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Comparison of salt stress resistance genes in transgenic *Arabidopsis thaliana* indicates that extent of transcriptomic change may not predict secondary phenotypic or fitness effects

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Summary

Engineered abiotic stress resistance is an important target for increasing agricultural productivity. There are concerns, however, regarding possible ecological impacts of transgenic crops. In contrast to the first wave of transgenic crops, many abiotic stress resistance genes can initiate complex downstream changes. Transcriptome profiling has been suggested as a comprehensive non-targeted approach to examine the secondary effects. We compared phenotypic and transcriptomic effects of constitutive expression of genes intended to confer salt stress tolerance by three different mechanisms: a transcription factor, CBF3/DREB1a; a metabolic gene, *M6PR*, for mannitol biosynthesis; and the Na^+/H^+ antiporter, *SOS1*. Transgenic *CBF3*, *M6PR* and *SOS1 Arabidopsis thaliana* were grown together in the growth chamber, greenhouse and field. In the absence of salt, M6PR and SOS1 lines performed comparably with wild type; CBF3 lines exhibited dwarfing as reported previously. All three transgenes conferred fitness advantage when subjected to 100 mM NaCl in the growth chamber. *CBF3* and *M6PR* affected transcription of numerous abiotic stress-related genes as measured by Affymetrix microarray analysis. *M6PR* additionally modified expression of biotic stress and oxidative stress genes. Transcriptional effects of *SOS1* in the absence of salt were smaller and primarily limited to redox-related genes. The extent of transcriptome change, however, did not correlate with the effects on growth and reproduction. Thus, the magnitude of global transcriptome differences may not predict phenotypic differences upon which environment and selection act to influence fitness. These observations have implications for interpretation of transcriptome analyses in the context of risk assessment and emphasize the importance of evaluation within a phenotypic context.

Introduction

Salt stress resulting from saline soils or irrigation water is a major factor limiting agricultural productivity worldwide (Yamaguchi and Blumwald, 2005; Shabala and Cuin, 2008; Munns and Tester, 2008). Increased irrigation, utilization of marginal crop land and increasing demand for food production are all anticipated to increase the rate of salinization, making salinity stress resistance an important goal for crop improvement. In recent years, genetic engineering of crops for environmental stress resistance has become increasingly important (Nickson, 2008; Beckie *et al.*, 2010; Grumet *et al.*, 2011). Field trials in the United States, for the crops engineered for resistances to drought, cold, heat and salt, increased from 23 in 2001 to 119 in 2010, and genetically engineered, drought-tolerant maize is approaching commercialization (USDA-APHIS records, http://www.isb.vt.edu/data.aspx; Edmeades, 2008).

Salt stress in plants is manifested as a combination of dehydration or osmotic-related stress effects owing to reduced water potential resulting from increased solute concentration and damage caused by toxic effects of excess sodium ions (Yamaguchi and Blumwald, 2005; Munns and Tester, 2008). Salt stress is also typically associated with oxidative stress (Hasegawa *et al.*, 2000; Miller *et al.*, 2010). Possible routes to counteract these negative effects include exclusion or sequestration of sodium ions or accumulation of compatible solutes or osmoprotectants. Compatible solutes or osmoprotectants have been suggested to osmotically balance stress-related decrease in water content, stabilize macromolecular structures and/or scavenge free radicals that accumulate in response to stress (Chen and Murata, 2002). Approaches to engineer salt stress resistance have included regulation of ion transport through introduction of Na^+/H^+ antiporters or H^+ pumps (Apse *et al.*, 1999; Gaxiola *et al.*, 2001; Shi *et al.*, 2003); synthesis of compatible solutes, e.g. mannitol (Zhifang and Loesher, 2003), proline (Kishore *et al.*, 1995) or glycine betaine (Chen and Murata, 2008); or the introduction of transcription factors regulating expression of stress-responsive genes (e.g. Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Zheng *et al.*, 2009).

While engineered salt stress resistance holds promise for agricultural productivity in impaired conditions, there has been considerable concern about the possible ecological impacts of release of transgenic crops. At the forefront are concerns about the risk of transgene escape into natural populations and

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potential effects on ecosystem balance (Conner *et al.*, 2003; Chandler and Dunwell, 2008; Craig *et al.*, 2008; Warwick *et al.*, 2009; Beckie *et al.*, 2010). The traits most likely to become established in natural environments are those that provide the greatest selective advantage (Hancock, 2003; Lu and Yang, 2009; Warwick *et al.*, 2009). Abiotic stress-related traits may fall in this category. Salt tolerance could provide a competitive advantage to recipient populations or allow a crop or wild relative to grow in areas that it could not previously colonize (Lu and Yang, 2009; Warwick *et al.*, 2009; Beckie *et al.*, 2010).

In addition to the selective advantages that may result from the primary intended effect of the transgene, e.g. ability to grow in saline environments, secondary changes in phenotype may also have reproductive or fitness effects. The first wave of transgenic crops primarily utilized genes whose protein product was directly responsible for the desired trait (e.g. Bt proteins confer insect resistance; herbicide resistance genes encode proteins that prevent binding of the herbicide or otherwise inactivate the herbicide; Carpenter *et al.*, 2002). These genes, as well as marker genes such as GUS or the kanamycin resistance gene, NPTII, have generally had minimal effects on fitness, except under the selective conditions (e.g. insect herbivory) for which they were developed (Crawley *et al.*, 2001; Pilson *et al.*, 2002; Snow *et al.*, 2003). They are largely inert with respect to other cellular functions as evidenced by minimal pleiotropic phenotypes and the results of global transcriptome and proteome studies (El Ouakfaoui and Miki, 2005; Ruebelt *et al.*, 2006; Cheng *et al.*, 2008; Zolla *et al.*, 2008; Little *et al.*, 2009). Indeed, transcriptome comparisons of single transgene differences vs. cultivar differences in wheat, rice, maize and soybean have shown greater differences among cultivars than as a result of transgene introduction (Baudo *et al.*, 2006; Batista *et al.*, 2008; Cheng *et al.*, 2008; Coll *et al.*, 2008). While introduction of a transgene, per se, may not cause extensive transcriptional modifications, the extent of changes is directly related to the nature of the introduced transgene and its biological function. It has been suggested that genes that are from distant biological sources or are novel to plants are less likely to interact with other plant processes than those that have specific plant-related functions (Miki *et al.*, 2009).

Many of the genes under consideration for abiotic stress resistance initiate subsequent changes within the cell that facilitate adaptive responses. They may cause the cell to produce compounds needed to survive, grow and respond to the environment. Such genes may encode transcription factors that regulate expression of other genes; signalling factors that initiate responses to perceived changes in the cellular environment; or metabolic pathway enzymes that result in the production of new cellular compounds. As a result of their downstream actions, these types of genes may have broader effects on plant metabolism, physiology and development, than genes for which the protein itself is the final product. Although the ability of a given gene to initiate a cascade of events can make it highly valuable for genetic engineering, such genes also have the potential to modify non-target phenotypes within the plant through pleiotropic or epistatic interactions (Wolfenbarger and Grumet, 2003; Little *et al.*, 2009; Miki *et al.*, 2009). These changes could, in turn, influence fitness of the recipient plant.

Therefore, different possible approaches to engineer salt stress resistance could have different secondary effects. In this work, we compared the phenotypic and transcriptomic effects of three types of genes intended to confer salt stress tolerance: a regulatory gene, *CBF3/DREB1a*, coding for the C-repeat binding factor/drought-responsive element binding transcription factor (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999); a metabolic gene, *M6PR*, coding for the mannose-6-phosphate reductase enzyme for mannitol biosynthesis (Zhifang and Loesher, 2003); and a membrane protein gene, *SOS1*, encoding a plasma membrane Na^+/H^+ antiporter (Shi *et al.*, 2003).

CBF/DREB1 genes encode a family of transcription factors that promote expression of a group of abiotic stress-responsive genes (Van Buskirk and Thomashow, 2006; Chinnusamy *et al.*, 2007). Transgenic *CBF/DREB1*-overexpressing *Arabidopsis* plants exhibit increased tolerance to freezing, drought and salinity stress (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2004). Constitutive expression of CBF/DREB transcription factors in *Arabidopsis* leads to the expected increase in CBF/DREB target genes (the CBF/DREB regulon) (Seki *et al.*, 2001; Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Zhang *et al.*, 2004; Vogel *et al.*, 2005;). The induced genes include those that likely function in stress tolerance (e.g. LEA, dehydrin, antifreeze and galactinol/raffinose synthesis) as well as factors involved in signal transduction and gene regulation. The CBF/DREB-responding genes can be clustered into groups showing increased or decreased expression at different time periods following transfer to the cold, suggesting sequential induction by CBF, or activity of downstream CBF-induced transcription factors (Fowler and Thomashow, 2002; Vogel *et al.*, 2005). Constitutive *CBF* expression has been associated with growth reduction in the absence of stress and delayed reproductive development (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Achard *et al.*, 2008a).

M6PR is responsible for the conversion of mannose-6-phosphate to mannitol-1-phosphate, the first committed step in mannitol production in plants (Everard *et al.*, 1997; Zhifang and Loesher, 2003). Transgenic *Arabidopsis*-overexpressing *M6PR* showed increased resistance to salt stress as manifested by increased dry weight and seed yield and reduced inhibition of photosynthetic activity (Zhifang and Loesher, 2003; Sickler *et al.*, 2007; Chan *et al.*, 2011). *M6PR* did not confer resistance against drought stress (Sickler *et al.*, 2007). Laboratory analysis of *M6PR* transgenic *Arabidopsis* plants in the absence of salt stress did not show effects on growth, photosynthetic activity, time to bolting or seed set (Zhifang and Loesher, 2003; Sickler *et al.*, 2007; Chan *et al.*, 2011). Transcriptome analysis suggested that increased salt tolerance may be due, at least in part, to the expression of numerous stress-related genes prior to salt treatment (Chan *et al.*, 2011).

SOS1 is a plasma membrane–located sodium efflux carrier that functions to transport sodium ions out of the cell into the apoplast and so can reduce cytoplasmic sodium content (Qiu *et al.*, 2003; Shabala and Cuin, 2008). SOS1 acts in coordination with two other members of the SOS pathway, SOS2 and SOS3, to maintain cellular ion homeostasis (Shi *et al.*, 2003; Zhu, 2003). Transgenic *SOS1*-overexpressing *Arabidopsis* plants exhibited increased salt stress tolerance as indicated by higher per cent survival and less reduction in root growth, protein content, total chlorophyll and photosynthetic activity than the non-transgenic controls (Shi *et al.*, 2003). In the absence of salt stress, the transgenic plants did not show obvious differences in growth and development. To our knowledge, global transcriptional analyses have not been published for SOS transgenic plants.

Transcriptome or proteome profiling has been suggested as a comprehensive non-targeted approach to examine secondary

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effects resulting from the introduction of transgenes (Ruebelt *et al.*, 2006; Batista *et al.*, 2008; Chung *et al.*, 2008; Zolla *et al.*, 2008; Ricroch *et al.*, 2011). These authors also have emphasized the importance of interpreting results within the context of naturally occurring variation that may result from genotypic or environmental factors. In this study, we sought to compare the transcriptome effects of the *CBF3*, *M6PR* and *SOS1* transgenes and, furthermore, to examine their phenotypic and fitness-associated effects when grown side by side in the growth chamber, greenhouse and field in the presence and absence of salinity stress. Our results indicate that each transgene influenced a different set of genes and that *CBF3* and *M6PR* had considerably greater effects on the transcriptome than *SOS1*. The magnitude of transcriptome effects, however, did not correlate with phenotypic and fitness effects.

Results

Growth and development of the CBF3, M6PR and SOS1 transgenic lines

To compare the growth, development and transcriptional effects of the three transgenes, three sets of lines, each including two independent transgenic lines and their respective wild-type (WT) parental controls [(1) CBF3 A30, CBF3 A40 and WS-WT; (2) M6PR M2, M6PR M5, and Col-WT; (3)SOS1 1-1, SOS1 7-6 and Col(gl)-WT], were grown together for their full life cycle in replicated experiments in the growth chamber, greenhouse and field. As has been reported previously for *CBF*-overexpressing plants in the growth chamber (Gilmour *et al.*, 2000; Vogel *et al.*, 2005), constitutive *CBF3* expression in *Arabidopsis* had negative effects on vegetative growth and reproductive development including delayed bolting and flowering (4 and 10 day delay to 50% flowering for A40 and A30, respectively; $P < 0.05$, Duncan's multiple range test; Figure S1), and reduced leaf number, rosette diameter, plant height, dry weight, and seed yield relative to the WS-WT parent genotype (Figure 1a; Table 1A; Figure S1). The CBF3 plants, especially line A30, which has a higher level of expression (data not shown), also exhibited significantly delayed flowering or maturation (Figure S1 and data not shown) and reduced dry matter in the greenhouse and seed production in the greenhouse and field (Figure 2).

The *M6PR* and *SOS1* genes had minimal effects on performance in the growth chamber. *M6PR* had somewhat positive effects on leaf number, dry weight and seed yield (Table 1A) and did not affect time to bolting or flowering (Figure S1). SOS1 1-1 exhibited some mild chlorosis, growth reduction and delay in flowering (Figure S1). M6PR and SOS1 plants exhibited some reduction in dry matter in the field, but not in the greenhouse; M6PR line M2 had increased seed yield in the field experiment (Figure 2).

Treatment with NaCl in the growth chamber experiments inhibited growth and development and caused progressive leaf injury symptoms of chlorosis and necrosis for all WT and transgenic genotypes (Figure 1; Table 1). Symptom development occurred more rapidly or to a greater extent on the WT lines than their transgenic counterparts as evidenced by CBF3 lines at 100 mM NaCl, M6PR lines at 100 mM and 200 mM NaCl, and SOS1 lines at 200 mM NaCl; 32 and 38 days after planting (DAP; $P = 0.05$, Duncan's multiple range). When treated with 100 mM NaCl, the transgenic lines (with the exception of CBF3 A30) had greater vegetative growth (stalk number, plant height, dry weight) and seed yield relative to their wild-type counterparts (Figure 1, Table 1A,B; Figure S1). Both M6PR lines and the SOS1 7-6 line also showed increased bolting and flowering relative to their wild-type parents (Figure S1). The average reduction in dry matter (25%) and seed yield (18%) of the transgenic lines (except CBF3 A30) was approximately half that of the WT lines (51% and 36% for dry matter and seed yield, respectively). The 200 mM NaCl treatment caused severe growth reduction or death for all genotypes, although several vegetative measures, e.g. leaf number, rosette diameter, plant height or dry weight were greater for the transgenic plants than for WT parents (Table 1). Only a few plants, however, produced inflorescences, and none set seed, except a few SOS1 7-6 plants (Table 1; Figure S1).

Comparative transcriptome effects of the *CBF3*, *M6PR* and *SOS1* transgenes

Microarray analyses were conducted to compare transgene effects on gene expression in the presence and absence of salt stress. The microarray signal data from any pair of biological replicates were highly reproducible for all pairs of comparisons ($R^2 > 0.95$ for normalized signal data; Figure S2), indicating strong reproducibility between the experiments. Microarray results were also partially verified using quantitative real-time polymerase chain reaction (qRT-PCR) assay for a set of 21 transcripts representing different categories of relative transcript abundance (i.e. increased, decreased or essentially unchanged). The expression ratios measured by microarray and qRT-PCR were highly correlated ($R > 0.90$ for 252 transcript/salt/transgene combinations; Figure S3).

Different threshold parameters were examined to determine meaningful differences in gene expression levels (Table 2). To minimize potential statistical biases and avoid incorrectly declaring gene expression to be influenced by one transgene and not another, modest criteria (2-fold difference and $P \le 0.05$), using both Bioconductor R package and GeneChip® Operating Software from Affymetrix as described in Materials and Methods, were used to declare differences. Comparable trends also were observed with more stringent criteria (3-fold cut-off or $P \le 0.01$; Table 2).

Effects of the three transgenes on the transcriptome in the absence of salt stress

As expected, in the absence of stress, transgenic SOS1 and CBF3 plants showed increased expression of *SOS1* and *CBF3* transcripts, respectively (Table 3A). *M6PR* is not a native gene and cannot be detected by microarray, but the expression was verified by Northern blot analysis (Figure S4). The CBF3 lines also showed elevated expression of a large number of CBF-target genes as identified in previous studies, including *COR, RD, LT, ERD, ZAT* and dehydrin genes (Seki *et al.*, 2001; Maruyama *et al.*, 2004; Zhang *et al.*, 2004; Vogel *et al.*, 2005; Magome *et al.*, 2008). About 70% (27/38) of *DREB1A* up-regulated genes in Maruyama *et al.* (2004) and 90% (35/40) of *CBF* regulon genes in Zhang *et al.* (2004) were also significantly changed by the *CBF3* transgene in this experiment (Table 3C). Collectively, these results provide confidence in the microarray analyses.

In the absence of salt stress, *CBF3* and *M6PR* transgenes affected a much larger number of transcripts than the *SOS1* transgene (Table 2, Figure 3a). The large number of changes observed for CBF3 plants was consistent with its function as a

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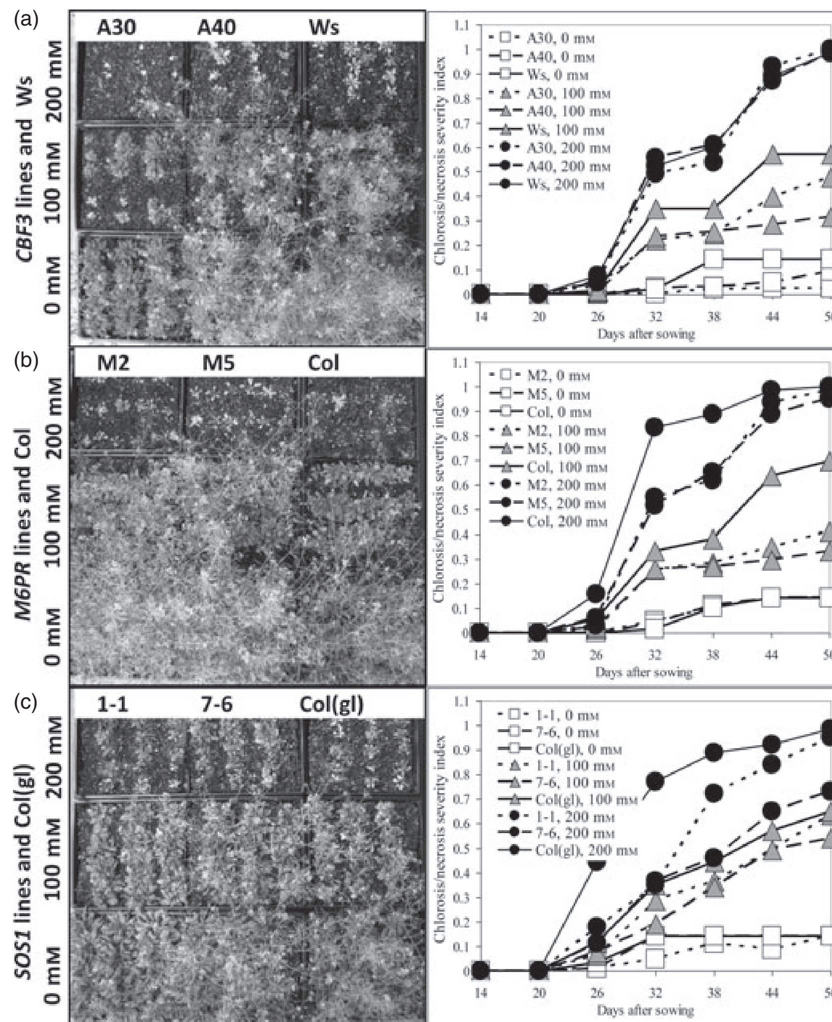

Figure 1 Growth and chlorosis/necrosis severity indices of transgenic and wild-type Arabidopsis in response to long-term salt stress in the growth chamber. (a) CBF3 and WS-WT; (b) M6PR and Col-WT; (c) SOS1 and Col(gl)-WT. The plants were photographed at 50 DAP. Chlorosis and necrosis were rated as: 0, no yellow or purple leaves; 1, older leaves turn yellow or purple; 3, younger leaves turn yellow or purple; 5, some leaves die; and 7, plants die. Chlorosis/necrosis severity indices were calculated as described in Experimental Procedures. Each value is the mean of three replicate trays, 18 plants/tray. White square, 0 mM NaCl; Grey triangle, 100 mM NaCl; Black circle, 200 mM NaCl.

transcription factor and with previous transcriptional analyses of *CBF* overexpressors (Seki *et al.*, 2001; Maruyama *et al.*, 2004; Zhang *et al.*, 2004; Vogel *et al.*, 2005; Magome *et al.*, 2008). Gene expression changes included direct members of the CBF regulon as indicated earlier, as well as a variety of other genes previously shown to be targets of the CBF regulon (Fowler and Thomashow, 2002; Cook *et al.*, 2004), including members of the stress-related categories of response to water deprivation, cold, osmotic stress and salt stress; numerous transport and minor carbohydrate metabolism genes, (e.g. pathway genes for the compatible solutes raffinose and trehalose); and numerous cell wall-associated genes (Table 3; Table S1; Table S2A,E,F). The CBF transgene influenced expression of several ABA-related genes, including up-regulation of several newly identified ABA receptors, [SNF1-related kinases (e.g. PYL5, SnRK2.2 and SnRK2.3)] and downstream ABA-responsive genes (Table S2D).

Expression of *M6PR* affected large number of the same genes as *CBF3*; approximately half (642, 49%; Figure 4A) of those up- or down-regulated were in common for the two transgenes. These included the members of the stress-related categories of water deprivation, cold, osmotic stress and salt stress, ABC and potassium transport, minor CHO metabolism (including raffinose and trehalose), and ABA-related and cell wall-associated genes (Table 4; Table S1; Table S2A–F). However, the total number of transcripts affected was greater for M6PR plants than for CBF3 plants (1719 vs. 1350; Table 2, Figure 3A). In addition to the stress-related gene categories influenced by *CBF3*, *M6PR* strongly affected expression of biotic, oxidative and heat stress-related genes including several pathogenesis-related (PR) or putative resistance gene analogues, and glutathione, thioredoxin, glutaredoxin family genes (Table 4; Table S2B,C). *M6PR* also caused more extensive changes in cell wall-associated genes including up-regulation of several arabinogalactan and xyloglucan-related protein genes and down-regulation of cellulose synthases (Table S1).

In contrast to *CBF3* and *M6PR*, many fewer transcripts were affected by the Na^+/H^+ antiporter *SOS1* (Figure 3). While the general categories showing biological enrichment were similar

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Table 1 Summary of transgene effects on growth parameters in the absence and presence of salt. The plants were harvested at 62 DAP. Leaf number, rosette diameter, stalk numbers and plant height were measured before harvest. Total dry weight and seed yield were measured after harvest. Each value is the mean of three replicate trays, 18 plants/tray. All data were analysed by ANOVA with SPSS 11.5 for windows (SPSS, Inc., Chicago, IL). Mean separations were performed by Duncan's multiple range test, $P < 0.05$. Detailed transgene effects on plant growth throughout the life cycle and on other growth parameters are shown in Figure S1

	0 mM						100 mM						200 mM					
	CBF3/Ws		*M6PR*/Col		*SOS1*/Col(gl)		*CBF3*/Ws		*M6PR*/Col		*SOS1*/Col(gl)		*CBF3*/Ws		*M6PR*/Col		*SOS1*/Col(gl)	
A. Growth relative to WT	A30	A40	M2	M5	1-1	7-6	A30	A40	M2	M5	1-1	7-6	A30	A40	M2	M5	1-1	7-6
Leaf number	0.64	0.81	1.05	1.17	0.93	1.10	0.86	1.04	1.55	1.64	1.26	1.62	0.63	0.77	1.19	1.26	1.90	3.22
Rosette diameter	0.86	0.86	1.08	1.10	1.00	1.01	0.78	0.86	1.03	1.18	1.12	1.06	0.51	0.85	1.13	1.15	0.94	1.31
Stalk number	0.89	1.00	1.05	0.98	0.90	0.98	0.57	1.38	1.47	1.47	1.29	1.52	0.00	2.65	/	/	/	/
Plant height	0.80	0.88	1.03	0.98	0.95	1.04	0.82	1.80	1.79	1.85	1.24	1.55	0.00	2.103	/	/	/	/
Dry weight	0.58	0.77	1.19	1.04	0.88	1.00	0.22	1.34	2.11	2.87	1.80	2.15	0.53	1.26	2.55	2.1	1.63	3.59
Seed yield	0.38	0.82	1.14	1.06	0.80	0.89	0.00	1.53	1.89	3.45	1.14	1.77	/	/	/	/	/	/

	100 mM/0 mM			200 mM/0 mM			100 mM/0 mM			200 mM/0 mM			100 mM/0 mM			200 mM/0 mM		
B. Growth relative to control plants without salt	Ws	A30	A40	Ws	A30	A40	Col	M2	M5	Col	M2	M5	Col(gl)	1-1	7-6	Col(gl)	1-1	7-6
Dry weight	0.29	0.11	0.51	0.03	0.03	0.05	0.22	0.39	0.61	0.02	0.05	0.05	0.25	0.51	0.53	0.05	0.09	0.17
Seed yield	0.20	0.0	0.38	0.0	0.0	0.0	0.16	0.26	0.51	0.0	0.0	0.0	0.20	0.28	0.39	0.0	0.0	0.04

Grey background—significant negative effect when compared to WT (Duncan's multiple range, $P < 0.05$); Black background—significant positive effect when compared to WT; white—no significant difference.

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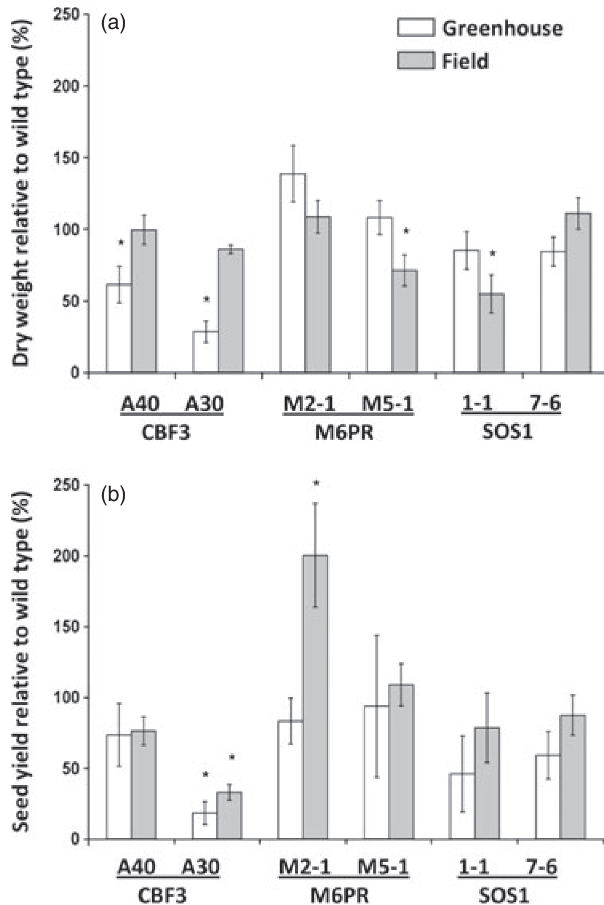

Figure 2 Dry matter accumulation (a) and seed yield (b) of greenhouse and field grown populations of transgenic CBF3, M6PR and SOS1 plants relative to wild-type parental populations. Plants were grown in the absence of salt stress. Values are expressed as per cent of wild type. Each value is the mean ± SE of five replicate trays. *Significant difference (ANOVA, Duncan's multiple range, $P < 0.05$) between transgenic and wild-type plants.

among all three transgenes (Table S3), very few of the specific genes affected by *SOS1* were in common with *CBF3* (7.7%) and *M6PR* (2.1%; Figure 4a). Only six expression changes were in common between all three. Microarray analysis indicated that *SOS1* overexpression influenced transcript levels of CBF3. However, the great majority of genes affected by CBF3 in the CBF3 transgenic lines were not affected in the SOS1 plants (Table 3). This may be due to the ~4-fold induction in SOS1 plants vs. ~200-fold induction for CBF3 plants. Of the stress-related categories, only response to oxidative stress was significantly overrepresented in the *SOS1*-influenced genes, but unlike M6PR plants, the majority of the affected redox-related genes in SOS1 plants were down-regulated, rather than up-regulated, especially in the glutaredoxin family (Table 4, Table S2C). These differences between *SOS1* effects relative to *CBF3* and *M6PR* are evident in the cluster analysis presented in Figure 5a.

Effects of the three transgenes on the transcriptome in the presence of salt stress

Imposition of salt stress affected a much smaller number of transcripts in the *CBF3* and *M6PR* plants than in the WT parental WS and Col plants (Table 2, Figure 3c,d). Indeed, a substantial portion of the transcripts modulated by salt stress in Col and WS WT plants, including numerous abiotic stress, biotic stress, redox, cell wall, minor carbohydrate metabolism, and transport genes, was affected by the *CBF3* and *M6PR* transgenes prior to salt treatment [33% (449) for *CBF3* and 47% (817) for *M6PR*] (Figures 5a,c and 6; Table S2A–F).

The small number of salt-induced gene changes in the parental Col(gl) plants appears to be associated with transcriptional effects of the *gl* mutation leading to constitutive induction of many stress-associated genes (Zhulong Chan, Rebecca Grumet and Wayne Loescher, unpublished) that may also mask some of the effects of SOS1 overexpression. Only 5.2% of changes associated with *SOS1* overlapped with those by affected by salt (Figure 6). Unlike CBF3 and M6PR plants, the salt-stressed SOS1 plants did not exhibit a reduction in the number of gene expression differences relative to the parental Col(gl) plants in the absence of salt stress (Figure 3). Many disease resistance–related protein and glutaredoxin genes continued to be down-regulated in SOS1 plants relative to Col(gl)-WT, even in the presence of salt (Table S2A,B).

Pathways showed differences for all three transgenes relative to their WT parents in the presence of salt included hormone, secondary, and cell wall metabolism. Other pathways, like stress, transport, redox, minor CHO metabolism, S-assimilation and amino acid metabolism were enriched in salt-stressed CBF3 and SOS1 lines but not M6PR lines (Table S3; Table 4). Despite similar categories of genes, there was little overlap among the specific genes that differed in each transgene-WT comparison (Figure 5b). This is in contrast to the substantial overlap between transcripts affected by CBF3 and M6PR in the absence of salt stress; 47.5% without salt vs. 19.5% in the presence of 100 mM NaCl (Figure 4a,b). The transcriptional differences between CBF3 and WS-WT in the presence of salt continued to include a large number of CBF-target, ABA-related, and other abiotic stress-related genes, possibly reflecting continued effect of CBF3 overexpression in the presence of salt stress (Table 3, Table S2A–D). While salt treatment induced expression of many of these genes in both WS-WT and Col-WT plants, the level of increase was not great as was caused by the CBF3 overexpression.

As occurred in the absence of salt, a larger portion of overlap was found between the *CBF3*- and *M6PR*-affected transcripts, than for *CBF3* or *M6PR* with *SOS1* (Figure 4b). In the presence of salt stress, the SOS1 plants exhibited down-regulation of numerous disease resistance-related genes that did not occur in the absence of salt stress or in the other transgenic lines (Table S2B).

Discussion

Several recent studies have compared the magnitude of transcriptional or proteomic changes caused by a transgene with those observed following introgression of a specific trait, among cultivars resulting from conventional breeding, or as a result of environmental effects (e.g. Corpillo *et al.*, 2004; Baudo *et al.*, 2006; Ruebelt *et al.*, 2006; Albo *et al.*, 2007; Batista *et al.*, 2008; Cheng *et al.*, 2008; Coll *et al.*, 2008; Zolla *et al.*, 2008). The general conclusion from these studies is that fewer changes are observed for the transgene than by conventional breeding, and those that are observed, fall within the range of natural variation. These modest effects were attributed to the single

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Table 2 Total numbers of changed transcripts by the three transgenes or salt stress at different cut-off values. All microarray data were normalized and analysed together using affylmGUI running on R package. The full list of genes is provided in Table S1

Comparisons	Fold change ≥2 and *P*-value <0.05		Fold change ≥3 and *P*-value <0.05		Fold change ≥2 and *P*-value <0.01	
	Up	Down	Up	Down	Up	Down
A. Transgene effects minus salt						
CBF3-0 mM_v_WS-0 mM	758	592	404	194	606	511
M6PR-0 mM_v_Col-0 mM	986	733	495	286	874	656
SOS1-0 mM_v_Col(gl)-0 mM	233	386	78	85	139	272
B. Transgene effects plus salt						
CBF3-100 mM_v_WS-100 mM	466	571	205	145	345	419
M6PR-100 mM_v_Col-100 mM	244	757	45	199	137	498
SOS1-100 mM_v_Col(gl)-100 mM	367	478	110	163	284	373
C. Salt effects on transgenic lines						
CBF3-100 mM_v_CBF3-0 mM	98	25	36	2	57	5
M6PR-100 mM_v_M6PR-0 mM	153	323	72	37	87	224
SOS1-100 mM_v_SOS1-0 mM	47	88	9	24	27	34
D. Salt effects on wild types						
WS-100 mM_v_WS-0 mM	774	319	327	80	572	231
Col-100 mM_v_Col-0 mM	1615	937	688	287	1423	843
Col(gl)-100 mM_v_Col(gl)-0 mM	80	58	29	19	48	38

gene change for the transgene vs. multiple changes that occur as a result of conventional breeding, even in a near-isogenic background, as well as the transcriptional flexibility widely exhibited by plants in response to variable environments. In most cases, the transgenes assessed encoded simple traits that were the direct product of the protein produced, such as endosperm seed storage protein in wheat (Baudo *et al.*, 2006); glyphosate tolerance in soybean (Cheng *et al.*, 2008); Bt protein in maize (Coll *et al.*, 2008); and selectable marker genes encoding kanamycin, biaphalos or glufosinate resistance (El Ouakfaoui and Miki, 2005; Abdeen and Miki, 2009; Miki *et al.*, 2009).

However, it is not unusual for introduction or deletion of a gene, especially those encoding transcription factors or proteins involved in signalling, to influence a cascade of gene expression changes, as has been noted for several stress response–related pathways (e.g. Vogel *et al.*, 2005; Perera *et al.*, 2008; Schramm *et al.*, 2008; Zhang *et al.*, 2011). Similarly, metabolic genes, such as chloroplast-targeted choline oxidase gene for glycine betaine synthesis introduced to engineer drought stress resistance in rice, can alter expression of many genes involved in stress responses, signal transduction, gene regulation, hormone signalling and cellular metabolism (Kathuria *et al.*, 2009). Thus, single gene modifications can have broad effects.

Here, we compared the phenotypic and transcriptomic effects of three alternate transgenic approaches to confer salt stress resistance with regard to implications for environmental risk assessment associated with genetically engineered crops. While it was anticipated that the transcription factor CBF3 would have the greatest effects, both the metabolic enzyme, M6PR, and ion transport protein, SOS1, could potentially affect a variety of cellular processes. The majority of published experiments have looked at short-term stress and early transcriptional responses and signalling. Here, we were particularly interested in long-term phenotypic impacts as observed throughout the life cycle, including effects on fecundity and fitness, as well as long-term transcriptional adjustments. Long-term assessment is particularly important for salt stress, which most often results from saline soils or irrigation water, and so is less likely to be episodic than stresses such as cold, heat or drought.

Comparative phenotypic and fitness effects of the three transgenes

In the absence of salt stress, the transgenic M6PR and SOS1 lines performed comparably with their WT parental genotypes, indicating limited obvious secondary or fitness effects in the growth chamber or greenhouse. Reduced growth and development for CBF3 overexpressing plants, as has been observed previously in the growth chamber (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Achard *et al.*, 2008a), also was seen in these experiments, resulting in reduced fecundity in the growth chamber, greenhouse and field, especially for A30. Thus, consistent with reports of the transgene effects from growth chamber or agar plate studies in separate labs, significant negative effects on growth were observed for the CBF3 lines but not, or minimally, for M6PR and SOS1 lines (Shi *et al.*, 2003; Zhifang and Loesher, 2003; Sickler *et al.*, 2007; Chan *et al.*, 2011).

The dwarf phenotype in *CBF/DREB* overexpressing plants has been linked to changes in GA metabolism and response (Munns, 2002; Achard *et al.*, 2008a,b; Magome *et al.*, 2008). Increased expression of the negative regulator of GA response, *RGL3* (RGA-like protein 3; At5g17490), occurred in the CBF3 lines, but not in the M6PR or SOS1 lines. In contrast, in control and salt-stressed SOS1 plants and salt-stressed M6PR plants, there was an increase in *GA3ox1* (At1g15550) transcript for a key enzyme in production of the bioactive forms of GA, GA_1 and GA_4 (Yamaguchi, 2008). Another difference observed only in the CBF3 lines that may influence growth was altered expression of two guard cell localized potassium channels that func-

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Table 3 Microarray data (log2 values) for overexpressed genes and selected CBF/DREB target genes ($P \leq 0.05$). Bold fonts indicate fold change ≥2.0. CBF/DREB target genes were chosen based on the study by Maruyama *et al.*, 2004; Seki *et al.*, 2001; Vogel *et al.*, 2005; and Zhang *et al.*, 2004

			CBF3 and wild type				*M6PR* and wild type				*SOS1* and wild type			
Probe Set ID	AGI	Description	CBF3-0 mM vs. Ws-0 mM	CBF3-100 mM vs. Ws-100 mM	CBF3-100 mM vs. CBF3-0 mM	Ws-100 mM vs. Ws-0 mM	M6PR-0 mM vs. Col-0 mM	M6PR-100 mM vs. Col-100 mM	M6PR-100 mM vs. M6PR-0 mM	Col-100 mM vs. Col-0 mM	SOS1-0 mM vs. Col(gl)-0 mM	SOS1-100 mM vs. Col(gl)-100 mM	SOS1-100 mM vs. SOS1-0 mM	Col(gl)-100 mM vs. Col(gl)-0 mM
A. Over-expressed genes														
265252_at	At2g01980	SOS1	–	–	–	–	–	–	–	–	**1.85**	**1.87**	–	–
254066_at	At4g25480	CBF3/DREB1a	**6.84**	**6.87**	–	–	**−1.60**	–	**1.27**	–	**2.10**	**2.41**	–	–
B. CBF/DREB genes														
254074_at	At4g25490	CBF1/DREB1b		0.987	–	–	–	**−1.63**	0.87	**2.38**	–	–	–	–
254075_at	At4g25470	CBF2/DREB1c	**1.36**	–	–	–	–	–	**1.12**	**2.05**	**1.70**	**1.13**	–	–
C. CBF/DREB target genes														
264511_at	At1g09350	ATGOLS3	**6.19**	**6.17**	–	–	–	0.81	0.83	–	**1.14**	**1.37**	–	–
266225_at	At2g28900	AtOEP16	**2.97**	**2.31**	–	0.72	0.83	–	–	**1.20**	–	–	–	–
263789_at	At2g24560	GDSL-like Lipase	**4.16**	**3.80**	−0.69	−0.32	–	–	–	–	–	–	–	–
260556_at	At2g43620	chitinase, putative	**2.58**	**2.68**	–	–	**1.41**	–	–	**2.33**	–	–	–	–
254232_at	At4g23600	COR13/JR2	**1.05**	–	0.77	**1.77**	–	–	–	–	–	–	0.63	–
263497_at	At2g42540	COR15a	**3.27**	**1.66**	–	**1.55**	–	–	**1.30**	0.78	–	0.65	0.60	–
263495_at	At2g42530	COR15b	**5.57**	**4.14**	–	**1.44**	0.87	–	0.84	**1.78**	–	–	–	–
256114_at	At1g16850	COR17	**5.27**	**3.40**	1.00	**2.87**	–	–	**1.50**	**2.23**	–	–	**1.14**	–
262452_at	At1g11210	COR35	**3.32**	**3.72**	–	–	**1.67**	–	–	**2.65**	–	–	–	–
265480_at	At2g15970	COR413-PM1	**2.40**	**1.80**	–	0.60	–	–	–	–	–	0.34	–	0.35
259789_at	At1g29395	COR414	**4.79**	**4.03**	–	0.59	–	**1.16**	–	**−1.16**	–	–	–	−0.73
253595_at	At4g30830	COR42	**1.04**	**1.61**	0.66	–	–	–	–	–	–	–	–	–
259570_at	At1g20440	COR47/RD17	**4.41**	**2.26**	–	**2.23**	**1.14**	–	–	**1.06**	–	–	–	–
246481_s_at	At5g15960	COR6.6/KIN2	**3.60**	**1.48**	–	**2.07**	0.61	–	–	**1.06**	–	–	–	–
248337_at	At5g52310	COR78/RD29A	**5.48**	**2.82**	–	**2.97**	–	–	**1.06**	0.92	–	**1.12**	–	–
267261_at	At2g23120	COR8.5	**2.47**	0.99	–	**1.24**	–	–	–	–	−0.48	–	–	–
261749_at	At1g76180	Dehydrin	0.86	0.39	–	0.44	–	–	–	–	–	–	–	–
252137_at	At3g50980	Dehydrin (Xero1)	**1.07**	**1.78**	–	–	–	–	–	–	–	–	–	–
245523_at	At4g15910	Di21	–	**1.14**	0.92	–	–	**−1.52**	0.95	**2.30**	–	**1.31**	0.67	–
259516_at	At1g20450	ERD10	**3.02**	**1.49**	–	**1.61**	–	−0.32	–	0.39	–	0.79	0.40	−0.50
256310_at	At1g30360	ERD4	**2.45**	**2.03**	–	0.42	**1.08**	–	–	0.52	–	–	–	–
264787_at	At2g17840	ERD7	**3.23**	**2.38**	–	**1.55**	**1.07**	–	–	**1.72**	–	–	–	–
265119_at	At1g62570	Flavin	**3.64**	**3.05**	–	–	−0.65	−0.78	–	–	–	–	0.95	–
245427_at	At4g17550	Transporter-related	0.80	**1.56**	–	–	**−1.66**	–	0.66	−0.93	–	0.93	–	–
247478_at	At5g62360	Invertase	**6.28**	**5.69**	–	–	**3.62**	–	–	**2.50**	–	–	–	–

Table 3 Continued

Probe Set ID	AGI	Description	*CBF3* and wild type				*M6PR* and wild type				*SOS1* and wild type			
			CBF3-0 mM vs. Ws-0 mM	CBF3-100 mM vs. Ws-100 mM	CBF3-100 mM vs. CBF3-0 mM	Ws-100 mM vs. Ws-0 mM	M6PR-0 mM vs. Col-0 mM	M6PR-100 mM vs. Col-100 mM	M6PR-100 mM vs. M6PR-0 mM	Col-100 mM vs. Col-0 mM	SOS1-0 mM vs. Col(gl)-0 mM	SOS1-100 mM vs. Col(gl)-100 mM	SOS1-100 mM vs. SOS1-0 mM	Col(gl)-100 mM vs. Col(gl)-0 mM
247450_at	At5g62350	Invertase	**1.86**	0.88	–	0.96	0.33	–	0.27	0.61	0.36	0.29	–	–
259426_at	At1g01470	LEA protein	**1.97**	**1.57**	–	0.74	–	–	–	–	–	–	0.45	–
252102_at	At3g50970	LTI30/XERO2	**8.55**	**6.11**	–	**2.46**	**2.01**	**−1.08**	**1.66**	**4.75**	–	**1.03**	**1.92**	–
253627_at	At4g30650	LTI6A/RCI2A	**5.03**	**2.82**	–	**1.98**	**1.23**	–	–	**2.26**	–	–	–	–
254818_at	At4g12470	pEARLI 1-like	**3.19**	**2.60**	–	–	**1.80**	–	–	**1.90**	–	–	–	–
245807_at	At1g46768	RAP2.1	**1.50**	**1.59**	–	–	–	–	–	–	–	–	–	–
264415_at	At1g43160	RAP2.6	–	–	–	**3.69**	–	–	–	**4.27**	–	–	–	–
259364_at	At1g13260	RAV1	**3.37**	–	–	**3.29**	**2.68**	–	–	**3.00**	–	–	**−1.46**	–
252927_at	At4g39090	RD19A	**2.64**	**1.47**	–	**1.53**	**1.27**	–	–	0.69	–	–	–	–
253872_at	At4g27410	RD26	**2.18**	–	**1.25**	**2.61**	–	–	–	–	–	–	–	–
248352_at	At5g52300	RD29B	**1.78**	**1.96**	**2.46**	**2.29**	–	–	–	–	–	–	–	–
262440_at	At1g47710	Serpin, putative	**1.71**	**1.33**	–	0.58	–	–	0.60	0.51	–	0.37	0.47	–
264516_at	At1g10090	Similar to RXW8	**1.59**	**2.31**	–	–	–	–	–	**−1.56**	–	–	–	–
264989_at	At1g27200	Similar to zinc fing	**3.13**	**2.64**	–	0.57	0.89	0.60	–	–	–	–	–	−0.57
264654_s_at	At1g08900	Sugar transporter	**1.85**	**2.41**	–	–	0.73	0.70	–	–	–	–	–	–
252591_at	At3g45600	TETRASPANIN	0.92	**1.06**	–	–	−0.70	0.66	–	−0.99	–	–	–	–
262881_at	At1g64890	Transporter	**2.84**	**2.20**	–	0.84	–	–	–	–	–	–	0.63	–
261648_at	At1g27730	ZAT10	**2.59**	–	–	**3.01**	**3.32**	–	–	**4.90**	–	–	**−1.65**	–
247655_at	At5g59820	ZAT12	**1.18**	**−1.16**	–	**3.19**	**1.57**	**−1.24**	–	**3.44**	–	–	−0.9	–
245711_at	At5g04340	ZAT6/CZF2	**2.76**	**2.36**	–	**1.28**	–	–	–	**2.51**	–	–	**−1.78**	–

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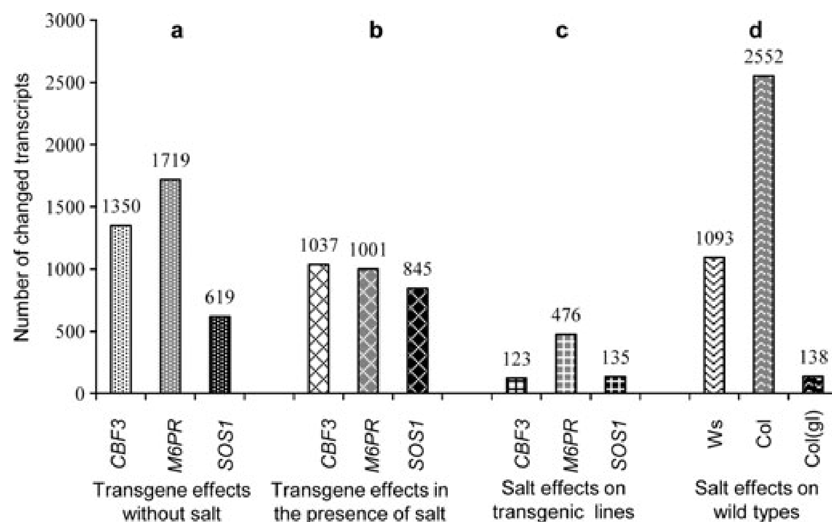

Figure 3 Total number of changed transcripts by three transgenes or salt stress. All microarray data were normalized and analysed together using affylmGUI running on R package. Transcripts level deemed significantly different were those with a fold change ≥2; a *P*-value ≤0.05, and a detection call of 'Present' in duplicate with the Affymetrix GCOS. The full list of genes is provided as Table S1.

tion reciprocally to drive stomatal closing and opening (Gambale and Uozumi, 2006; Ward *et al.*, 2009). Similar to the observations by Vogel *et al.* (2005), the *CBF*-overexpressors exhibited 4-5-fold increased transcription of the guard cell outwardly rectifying potassium channel, *Shaker*-type *GORK1* (At5g37500) gene and 4-5-fold decreased transcription of the inwardly rectifying *KAT1* potassium channel (At5g46240) gene (Table S2E), thereby potentially increasing stomatal closure, while decreasing rate of water loss, photosynthetic capacity, and growth.

With the exception of the more severely dwarfed line, CBF3 A30, all transgenic lines exhibited a significant fitness advantage relative to their wild-type parents when subjected to moderate (100 mM) salt stress throughout their life cycle verifying that all three transgenes can confer tolerance to the long-term salt stress imposed in these experiments. Seed production ranged from 114% to 345% of salt-stressed WT genotypes. SOS1 conferred the greatest salt tolerance as measured by reduced salt injury effects and greater survival at 200 mM NaCl. M6PR plants, however, had the greatest fecundity at 100 mM NaCl. The M6PR plants also exhibited enhanced seed yield in the field, possibly reflecting better adaptation to environmental stresses that can be experienced in field conditions. Preliminary results of direct competition experiments between each transgenic line and corresponding parental genotype in field tests also showed strong negative fitness effects for CBF3 plants and somewhat negative effects for SOS1, while M6PR had somewhat positive effects (Bigelow *et al.*, 2010). Thus, relative fitness advantages or disadvantages caused by the transgenes varied depending on the presence, absence and level of stress.

Comparative transcriptome effects of the three transgenes

In contrast to the minimal effects of M6PR on phenotype and fitness in absence of salt stress, the global transcriptome effects of *M6PR* were at least as great as those of *CBF*3 and included many changes in common. Among the changes induced by *M6PR* was strong activation of three recently identified ABA receptor genes (*PYL4*, *PYL5* and *PYL6*; Ma *et al.*, 2009; Park *et al.*, 2009) (Table S2C) and down-regulation of two ABA signalling inhibitor genes, type 2C protein phosphatases (PP2C), *ABI1* and *ABI2* (Table S2C). Increased ABA signalling may contribute to the broad range of stress-related gene expression and commonality in many expression responses between M6PR and CBF3 plants.

There were also numerous gene expression changes in the M6PR plants not seen in the CBF3 plants, especially with respect to biotic stress and oxidative stress-related genes including many disease resistance-related proteins, and glutaredoxin and thioredoxin family protein genes. As many pathogenic fungi produce mannitol during the infection process (Vogele *et al.*, 2005; Cheng *et al.*, 2009), the endogenous mannitol production may be perceived by the M6PR plants as a signal of pathogen attack to stimulate expression of biotic stress-related genes. Work of the past decade has led to increasing recognition of extensive crosstalk between biotic and abiotic stress responses, including ABA- and reactive oxygen-mediated signalling (Garg and Manchanda, 2009; Klinger *et al.*, 2010). Indeed, several of the disease-resistance-related genes whose expression was up-regulated by *M6PR* were also induced by salt treatment of the WT Col plants. Similarly, if mannitol is perceived as a sign of pathogen attack, the resultant defences may include abiotic responses in common with those induced by CBF3.

Transcriptional effects of the *SOS1* transgene in the absence of salt stress were considerably smaller than for *CBF3* or *M6PR*. These results are consistent with the apparent independence of the SOS signalling pathway from CBF, ABA and MYC/MYB pathways as was observed for *sos2* and *sos3* mutants of *Arabidopsis* (Kamei *et al.*, 2005). The small number of gene expression changes may also be influenced by the Col(gl) background (Zhulong Chan, Rebecca Grumet and Wayne Loescher, unpublished) leading to a partial masking of *SOS1* effects. The *SOS1* transgene, did however, have substantial effects on oxidative stress or redox-related genes, resulting in down-regulation

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of numerous transcripts. Direct interplay between the SOS pathway and redox signalling has been observed by interaction between SOS2 and the redox signalling pathway proteins, nucleoside diphosphate kinase 2 (NDPK2) and catalases, and between SOS1 and RCD1, a regulator of oxidative stress (Katiyar-Agarwal *et al.*, 2006; Verslues *et al.*, 2007). Mutants of *sos2* had increased sensitivity to oxidative stress (Zhu *et al.*, 2007). However, consistent with reduced expression of redox-related genes in the *SOS1* overexpressors, *sos1* mutants had increased tolerance to oxidative stress induced by methyl viologen, indicating that *SOS1* expression can act to make plants more sensitive to oxidative stress (Katiyar-Agarwal *et al.*, 2006; Chung *et al.*, 2008).

The relative impacts of the transgenes on global transcription changed in the presence of salt. While the differences between the transgenic CBF3 and M6PR plants and their WT parents were greater without salt, the differences for SOS1 plants relative to WT parents were greater in the presence of salt, both in terms of numbers of genes affected and level of induction or repression (Figure 4). Similarly, significant enrichment for modified expression of genes associated with response to osmotic stress and salt stress only occurred for SOS1 plants when subjected to salt stress (Table 4). These observations are consistent with studies showing stabilization of the SOS1 protein and increased ion exchange activity in response to salinity (Qiu *et al.*, 2003, 2004; Chung *et al.*, 2008). Similar results were observed with transgenic *Arabidopsis* overexpressing the drought-related transcription factor, ABF3, wherein extensive transcriptional differences were only observed after application of drought stress (Abdeen *et al.*, 2010). Minimal changes in gene expression in the absence of drought stress were attributed to lack of activation of ABF3 by SnRK2-mediated phosphorylation that is normally induced in response to abscisic acid.

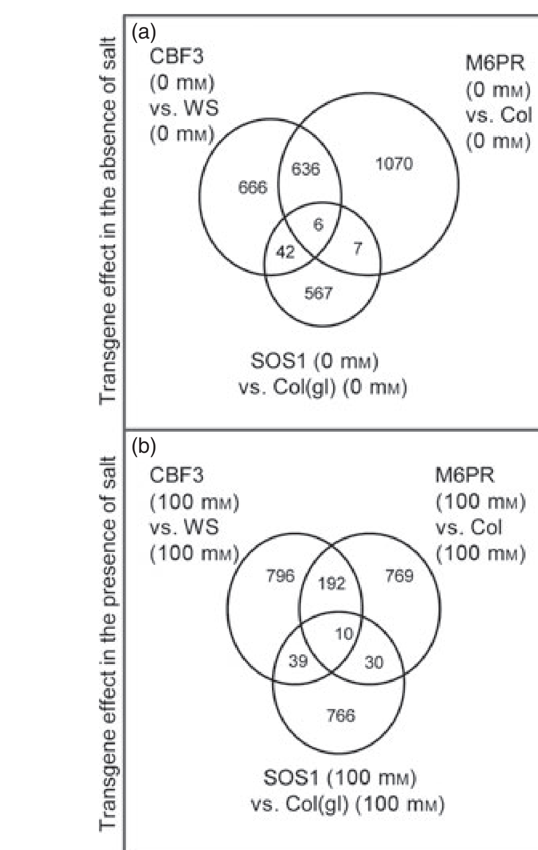

Figure 4 Venn diagrams showing overlapping transcripts (*P*-value ≤0.05 and fold change ≥2.0) affected by the three transgenes in the absence (a) and presence (b) of salt stress (100 mM NaCl).

Relationship between transcriptome and phenotype

The transcriptome data show that transgenes intended to confer salt stress tolerance can have extensive and variable effects on the transcriptome. The three transgenes affected different pathways or groups of pathways consistent with their different functions as summarized in Figure 7. The global transcriptional differences as measured by number of genes affected by each transgene, however, did not correlate with changes in phenotype (Figure 8). Furthermore, while the effects of the transgenes on plant growth and effects on global gene expression varied in response to salt stress, they did not vary in parallel. In the absence of salinity, despite a range of transcriptional differences, performance differences [as measured by average difference in dry weight or seed yield between the transgenic and WT counterparts (with the exception of CBF A30)] were rel-

Table 4 Stress-related GO term enrichment analysis. Term enrichment analysis was performed using AmiGO software

	CBF3-0 mM vs. Ws-0 mM		M6PR-0 mM vs. Col-0 mM		SOS1-0 mM vs. Col(gl)-0 mM		CBF3-100 mM vs. Ws-100 mM		M6PR-100 mM vs. Col-100 mM		SOS1-100 mM vs. Col(gl)-100 mM	
GO Terms	FC	*P*-value	FC	*P*-value	FC	*P*-value	FC	*P*-value	FC	*P*-value	FC	*P*-value
GO:0009414 response to water deprivation	5.12	0.000	3.09	0.000	–	–	6.22	0.000	–	–	–	–
GO:0009409 response to cold	4.50	0.000	2.87	0.000	–	–	4.89	0.000	–	–	–	–
GO:0006970 response to osmotic stress	3.15	0.000	2.59	0.000	–	–	3.76	0.000	–	–	2.87	0.000
GO:0009651 response to salt stress	3.10	0.000	2.56	0.000	–	–	3.52	0.000	–	–	3.00	0.000
GO:0006979 response to oxidative stress	–	–	2.72	0.000	3.68	0.001	2.85	0.003	–	–	3.63	0.000
GO:0009408 response to heat	–	–	2.78	0.048	–	–	5.96	0.000	–	–	–	–
GO:0009607 response to biotic stimulus	2.42	0.000	2.26	0.000	2.58	0.000	2.90	0.000	–	–	2.84	0.000

FC: enriched fold change was calculated as frequency of transcripts from the functional category relative to total changed transcripts/background frequency of that functional category in the Arabidopsis genome. Colour scales represent fold enrichment >6.0 5.0–6.0 4.0–5.0 3.0–4.0 2.0–3.0 <2.0.

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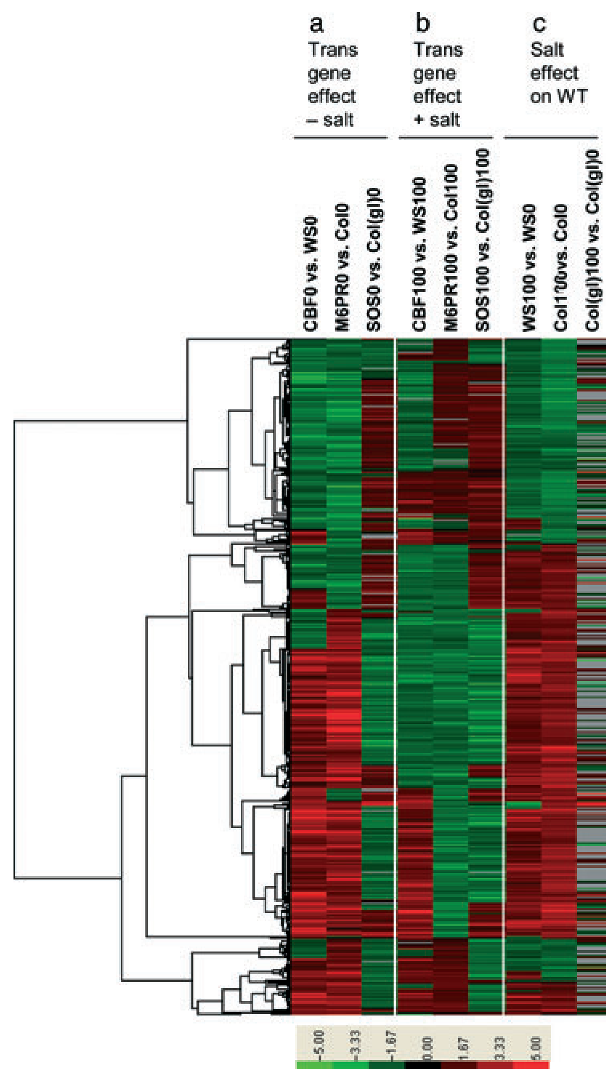

Figure 5 Cluster analysis of transcripts with expression levels significantly affected (*P*-value ≤0.05) by transgenes or salt. Red, black and green scales indicate fold change for genes with significant changes. Red, up-regulation; green, down-regulation. Gray, transcription levels were not significantly changed for that comparison. Hierarchical cluster analysis was performed with Cluster 3.0 software. Resulting tree figures were displayed using the software package, Java Treeview. The detailed gene IDs and fold changes are listed in Table S1.

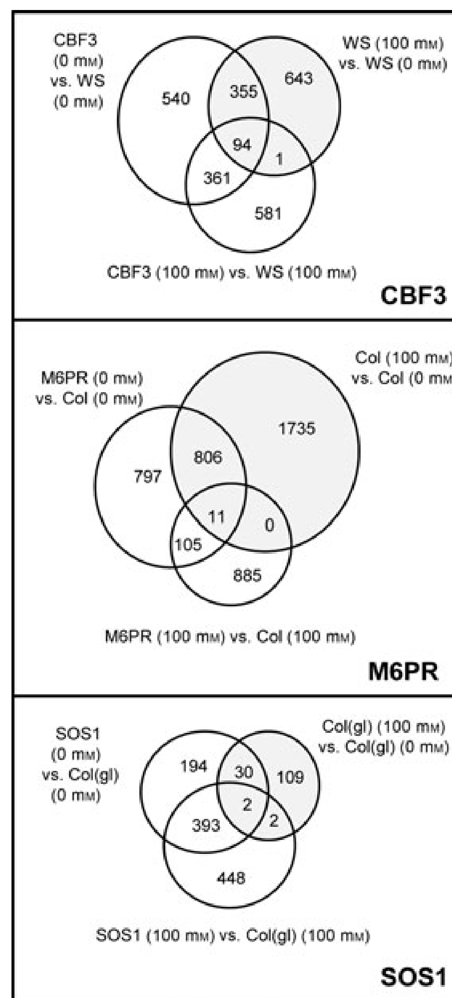

Figure 6 Venn diagrams showing overlapping transcripts affected by salt stress (shaded) and the *CBF3*, *M6PR* and *SOS1* transgenes in the presence and absence of salt (*P*-value ≤0.05 and fold change ≥2.0). Comparisons are indicated around the circle.

atively modest (average transgene effect was 85%–110% of WT seed yield). In the presence of salt stress, the range of transcriptional differences between the transgenic lines was quite small, but the performance differences, relative to each other and to WT plants, were considerably increased (average transgene effect was 146%–267% of WT seed yield).

These results suggest that depending on genotype and environment, extensive transcriptional changes may serve different functions. They could facilitate adaptive expression of fitness-associated traits, or they may reflect response to injury. They may also be an adaptive response to buffer the effects of genetic perturbation. Evolutionarily conserved buffering systems modifying gene expression have been observed across organisms and are hypothesized as an adaptive mechanism to minimize potential negative impacts of mutation on fitness (Boerjan and Vuylsteke, 2009; Fu *et al.*, 2009). Studies in *Arabidopsis* have shown that only a handful out of thousands of expression differences are observed at the phenotype level, indicating that much of the genetic variation in gene expression is hidden by non-linearity in response functions (Fu *et al.*, 2009). It was proposed that such robust system properties serve to keep traits within acceptable limits, thereby preventing dysfunction of the organism.

Lack of correspondence between magnitude of transcriptional differences and performance differences indicates that extent of global transcriptome differences may not predict phenotypic differences upon which environment and selection act in influencing fitness and fecundity. These observations have implications for the use of global gene expression data for purposes of risk assessment. The sorts of changes identified, however, may provide guidance for risk assessment analyses. For example, given the transcriptional changes for biotic stress-related genes, do the M6PR plants show altered disease responses? Collectively these observations emphasize the importance of evaluation of the transcriptomic effects of transgene within a phenotypic context.

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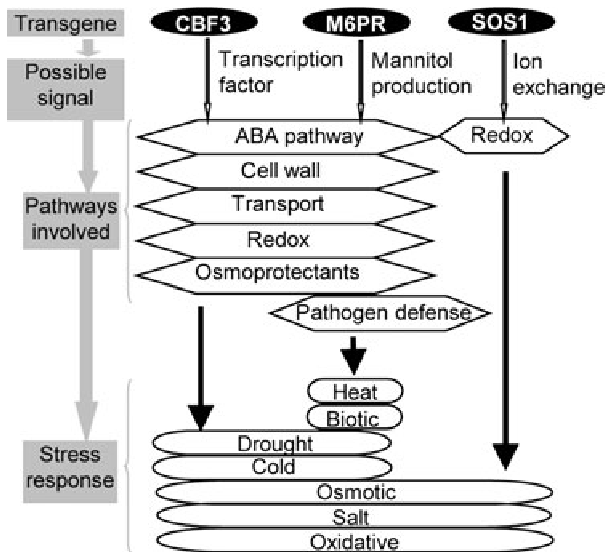

Figure 7 Model of relative transgene effects of *CBF3*, *M6PR* and *SOS1* on Arabidopsis gene expression and stress responses.

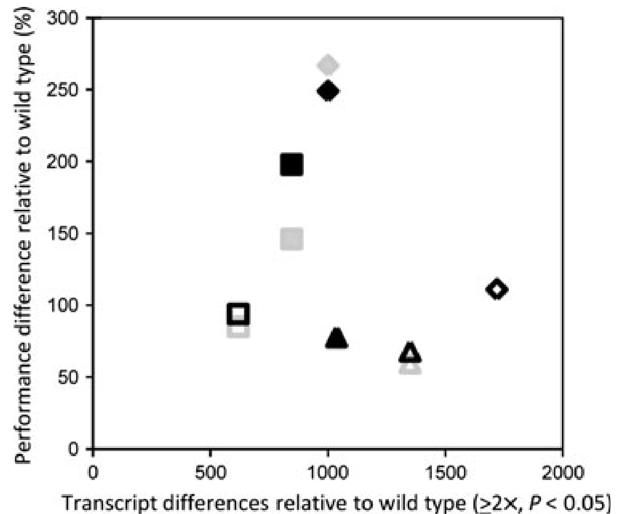

Figure 8 Lack of relationship between magnitude of transcriptional effects of the CBF3 (triangles), M6PR (diamonds) and SOS1 (squares) transgenes and effect of the transgenes on vegetative (dry weight, black symbols) and reproductive (seed production, grey symbols) performance in the growth chamber. Open symbols, 0 mM NaCl; closed symbols, 100 mM NaCl. Performance values are averaged over the two lines for each transgene. Magnitude of transcriptional effects was number of significantly changed transcript levels for the transgenic lines vs. wild type. Performance difference refers to above-ground dry weight or seed yield for the transgenic lines relative to wild type, expressed as a per cent of wild type.

Experimental procedures

Plant materials

Arabidopsis thaliana L. (Heynh) plants overexpressing three abiotic stress resistance genes under the control of CaMV 35S promoter, as well as their wild-type parents, were used in this experiment. Two lines were used for each transgene. A30 and A40 transgenic lines overexpressing C-repeat/DRE binding factors (*CBF3*) and Wassilewskija (Ws) background were kindly provided by Michael F. Thomashow (Gilmour *et al.*, 2000). M2 and M5 lines overexpressing celery mannose-6-phosphate reductase (*M6PR*) in the Columbia (Col) background were produced by Zhifang and Loesher (2003). Two plasma membrane Na^+/H^+ antiporter (*SOS1*) transgenic lines (#1-1 and #7-6) and Columbia-glabrous (*gl1-1*) (Col(gl)) background were generously provided by Huazhong Shi (Shi *et al.*, 2003). All transgenic and wild-type lines were verified for the presence and expression of the relevant transgenes by Southern and Northern blot analyses prior to initiation of the experiments (data not shown).

Growth conditions and salt treatment in the growth chamber

Seed production, planting and growth conditions were as described by Chan *et al.* (2011). Salt treatment was initiated at 14 DAP (6 true leaf stage). Plants subjected to salt stress were sub-irrigated to field capacity with NaCl solution dissolved in ½ strength Hoagland solution and then sprayed with the same concentration of NaCl solution from the top, ensuring adequate leaching and preventing excess salinity. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 2 days for each line, to the indicated maximum (0, 100, or 200 mM). Plants were then watered every 2 days at the indicated concentrations. The pots were rotated in the growth chamber everyday to minimize the effect of environment. All genotype–salt combinations were grown together in the growth chamber at the same time.

Measurement of growth parameters including bolting and flowering time, leaf number, rosette diameter, plant height, stalk number, dry weight, and seed yield were taken as described by Chan *et al.* (2011). Chlorosis/necrosis severity indices, leaf numbers and rosette diameters were measured every 6 days. Chlorosis/necrosis severity was rated as follows: 0, no yellow or purple leaves; 1, older leaves turn yellow or purple; 3, younger leaves turn yellow or purple; 5, some leaves die; and 7, plants die. Severity indices were calculated analogous to the disease severity index of Piccinni *et al.* (2000) as follows: Σ (number of plants with each score $\times$ score value)]/(total number of plants $\times$ highest score). The plants were photographed at 50 DAP and harvested at 62 DAP when most of them reached maturity. The complete experiment was repeated three times. All data were analysed with SPSS 11.5 for windows (SPSS, Inc., Chicago, IL). Mean separations were performed by Duncan's multiple range test. Differences at $P < 0.05$ were considered to be significant.

Growth conditions and parameters in the greenhouse and field

Seed from verified growth chamber grown plants of all transgenic and wild-type lines were counted into 5 replicate batches per line for the greenhouse and field experiments. Each batch contained approximately 180 seeds. All batches of seeds were stratified as described previously, mixed with sterile sand and randomly scattered onto 26 × 26 × 6 cm pots filled with a standard planting medium (Baccto, Houston, TX) mixed with 2.1 kg/m^3 Osmocote Classic 14-14-14 slow release fertilizer (The Scotts Miracle-Gro Company, Marysville, OH). All plant populations were germinated and grown in the greenhouse with supplemental lighting providing 12 h light/12 h dark. Pots were sub-irrigated as required. Greenhouse populations were rotated biweekly to minimize location effects.

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At the 6 true leaf stage, the pots for the field experiment were moved from the greenhouse and placed into anchored 52 × 26 × 6 cm trays placed atop weed barrier plastic in the field. Trays were spaced every 0.6 m and watered as needed by trickle hose to allow for sub-irrigation of the pots. The plants were maintained in the field until they approached senescence and then returned to the greenhouse to complete senescence and dry down. Total above-ground dry weight and seed yield were measured after harvest in the greenhouse. All data were analysed with SAS 9.2 for windows (SAS Institute Inc., Cary, NC). Statistical tests were performed as described before.

Plant growth and salt treatment for microarray experiment

Seeds of three transgenic lines and their wild-type plants were sowed as described previously with two replicate pots for each genotype and salt combination (0 or 100 mM). Two replications were performed in different growth chambers on different dates with 36 plants/replicate pot for each genotype and salt combination. Plants were grown at 23/18 °C in the growth chamber 10-h light/14-h dark cycle at 350 μmol/m^2/s and 70% relative humidity. Salt treatments were initiated at 14 DAP and applied as described previously. Sampling was performed at 20 DAP by collecting fully developed but not senescent leaves (about 0.5 cm width × 1.5 cm length) from at least 15 seedlings/treatment.

RNA isolation, GeneChip® hybridization and microarray analysis

RNA isolation and GeneChip® hybridization for microarray experiments was performed as described by Chan *et al.* (2011). Total RNA was extracted and purified from leaves of at least 15 plants per genotype and salt treatment combination. Two biological replicates from different growth chambers were prepared for each genotype and salt combination. To minimize the transgene position effects, equal amounts of total RNAs from the two lines for each transgene (A30 and A40 for *CBF3*, M2 and M5 for *M6PR*, and 1-1 and 7-6 for *SOS1*) were pooled for biotin labelling.

The reproducibility of the microarray experiments was characterized by comparing each set of data generated from the duplicated experiment with Affymetrix GCOS software. Raw signal data from two biological replicates were compared, and a correlation coefficient was calculated between the duplicate experiments. All biological replicates had a coefficient of determination (R^2) larger than 0.91 (Figure S2). All the Affymetrix data files produced with Affymetrix GCOS software (*.CEL files) were analysed using Bioconductor, a public source software for the analyses of genomic data rooted in the statistical computing environment R (Gentleman *et al.*, 2004). The data were normalized by robust multiarray normalization of probe-level data with RMA and analysed using affylmGUI running on R software (Wettenhall *et al.*, 2006). To determine meaningful differences between samples, modest threshold parameters were applied in this study to minimize any potential statistical biases. Transcript levels deemed significantly different were those with (i) a fold change larger than 2; (ii) a *P*-value smaller than 0.05; and (iii) a detection call of 'Present' in duplicate with the Affymetrix GCOS. Microarray data are available online in Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accessions number (GSE26983). The M6PR-Col WT microarray data were previously deposited to the Gene Expression Omnibus (GEO) online database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE18217 and published by Chan *et al.* (2011).

Quantitative real-time PCR

Total RNAs extraction, cDNA synthesis and PCR amplification were performed as described by Chan *et al.* (2011). All reactions were run in duplicates, and the average values were calculated. Quantification was performed with at least two independent experiments. The housekeeping F-actin gene (At3g05520) was used as endogenous control. Relative expression levels of target genes and SD values were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Twenty genes with at least one significant sample difference from nine comparisons based on microarray data were selected for qRT-PCR analyses, along with a single gene that did not (At3g63490). Log2 values for each replicate and their averages and standard errors were calculated. Primers used for real-time PCR are listed in Table S4.

Biological enrichment and metabolic pathway analyses

All transcripts with *P*-value ≤0.05 and fold change ≥2 were loaded and annotated in the Classification SuperViewer Tool w/Bootstrap web database (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) (Provart and Zhu, 2003). MapMan was used as the classification source to assign functional categories for each gene (Thimm *et al.*, 2004). The absolute values and normalized frequency relative to the Arabidopsis genomic set of each functional category were then calculated as described by Chan *et al.* (2011). For GO term enrichment analysis, all transcripts with *P*-value ≤0.05 and fold change ≥2 were loaded in 'Term enrichment' using AmiGO software (http://amigo.geneontology.org) (Carbon *et al.*, 2009). Enriched fold change of each functional category was calculated as following: enriched fold change = sample frequency of each category in this experiment/background frequency of each category in the Arabidopsis genome. Hierarchical cluster analyses was performed on selected sets of genes using the CLUSTER program (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/) (deHoon *et al.*, 2004) by the uncentred matrix and complete linkage method. Resulting tree figures were displayed using the software package, Java Treeview (http://jtreeview.sourceforge.net/).

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Supporting information

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

Figure S1 Transgene effects on growth parameters during the whole life cycle.
Figure S2 Comparisons of Affymetrix chip signals from biological replicates.
Figure S3 Comparison of relative transcript abundance measured by q RT-PCR versus microarray analysis.
Figure S4 Northern blot analysis of M6PR gene expression.
Table S1 Transcript levels changed by transgenes or salt.
Table S2 List of abiotic, biotic, stress, redox, abscisic acid, transport and minor carbohydrate-related gene expression significantly affected by transgenes.
Table S3 Summary of the classification information and biological enrichment analysis of transcripts changed by transgene or salt.
Table S4 Detailed primer sequences used for qRT-PCR.

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