarkers. Our results revealed the ABA-dependent specific upregulation of the gene encoding an ACC oxidase in tomato roots (*Solyc07 g049530*) and suggested an inhibitory role of increased ethylene levels on primary root growth in response to a mild salt stress in an analogous way to that proposed in *Arabidopsis* (Luo et al. 2014). The availability of tomato mutants impaired in ethylene biosynthesis or ethylene perception (Negi et al. 2010) will allow testing this hypothesis. Another gene strongly upregulated by salt and ABA was *Solyc02 g084850*, encoding the Tas.14 dehydrin (Godoy et al. 1994). Tomato plants overexpressing *Tas.14* achieved improved long-term drought and salinity tolerance without affecting plant growth under non-stress conditions, which might be associated with their ability to rapidly increase ABA levels after perceiving drought stress (Muñoz-Mayor et al. 2012).

As it was previously shown in *Arabidopsis*, salt stress transiently inhibited root growth through cell cycle inhibition in the meristem and when the meristem reached the appropriate size for the given

conditions, cell cycle duration returned to its default (West et al. 2004). Our results on *CYCD3*;*2* (*Solyc01 g107730*) expression suggest that similar regulation might take place in tomato roots in response to salt stress. Among the early upregulated genes by salt stress, *Solyc01 g079200*, encoding a putative GA2 oxidase-3 (GA2ox-3) involved in GA catabolism (Rieu et al. 2008), was particularly interesting. A recent report in hybrid poplar (*Populus tremula* × *Populus alba*) identified GA2ox-encoding genes as targets mediating the shoot growth inhibition and physiological adaptation in response to drought stress in these species (Zawaski and Busov 2014). Our results are in agreement with the hypothesis that GA metabolism and signalling constitute a major regulatory circuit mediating growth restraint and physiological adaptation to unfavourable conditions. However, *Solyc01 g111660*, which encodes the PIP1.2 aquaporin (Reuscher et al. 2013), was found significantly downregulated by salt stress. The expression of other tomato aquaporins in response to salt stress has not been so far investigated, but in *Arabidopsis* aquaporin gene expression in roots dramatically decreased between 2 and 4 h after the salt treatment (Boursiac et al. 2005). It has been proposed that coordinated transcriptional downregulation and subcellular localisation of several aquaporins might contribute to the short- and long-term regulation of root water transport in response to salt stress, by limiting water symplastic transport (and hence transpiration) which prevents fast wilting under water stress (Boursiac et al. 2005). Besides, although the *GH3.3* (*Solyc01 g107390*) gene has been previously shown to be induced by salt stress in tomato (Kumar et al. 2012), its *Arabidopsis* homologue is known to be required for adventitious root development by modulating JA catabolism downstream of the auxin signal in this species (Gutierrez et al. 2012). Hence, further experiments will be required to uncover the link between salinity stress and *GH3.3* expression and whether this gene affects root architecture in tomato. We found that most of the studied genes were similarly upregulated by single (salt) or combined (low P and drought) stresses. In contrast, *P5CS* was significantly upregulated only by salt. Indeed, proline accumulation is a common physiological adaptive response to salinity in many plant species. Proline is mainly synthesised from L-glutamic acid by pyrroline-5-carboxylate synthetase (P5CS) (Verbruggen and Hermans 2008), and *P5CS* expression has been shown to be positively regulated by salt in rice (Bagdi et al. 2015).

In conclusion, by screening available microarray data obtained from different plant species and under different stresses, we identified several genes showing differential expression in tomato roots in response to one or several stresses. We validated 10 of these genes as reliable biomarkers whose expression levels are related to signalling pathways involved in adaptive stress responses. These genes could be used to evaluate, at the molecular level, the stress responses of tomato cultivars that differ in stress tolerance. Due to conservation of genes and their downstream responses, they could also be used to evaluate the stress responses in different species where full transcriptomic information is not yet available.

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Fig. 1. Experimental validation of conserved salt-stress responsive genes in tomato (*Solanum lycopersicum*) roots. (*a*) A four-way Venn diagram showing the number of tomato homologues of conserved salt-stress genes from other species that showed differential expression by salt and drought stress from available microarray data. For 12 of the 85 genes selected there was no significant alteration of expression in this dataset. (*b*, *c*) Real-time PCR quantification of the expression of selected genes in tomato roots in response to (*b*) salt stress or (*c*) ABA treatment. (*d*) Real-time PCR quantification of the expression of selected genes in wild-type and *flc* roots. Relative expression levels were normalised to the *SlACTIN7* gene. Bars indicate the relative expression levels ($2^{-\Delta\Delta Ct}$ method) ± s.e. Significant differences between treatments are indicated: *, $P \leq 0.05$ (75 mM NaCl or 1 µM ABA vs mock) or genotypes (*flc* vs AC).

Fig. 2. Identification of abiotic stress-responsive genes from public microarray data. (*a*) Scatter plots of differentially expressed genes (DEGs) from microarray experiments of different abiotic stresses in tomato species. Numbers indicate the coefficient of determination (r^2) of simple linear regression between pairs of experiments. (*b*) Two-way Venn diagram showing the common DEGs between salt sensitive (red) and salt-

tolerant (green) tomato species. (*c*) Scatter plots of DEGs in response to different stresses in tolerant tomato species. (*d*) Gene ontology (GO) enrichment analysis of shared DEGs between different stresses (salt-, drought- and heat-stress). Numbers between brackets in (*b*) and (*d*) indicate the *P*-value of the GO term shown.

Fig. 3. Clustering of the DEGs in response to several abiotic stresses in tomato. Each row in the column corresponds to a single gene, and the colour scale corresponds to the ratio of expression from red (downregulated genes) to white (upregulated genes). Euclidean distance matrixes between genes (rows) and experiments (columns) were calculated to build the dendrograms. Numbers in the dendrogram indicate the gene clusters identified (see text).

Fig. 4. Experimental validation of abiotic stress-responsive genes in tomato roots. (*a*) Real-time PCR quantification of the expression of selected genes in tomato roots in response to salt stress. (*b*) Real-time PCR quantification of the expression of selected genes in wild-type and *flc* roots. (*c*) Real-time PCR quantification of the expression of selected genes in tomato roots in response to ABA. Relative expression levels were normalised to the *SlACTIN7* gene. Bars indicate the relative expression levels ($2^{-\Delta\Delta Ct}$ method) ± s.e. Significant differences between treatments are indicated: *, $P \leq 0.05$) (75 mM NaCl or 1 μM ABA vs mock) or genotypes (*flc* vs AC).

Fig. 5. Experimental validation of biomarkers for abiotic stress responses in tomato roots. (*a*) Representative scanned images of wild-type tomato roots grown in control conditions and 100 mM NaCl for 11 days. Scale bar: 1 cm. (*b*) Real-time PCR quantification of the expression of selected biomarkers in tomato roots in response to long-term salt stress (red bars) compared with microarray data from combined drought and low phosphorus conditions (orange bars). The blue bar represents the non-stressed treatment and is set to unity for each of the two experiments. Relative expression levels were normalised as regards to that of the *SlACTIN7* gene. Bars indicate the relative expression levels ($2^{-\Delta\Delta Ct}$ method). Significant differences between treatments are indicated: *, $P \leq 0.05$). Abbreviations: AC, Ailsa Craig; A4, tomato RIL (*c*) Proposed model for the selected biomarkers' expression in response to abiotic stresses and their putative role in adaptive stress responses.

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Table 1. Putative tomato salt-responsive genes selected from the literature review

Gene locus	Protein product (synonyms)	Oligonucleotide sequences (5′ to 3′)		Product (bp)	Cluster
Solyc01 g067710	(Sodium/potassium)/proton exchanger 3 (NHX3)	CTTTGCACTGGAGTTGTCATTC	TCACCTGAAACCTGCCAGCAT	130	II
Solyc01 g094690	Water channel protein (PIP1.2, AQP2)	TGTATTGACTGTTATGGGTTATTC	GTTAATGTGTCCACCTGATATG	139	V
Solyc02 g084240	H1 histone-like protein	CAAGGTTAAGGAGCAGGCTTC	AGAGCCTCCTTAATCATCTGAA	135	II
Solyc04 g078840	BZIP transcription factor (AREB1)	GGAGAATGATAAAAAATAGAGAGTC	CATTTCTAACATTTCTTCCTGTTTC	143	II
Solyc06 g005170	Mitogen-activated protein kinase 3 (MPK3)	GAATGAGATGGTTGCAGTTAAG	CATCTCTTAAACCAATGACGTTTTC	128	I
Solyc06 g048410	Superoxide dismutase	GCTTACAATGGAGAACCCAAAAG	TGAGGCTCCAAAGCATCCATTG	115	VI
Solyc07 g062970	Serine/threonine phosphatase family protein (DIG3)	GAACTTGGTCTATTTGCAATATTTG	GCCCAGAAGTTAGGCTCATTG	107	II
Solyc08 g043170	Delta 1-pyrroline-5-carboxylate synthetase (P5CS, PRO2)	TTAGAGATCCAGATTTTAGGAGAC	CAAAATATTCCAGAAGAGTCCTCAT	139	VII
Solyc08 g081540	1-aminocyclopropane-1-carboxylate synthase (ACS1A)	CCAAGAATGGATGGTGAATAAT	TAAACCTTGCAACTGCTTGTCTA	131	III
Solyc09 g015770	WRKY transcription factor (WRKY70, WRKY6)	GTTATAAACAATTCTGATGTCGTCG	TCTGATTCTGAAGTTTTCCTTCTC	131	II
Solyc11 g011050	MYB-related transcription factor (THM16, ODORANT1)	ATGGGGAGACAACCTTGTTGTG	TTCCACATCTCAATAACCCTGCTA	151	I
Solyc11 g069030	MYB-related transcription factor (BLIND)	CTCCATGTTGTGATAAAGCAAATG	CCACATCTCCTTAGTCCTGCTT	142	-
Solyc12 g013620	NAC domain-containing protein (JA2)	TATTTATGTAAGAAAGTTGCTGGAC	CCAAATGTCGCCTTACTAGGTA	107	II

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Table 2. Tomato stress-responsive genes selected from microarray data analysis

Gene locus	Protein product (synonyms)	Oligonucleotide sequences (5′ to 3′)		Product (bp)	Cluster
Solyc01 g079200	Gibberellin 2-β-dioxigenase 2 (GA2ox-3)	TCAATGGAGATAAAGGTGATCTTG	GTAATCATTTGTCACCGAGCTGAA	122	12
Solyc01 g107390	Auxin and ethylene responsive GH3-like protein	CCGGTCGTAACTTATGAAGATC	CTGACGTTCCAGAGCTAGTG	118	12
Solyc01 g107730	D-type cyclin (CYCD3;2)	GACTCAACTTGCTGCTGTCAC	CATATTTTGCATCCTCCACTTGGA	108	8

Solyc01 g111660	Aquaporin/plasma membrane intrinsic protein 3	GACGGAGTTGGTATTCTCGG	TGGGTTAATATGTCCACCAGAG	99	11
Solyc02 g064830	Auxin-responsive GH3 family protein	AGGAAATTCAACCTGATATTCAACG	GCAGATGTCCCCGAGCTGGT	103	12
Solyc02 g069490	FAD linked oxidase domain protein (DWARF1, DIM)	CCACACAAAGTGAGGCTATTAG	CAGCCAAATTGGATATACCTCCA	134	11
Solyc02 g084850	Dehydrin-like protein (Tas.14/RAB18)	GCACTGGTGGAGAATATGGAAC	TCCATCATCCTCCGACGAGC	110	13
Solyc03 g006880	Gibberellin 20-oxidase (GA20ox)	CACTCTCTTTTCGTTACTCCG	AATATTCTTGATAAACATTCCCGAG	114	10
Solyc03 g095510	Protein kinase 2 (KIN2)	GATTTTGGAGAAAGATCACGCTG	GGTATAGTCTGTATTTGGTCTGGA	119	18
Solyc03 g096460	Lipoxygenase homology domain-containing protein 1	GGAGTAGCAGCTCAAGTTAAC	TGTGTAAACACAATCTTCAGCAG	99	8
Solyc05 g007180	Homeobox-leucine zipper protein (ATHB13, HAT7, JA1)	CAAATTTCATGCTACAAACTCCTC	CCCAAAAATGAAGCAATACCATGG	118	9
Solyc07 g043130	Root phototropism protein 2	TGGTGCACTTGTTGTGTTAAAGTC	CGCCTCCACACACGCCTTAG	112	10
Solyc07 g049530	1-Aminocyclopropane-1-carboxylate oxidase (ACCox)	TTAACTTGGAAAAGCTCAATGGAG	GAATTCCATGGTTCACCAACTCAA	105	22
Solyc11 g071620	Aldehyde oxidase	GTTGCCATCCGTTGATCCTTC	CAAGCTCCACAACCACCTTC	105	1

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