Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant

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Summary

The phytohormone abscisic acid (ABA) plays important regulatory roles in many plant developmental processes including seed dormancy, germination, growth, and stomatal movements. These physiological responses to ABA are in large part brought about by changes in gene expression. To study genome-wide ABA-responsive gene expression we applied massively parallel signature sequencing (MPSS) to samples from *Arabidopsis thaliana* wildtype (WT) and *abi1-1* mutant seedlings. We identified 1354 genes that are either up- or downregulated following ABA treatment of WT seedlings. Among these ABA-responsive genes, many encode signal transduction components. In addition, we identified novel ABA-responsive gene families including those encoding ribosomal proteins and proteins involved in regulated proteolysis. In the ABA-insensitive mutant *abi1-1*, ABA regulation of about 84.5% and 6.9% of the identified genes was impaired or strongly diminished, respectively; however, 8.6% of the genes remained appropriately regulated. Compared to other methods of gene expression analysis, the high sensitivity and specificity of MPSS allowed us to identify a large number of ABA-responsive genes in WT *Arabidopsis thaliana*. The database given in our supplementary material (http://jcs.biologists.org/supplemental) provides researchers with the opportunity to rapidly assess whether genes of interest may be regulated by ABA. Regulation of the majority of the genes by ABA was impaired in the ABA-insensitive mutant *abi1-1*. However, a subset of genes continued to be appropriately regulated by ABA, which suggests the presence of at least two ABA signaling pathways, only one of which is blocked in *abi1-1*.

Key words: ABA, Gene expression, *abi1*, MPSS, Stress response, Growth

Introduction

The phytohormone ABA regulates plant growth and developmental processes in response to changes in water status. This is manifested at the physiological level, for example, by controlling germination, stomatal movements, and growth. At the molecular level ABA-dependent changes in gene expression and post-translational modifications underpin these processes (Leung and Giraudat, 1998; Schroeder et al., 2001). As water deficit is one of the most important factors that limit plant productivity, the elucidation of ABA signal transduction pathways offers the possibility to genetically improve water stress tolerance.

Analysis of ABA mutants has identified some components of the signaling network. Three classes of mutants: ABA-deficient, -hypersensitive, and -insensitive mutants have been characterized. In ABA-deficient mutants, ABA-induced stomatal closure and expression of some genes are impaired leading to a wilty phenotype (De Bruxelles et al., 1996; Seo et al., 2000; Taylor et al., 2000; Xiong et al., 2001; Xiong et al., 2002). Several *Arabidopsis* mutants display ABA hypersensitivity resulting in diminished germination rates at low ABA concentrations and reduced water loss due to enhanced ABA-induced stomatal closure (Cutler et al., 1996; Lu and Fedoroff, 2000; Hugouvieux et al., 2001; Steber and McCourt, 2001). ABA-insensitive mutants including *abi1* to *abi5* and *gpa1* are affected in ABA-mediated inhibition of germination and growth and they are also impaired in stomatal movement (Leung and Giraudat, 1998; Koornneef et al., 1984; Wang et al., 2001). Changes in ABA-regulated gene expression result from mutations in the putative transcription factors ABI4 and ABI5 (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Söderman et al., 2000). *ABI1* and *ABI2* encode protein phosphatases 2C. Although no target protein has yet been identified, an impaired expression of a few reported genes in *abi1-1* and *abi2-1* suggests that dephosphorylation events may regulate ABA-mediated gene expression (Gilmour and Thomashow, 1990; Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1995; Strizhov et al., 1997; Uno et al., 2000). It is, however, not conclusively known whether the dominant *abi1-1* and *abi2-1* mutations are neomorphic or whether ABI1 and

ABI2 are indeed components of ABA signaling because no null allele lacking phosphatase protein expression has yet been isolated. Although suppressor mutants of *abi1-1* lack phosphatase activity, they still produce mutant proteins that may have a signaling function, which is independent of its phosphatase activity (Gosti et al., 1999).

In addition to the analysis of ABA signaling mutants, the use of microinjection identified cyclic ADP ribose (cADPR) as an early mediator of ABA responses. The ABA signal is transduced and sustained by increases in cytosolic [Ca^{2+}] stimulated by cADPR and phosphoinositides, respectively (Wu et al., 1997; Sanchez and Chua, 2001). Nevertheless, a link between the nature of [Ca^{2+}] oscillations and gene expression has not yet been shown in plants (McAinsh et al., 2000; Schroeder et al., 2001).

Despite substantial advances in the understanding of ABA-mediated transcriptional control, only a few regulatory components have been identified thus far. In view of the diversity and complexity of the signaling network underlying physiological responses to ABA and the potential crosstalk with other signaling pathways, many fundamental mechanisms remain to be resolved. One approach is the identification of ABA-regulated genes on a genome-wide scale. Whereas the regulation of single genes can be easily examined by RNA gel blot hybridizations or RT-PCR, DNA microarrays provide a tool to monitor changes in expression levels of a larger number of genes simultaneously (Desprez et al., 1998; Schaffer et al., 2001; Schenk et al., 2001; Seki et al., 2001). However, plant DNA microarrays represent closed systems that prevent whole genome scans of gene expression. Prior to the very recent release of the Affymetrix GeneChip Arabidopsis ATH1 (approximately 24,000 genes represented), the only available plant DNA microarrays carried a limited number of genes. To monitor ABA-dependent gene expression on a genome-wide scale, we used MPSS on WT and *abi1-1* samples. The MPSS technique combines the physical separation of complex nucleic acid samples by in vitro cloning of templates on microbeads with ligation-based signature sequencing (Brenner et al., 2000a; Brenner et al., 2000b). Starting from the GATC site closest to the polyA tail, 17-base long sequences are collected from the cloned cDNA molecules and the frequency of occurrence of each sequence is counted to determine the transcript abundance. The availability of the *Arabidopsis* genomic sequence and information on numerous expressed sequence tags allowed us to assign the signature sequences to annotated genes and obtain the transcript level of all expressed genes.

Here, we report a genome-wide gene expression study identifying 1354 ABA-responsive genes in *Arabidopsis*. We have uncovered gene subclasses previously not known to be regulated by ABA. In the ABA-insensitive mutant *abi1-1*, the ABA response of about 84.5% of the identified genes was dramatically altered. Nearly 9% of the ABA-responsive genes are regulated by a separate pathway that is insensitive to the *abi1-1* mutation. The gene expression in *abi1-1* was impaired in the absence of ABA suggesting that the dominant negative mutation also acts independently of exogenous ABA.

Materials and Methods

Plant material and RNA isolation
Seeds of WT (ecotype Landsberg) and *abi1-1* were grown on agar plates as previously described (Sanchez and Chua, 2001). Four-week-old seedlings were transferred to a hydroponics system containing 50% Murashige and Skoog salt base (pH 5.7) and 1% sucrose and grown for 2 days under long-day conditions (16 hours of light/ 8 hours of dark). Fresh medium containing ABA (Sigma, St Louis, MO) dissolved in 100% methanol to give a final concentration of 50 µM was added between 08.00 and 09.00 hours. The same volume of methanol was added to the medium of control plants. The final concentration of methanol in both treatments was 0.05%. Plants were removed at the designated times and frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Gibco BRL, Grand Island, NE). PolyA RNAs were isolated using a purification kit by following the manufacturer's protocols (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA gel blot analysis, RT-PCR and expression of GFP fusions in onion cells
RNA gel blot analysis was performed as described (Ausubel et al., 1994). Each lane contained 10 µg total RNA. 200-250 bp fragments from the 5′-end or 3′-regions of the indicated genes were amplified using HotStarTaq DNA Polymerase (Qiagen, Valencia, CA). The fragments were purified using the Qiaquick Gel extraction protocol (Qiagen), verified by sequencing analysis and labeled with ^{32}P-dCTP and ^{32}P-dATP by random priming (Amersham, Arlington Heights, IL). Hybridization signals were monitored using the STORM phosphoimager system and analyzed using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RT-PCR was performed using the Titan one tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocols. 20 cycles of PCR amplification were used for each of sample except the control actin-2, which was detected at 15 cycles. The primers used for PCR amplification of At5g57520 were 5′-GGACTACCAGCCAAACACATCCCTACGTC-3′ and 5′-CCACTCTCCGGCACACGGCGGGC-3′; primers used for At2g46510 were 5′-CTCCTCGGCCACGATGTCTCTCCGC-3′ and 5′-CATAATCCGCCAAAATCTCTTCCATTCCTTC-3′; primers used for At2g35940 were 5′-CACGATGAAGATTCTAGAAGAACGG-CAAGGG-3′ and 5′-CGGTTTCTCCTTCGAGAGAGATGGGTT-TATGC-3′; primers used for At4g18160 were 5′-GAGTAAAGC-AGAGTATGTGATATACAAACTGAAGGAGATGG-3′ and 5′-GAT-GTGAATCCAGTGAATCCTGCTTAACCAATTATGC-3′; primers used for At3g43600 were 5′-CCACCCGATTCACAAGCGGTT-ATCCGG-3′ and 5′-CCTCTGAGCCATTCACAAGGTTTCCACC-3′; primers for actin-2 (U37281) were 5′-GCCGGACTTACCGTTG-TATGTACCGTCC-3′ and 5′-ACAATGGAACTGGAATGGTGAA-GGCTGG-3′. Amplified DNAs were separated in a 1.6% agarose gel. All DNA bands resulting from the RT-PCR reaction were tested for RNAase A sensitivity.

For transient expression in onion epidermal cells, the *GFP* coding sequence was fused in-frame to the 3′ ends of the *ABI1* and *abi1-1* cDNAs as previously described (Kost et al., 1999). *ABI1* and *abi1-1* cDNAs were generated by using primers to amplify the coding region from the ATG start codon (5′-CGACTTCTCGAGATGGAGGA-AGTATCTCCGGC-3′) with an additional *XhoI* site, to the TGA stop codon (5′-GCGAACTGAGGTACCGTTCAAGGGTTTGCTCTTG-3′) thus adding a *KpnI* site and deleting the stop codon. GFP localization was monitored 14-16 hours after transfection of onion cells.

### Data acquisition and analysis
In vitro cloning of polyA RNA, formation of microbead libraries, sequencing of DNA on microbeads and base calling were performed as described (Brenner et al., 2000a; Brenner et al., 2000b). The number of signatures collected (corresponding to the number of mRNA molecules analyzed) was 1,477,653 for the WT control

sample, 1,266,435 for the WT treated sample, 1,656,984 for the *abi1-1* control sample, and 1,241,725 for the *abi1-1* treated sample. Four separate sequencing runs were performed for each sample to produce the total number of signatures. Abundance for each distinct signature was counted and normalized in parts per million to estimate transcript abundance. Differences in expression levels were deemed significant when the ratio of the abundances was at least 3 and/or statistically significant at the 0.0005 level as determined on the basis of the total number of signatures collected in each sample using a formula previously described (Audic and Claverie, 1997). When the lower parts per million (ppm) count was less than 10, larger fold ratios are required in order to be statistically significant. For comparison of ABA-responsive gene expression in the *abi1-1* mutant to WT we considered a twofold change at the 0.0005 level to be statistically significant in *abi1-1*. In the supplementary data (http://jcs.biologists.org/supplemental) 'induced.xls' and 'repressed.xls' we marked each gene with one of the following codes (column J): code 0, if the expression of the particular gene is ABA-responsive in WT but ABA-insensitive in *abi1-1* [e.g. the induction factor in *abi1-1* (column G) is not significant (n.s.)]; code 1 for comparable regulation in WT and *abi1-1*, if the quotient of the induction factor in WT (column D) and the induction factor in *abi1-1* (column G) is in a range between 0.5 and 2; code 2 for similar regulation, if the particular gene is ABA-sensitive in both WT and *abi1-1* but to a different extent. The quotient of the induction factor in WT (column D) and the induction factor in *abi1*-1 (column G) is greater than 2 or less than 0.5; code 3 for genes that are induced by ABA in WT but repressed in *abi1-1* or vice versa. In this case, the induction factor in *abi1-1* (column G) is less than 0.5.

Results

Genome-wide identification of ABA-responsive genes

To investigate ABA-responsive gene expression at the whole-genome level, we applied MPSS to samples from 4-week-old *Arabidopsis thaliana* seedlings (ecotype Landsberg erecta). Plants were treated with 50 µM ABA or a mock solution. To minimize the effect of biological variations among single plants, more than 250 seedlings were treated for each sample. Under the conditions used, many known ABA-responsive genes displayed a significant induction in their expression level 3-6 hours after ABA addition, as determined by RNA gel blot experiments (not shown). Total RNAs isolated from tissue samples after treatments for 3 hours and 5 hours were therefore pooled. The transcriptional upregulation of the ABA-responsive marker gene *KIN2* was monitored by RNA gel blot hybridization to verify the effectiveness of the ABA treatment (not shown). Using the MPSS technique we identified 31221 and 29475 unique signature sequences of 17 bases each for the control and the ABA-treated sample, respectively. For a unique signature sequence the normalized number of the frequency of occurrence in parts per million (ppm) represents a measure of the expression level of the corresponding transcript. To investigate differential gene expression, we plotted the signature abundance for the ABA-treated sample versus that of the control sample (Fig. 1). Based on a 0.0005% significance level a large number of signatures were up- or downregulated (Audic and Claverie, 1997). For the following analyses we considered a threefold induction or repression to be biologically significant (except when it was not statistically significant, where we used a 0.0005% significance level) and identified 1191 signatures to be upregulated and 1128 signatures to be downregulated by ABA. A BLAST search was
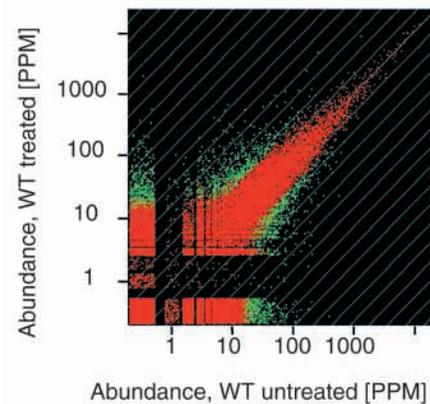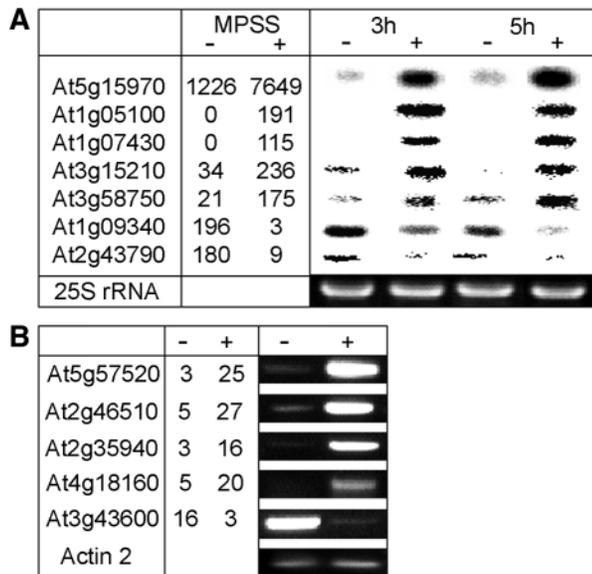

Fig. 1. ABA-responsive gene expression in *Arabidopsis* WT. The normalized abundance in ppm of each signature derived from the ABA-treated sample (y axis) was plotted on a logarithmic scale versus that derived from the untreated sample (x axis). The log scaling ends at 1 and signatures with abundance 0 are presented in the lower left corner. For each abundance (ppm) value, points are not placed on a line but allocated to a bar of a certain width that decreases with increasing abundance. Thus, data points are scattered in bi-dimensional space to better reflect density. This translates into a wide gap as seen at around 1 ppm because there were few signatures with both 1 or 2 ppm that would normally occupy a certain space either along the x- or y-axis. Up- or downregulated genes, as defined on the basis of a threefold ratio and/or a 0.0005% significance level, are shown in green. Genes unaffected by ABA are shown in red.

performed for each of these signature sequences and hits in the genomic sequence of *Arabidopsis thaliana* (ecotype Columbia) were found for 813 upregulated and 876 downregulated signatures. The fact that not all signature sequences were found in the *Arabidopsis* genomic sequence might be due in part to DNA sequence variations between the Landsberg and Columbia ecotypes because only perfect matches were accounted for. In addition, some differences may arise from sequencing errors and/or signature sequences that are unpredictable from the genomic sequence because of exon-exon junctions. We identified 660 induced and 694 repressed annotated genes (see supplementary data: http://jcs.biologists.org/supplemental) following the prediction of genes provided by the Munich Information Center for Protein Sequences (MIPS) and The Institute for Genomic Research (TIGR). Whereas the expression of the majority of these genes changed by three- to tenfold, about 25% of the genes were induced or repressed by more than tenfold. For the remainder, 144 upregulated sequences and 178 downregulated sequences fall into genomic regions that did not include an annotated gene according to the prediction of MIPS and TIGR.

Verification of the MPSS data

To verify the results obtained with MPSS we isolated RNA samples from two independent but otherwise identical ABA treatments that were performed by different investigators. We confirmed the MPSS data (Fig. 2A) by RNA gel blot analyses, using as probes newly identified ABA-responsive genes encoding proteins with very different functions. These include a putative NPK1-related MAP kinase (At1g05100), a putative protein phosphatase 2C (At1g07430), the ethylene

A

	MPSS		3h		5h	
	−	+	−	+	−	+
At5g15970	1226	7649				
At1g05100	0	191				
At1g07430	0	115				
At3g15210	34	236				
At3g58750	21	175				
At1g09340	196	3				
At2g43790	180	9				
25S rRNA						

B

	−	+	−	+
At5g57520	3	25		
At2g46510	5	27		
At2g35940	3	16		
At4g18160	5	20		
At3g43600	16	3		
Actin 2				

Fig. 2. Confirmation of MPSS data using different techniques. (A) Induction or repression of seven genes was verified by RNA gel blot analyses. Total RNAs from an independent ABA (50 μM) treatment were isolated from WT plants after induction for 3 hours and 5 hours (+). Control plants were treated in parallel (−). The RNA gel blot was probed with DNA fragments of the indicated genes. (B) RT-PCR was used to confirm regulation of genes with low transcript abundance. 200-500 ng total RNA of samples from an independent 4 hours ABA (50 μM) treatment was used for each of the indicated RT-PCR reactions. The 25S RNA band stained with ethidium bromide served as a loading control. The names of genes shown in A and B are: *KIN2* (At5g15970), putative NPK1-related MAP kinase (At1g05100), putative protein phosphatase 2C (At1g07430), ethylene responsive element binding factor 4 (At3g15210), citrate synthase-like protein (At3g58750), putative RNA-binding protein (At1g09340), *MPK6* (At2g43790), *ZFP2* (At5g57520), putative bHLH transcription factor (At2g46510), putative homeodomain transcription factor (At2g35940), potassium channel-like protein (At4g18160), and *AAO2* (At3g43600).

responsive element binding factor 4 (At3g15210), a citrate synthase-like protein (At3g58750), a putative RNA-binding protein (At1g09340), and the MAP kinase AtMPK6 (At2g43790). For some ABA-regulated genes that were expressed at very low levels in both induced and control samples (3-27 ppm), the MPSS data were confirmed by RT-PCR (Fig. 2B). These included the zinc finger protein ZFP2 (At5g57520), a putative bHLH transcription factor (At2g46510), a putative homeodomain transcription factor (At2g35940), a potassium channel-like protein (At4g18160), and AtAAO2 (At3g43600). Besides confirmation of the results through other techniques, the MPSS data set was also confirmed by analysis of the regulation of the large number of ABA-upregulated genes previously reported in the literature. Table 1 shows a list of ABA-responsive genes including the prominent marker genes *KIN2, KIN1, COR47, RD20,* and *COR15A.* Note, that the expression of the actin genes *ACT2* and *ACT8,* which are frequently used as internal controls in gene expression experiments, was not affected by ABA.

**Table 1. Induction factors of known ABA-induced genes**

MIPS ID	Gene product	Induction factor
At5g06760	Late embryogenesis abundant protein LEA like (D113)	328
At5g52310	Low-temperature-induced protein 78, RD29A	58.3
At3g61890	Homeobox-leucine zipper protein AtHB-12	58
At3g17790	AtACP5, acid phosphatase type 5	29.2
At5g15960	AtKIN1	24
At5g66400	Dehydrin RAB18-like protein	77.6
At2g46680	Homeodomain transcription factor AtHB-7	27.7
At2g39800	AtP5C1, delta-1-pyrroline 5-carboxylase synthetase	22.3
At1g20450	AtERD10, hypothetical protein	18.4
At2g33380	AtRD20	18
At4g26080	AtABI1	16.4
At4g34000	AtABF3	13.5
At5g57050	AtABI2	12.9
At2g42530	Cold-regulated protein cor15b precursor	8.5
At1g77120	Alcohol dehydrogenase	8.5
At1g69270	Putative receptor-like protein kinase RPK1	8
At1g20440	Hypothetical protein, AtCOR47	6.6
At5g15970	Cold-regulated protein COR6.6 (KIN2)	6.2
At2g42540	Cold-regulated protein cor15a precursor	5.8
AB017160	ABA-responsive element binding protein 1 (AREB1)	4.6
At5g13200	ABA-responsive protein-like	4.1
At1g72770	Protein phosphatase 2C (AtP2C-HA)	3.7
At2g45960	PIP1B aquaporin	3.4

Functional classification of ABA-responsive genes

The classification of genes into groups of similar function is one approach to understanding gene reguation by ABA at the genomic level. We performed a functional classification based on the classification of the MIPS database. In addition, protein-protein BLAST analysis enabled the identification of characteristic protein motifs suggesting putative gene functions. Fig. 3 summarizes our classification of ABA-responsive genes into 9 different groups. In the *Arabidopsis* genome many genes still do not have a clearly defined function and are therefore referred to as 'unclassified'. The fraction of unclassified genes is more prominent among the downregulated genes. The proportion of genes induced or repressed in most categories did not differ. However, there were significant differences in the number of genes belonging to the 'Cell rescue, defense, ageing' class. Less than 1% of all downregulated genes was related to cell rescue and defense, whereas more than 5% of the upregulated genes belong to this group. This is consistent with the role of ABA in plant stress responses.
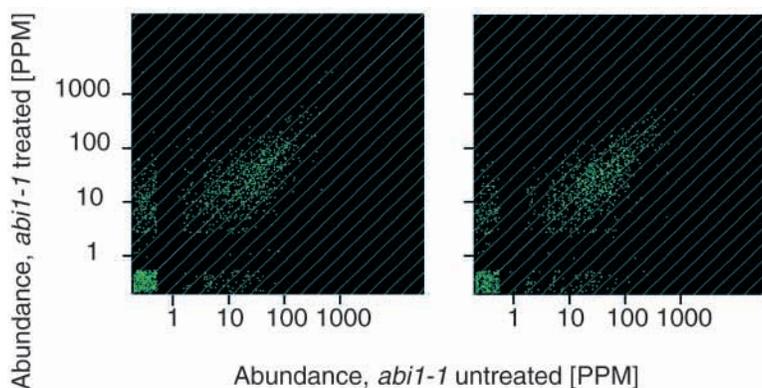
The ABA mutant *abi1-1* is largely affected in gene expression

Although the *abi1-1* mutant had been previously reported to be impaired in ABA-responsive gene expression, the analysis was limited to only a few genes (e.g. Gilmour and Thomashow, 1990; Leung et al., 1997; Leung and Giraudat, 1998). To extend this analysis to the whole *Arabidopsis* genome, we performed the same MPSS analysis on RNA samples isolated from *abi1-1* mutant plants. The majority of signature sequences, which were ABA-responsive in WT, were not differentially regulated in *abi1-1* (Fig. 4). Of the 1354 ABA-responsive genes 75.2% were unaffected and 9.3% showed inverse regulation by ABA in *abi1-1* (supplementary data, 'induced.xls' and 'repressed.xls', code 0 and 3, respectively). About 7% of the genes were still responsive to ABA in
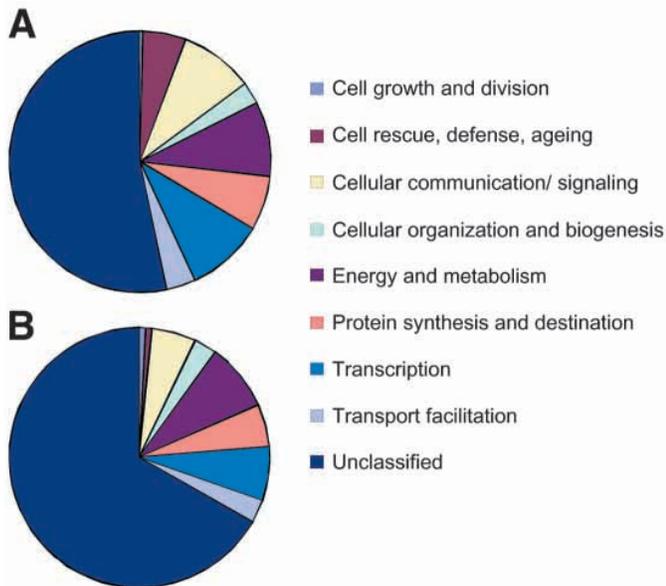
Fig. 3. Functional classification of ABA-induced genes. According to their known or suggested functions induced (A) and repressed genes (B) were grouped into the nine indicated categories. The segment sizes correspond to the number of genes in each category related to the total number of either up- or downregulated genes.

abi1-1 albeit with a diminished induction or repression (supplementary data, 'induced.xls' and 'repressed.xls', code 2). Nearly 9% of the genes such as the transcriptional activators *HB8* (At4g32880) and *NAC1* (At1g56010) showed very similar transcript levels and regulation in both *abi1-1* and WT indicating that their ABA-mediated expression is insensitive to *abi1-1* (supplementary data, 'induced.xls' and 'repressed.xls', code 1). Surprisingly, an induction of *ABI1* itself was observed in both WT (from 41 up to 671 ppm) and *abi1-1* (from 52 ppm up to 157 ppm), although to slightly different levels. This result is at variance with the impaired upregulation of *ABI1* in *abi1-1* after 55 hours of ABA incubation reported by Leung et al. (Leung et al., 1997). To investigate the time-dependent transcriptional control of *ABI1*, we performed a detailed kinetic analysis of ABA induction. RNA gel blot analyses demonstrated a dramatic upregulation of *ABI1* after 1 hour ABA but not after mock treatment (Fig. 5A). The transcription level reached a peak after 4 hours and decreased slightly after more than 5 hours. In the dominant mutant *abi1-1* the mutant

abi1-1 gene exhibited the same induction kinetics indicating that *ABI1* does not control its own early ABA-mediated expression. The homologous *ABI2* was ABA-responsive in WT like *ABI1*, but its induction was abolished in the *abi1-1* mutant (Fig. 5A).

In contrast to WT samples, only 24764 and 24044 unique signature sequences were found in ABA-treated and untreated *abi1-1* samples, respectively. This suggests that fewer genes were expressed in the mutant even under control conditions. We identified 194 genes that were expressed at a highly significant level in WT plants (ppm≥22), but not in *abi1-1* irrespective of the treatment (ppm=0). These 194 genes could be subdivided into two groups: (1) 71 genes induced by more than threefold by ABA; and (2) 123 genes the expression of which is independent of the hormone.
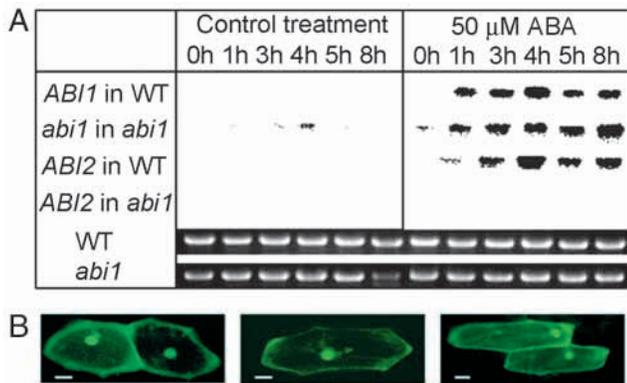
As the *abi1-1* mutation caused dramatic changes in gene expression, we studied the subcellular localization of the WT and mutant protein by introducing *ABI1-GFP* and *abi1-GFP* fusion genes into onion epidermal cells using a particle gun. Expression of both fusion proteins was observed in the cytoplasm and in the nucleus (Fig. 5B) indicating that the mutation did not affect the subcellular localization of the protein.

Discussion
Identification of ABA target genes in the *Arabidopsis* genome by MPSS

The plant hormone ABA mediates environmental stress signals resulting from drought, high salinity, and low temperature (Ingram and Bartels, 1996; Bray, 1997; Thomashow, 1999). A broad spectrum of genes is induced during water deficit conditions, and these genes encode not only proteins involved in the protection of cellular structures or in the biosynthesis of protective metabolites but also key regulatory proteins that amplify or attenuate the signal (Ramanjulu and Bartels, 1996). However, in view of the many ABA-dependent processes, the number of ABA-responsive genes identified so far is rather limited. In particular, few ABA-downregulated genes have been reported. We used MPSS to investigate gene expression on a genomic scale and identified 1354 genes differentially regulated by ABA by more than threefold (see supplementary data), including 694 downregulated genes.

Compared with cDNA-microchip experiments, MPSS offers several advantages. (1) The problem of cross-hybridization between highly homologous sequences in cDNA-microchip experiments is not encountered, thereby ensuring much higher gene specificity. In 97.2% of all the signatures, 17 bases were sufficient to uniquely identify the corresponding gene in the *Arabidopsis*

Fig. 4. ABA-responsive gene expression in *abi1-1*. The normalized abundances in ppm for the treated *abi1-1* sample were plotted against those for the untreated *abi1-1* sample. Representation is as described for Fig. 1. Displayed signatures were selected on the basis of either being upregulated (left panel) or downregulated (right panel) in WT following ABA treatment (c.f. Fig. 1, signatures in green above the diagonal and below the diagonal, respectively).

Fig. 5. (A) Kinetics of *ABI1* and *ABI2* expression. The time-dependent expression of *ABI1* and *ABI2* in the presence and absence of ABA in WT and *abi1-1* was monitored at the indicated time points. The 25S RNA band stained with ethidium bromide served as a loading control of the RNA gels. (B) Subcellular localization of ABI1 and abi1. GFP fluorescence of ABI1-GFP (left), abi1-GFP (middle) and GFP (right) in onion epidermal cells was observed 16 hours after biolistic bombardment. Representative cells are shown. Bars, 50 µm.

genome. This discrimination, even between members of highly homologous gene families, cannot always be obtained in microarray experiments. (2) The MPSS technique offers a high resolution through very deep sampling, thus providing information about genes expressed at very low levels (Brenner et al., 2000b). (3) Previous knowledge of genes is not required for MPSS. In microarray experiments DNA fragments of annotated genes are spotted as probes. The still evolving annotation of the *Arabidopsis* genome therefore results in a partial loss of information obtained from DNA microarrays whilst the complete information of the MPSS experiment survives this evolution.

In comparison with techniques that depend on hybridization of probes to microarrays followed by the detection of a fluorescent signal, techniques based on counting of signatures of DNA fragments [so called digital northern blots (see Audic and Claverie, 1997)] are statistically more robust, especially when the size of the sample that is being counted is very large. The counting statistics are well modeled by the Poisson distribution (Audic and Claverie, 1997) and confidence intervals that account for sampling errors can be assigned to the data without the need for repetitions. These confidence intervals have to be interpreted as the boundaries for abundance estimates that could be obtained by analysing the same starting material multiple times (i.e. they refer to the error in measuring the abundance of each transcript) but do not take into consideration possible variations arising from the use of different biological materials that are being investigated (i.e they do not address the possibility that the variation among two experiments is due to factors other than the treatments being applied to them). In view of the costs of MPSS experiments we addressed this issue in different ways. (1) The RNA samples were isolated after treatment of at least 250 seedlings for each sample, thus providing an average of plants at the same developmental stage. (2) We have selected a very stringent significance level (at least a threefold change and $P<0.0005$) in spite of the high sensitivity of the MPSS

technique. Changes lower than threefold were not regarded as biologically significant. (3) The regulation of several novel ABA-dependent genes was confirmed by RNA gel blot analyses or RT-PCR (Fig. 2). For these confirmations we performed two independent but otherwise identical ABA treatments that were performed by two different investigators. Following these independent treatments we were able to confirm ABA-induced changes in gene expression at very low transcript levels by RT-PCR (Fig. 2). In addition, we uncovered many genes whose ABA responsiveness has been previously reported, thus verifying our results (Table 1). This list is not exhaustive for different reasons. For example, some genes are ABA-regulated in embryos but not in seedlings and different ABA concentrations and incubation times used in other studies could yield different results. A detailed kinetic analysis of induction would be a prerequisite to relate changes at the transcriptional level to physiological responses. Transcripts in which the GATC sequence is either lacking (not clonable) or more than 1000 bases away from the polyA end (not amplified well) and those that form a double palindrome (not sequencable) would not be detected by MPSS.

Many new gene targets are regulated by ABA

By extending the study of ABA-responsive gene expression to the genomic scale, we have identified large numbers of ABA-responsive genes that encode putative ABA signaling components including transcription factors, kinases, and phosphatases (Fig. 3). In addition, we discovered gene families that have not previously been reported to be ABA-regulated. As examples, we discuss genes encoding ribosomal proteins and proteins involved in regulated proteolysis. (1) Transcription factors and DNA-binding proteins: protein biosynthesis is required for ABA induction of some genes (Shinozaki and Yamaguchi-Shinozaki, 1996). The proteins that need to be synthesized include transcription factors that in turn amplify gene expression in a secondary response. We identified about 100 genes that encode transcription factors or DNA-binding proteins whose expression was regulated by ABA. This suggests that a cascade of transcription factors may mediate stimulus-dependent gene expression in ABA responses. (2) Ribosomal proteins decorate the rRNA cores of each ribosome subunit to stabilize their tertiary structure and control the dynamics of protein biosynthesis. Individual ribosomal proteins may play roles in regulating cell growth, proliferation, and death (Naora, 1999; Maguire and Zimmermann, 2001). Protein expression studies in maize suggested that ribosomal subunit heterogeneity at the tissue or cellular level might regulate the efficiency of the translation machinery (Szick-Miranda and Bailey-Serres, 2001). In *Arabidopsis* none of the 249 genes encoding ribosomal proteins is single copy and most proteins might be encoded by three or four expressed genes thus producing ribosome heterogeneity (Barakat et al., 2001). Here, we show that ABA contributes to the regulation of ribosomal proteins at the transcriptional level. Whereas only five genes encoding ribosomal proteins were induced, 16 genes were downregulated by ABA treatment. This may result in a diminished overall protein biosynthesis or an altered pattern of protein expression in plants confronting stress. (3) Proteins involved in regulated proteolysis: regulated proteolysis

Table 2. ABA-responsive genes coding for proteins involved in regulated proteolysis

Induction			Repression		
MIPS ID	Gene	Factor	MIPS ID	Gene	Factor
At1g60190	Hypothetical protein containing RING finger	157	At1g79650	Ubiquitin-like protein	21
At3g46620	Putative protein containing RING finger	50	At2g16920	Putative ubiquitin-conjugating enzyme	17
At1g20780	Hypothetical protein containing U-box	38	At5g43420	Putative protein containing ring finger	7.2
At5g05810	Putative protein containing RING finger	25	At5g20880	Putative protein containing RING finger	5.9
At3g61060	Putative protein containing F-box	22	At1g77440	Putative 20S proteasome beta subunit PBC2	4.2
At4g02570	Putative cullin-like 1 protein	20	At4g12570	Polyubiquitin-like protein	4.1
At3g09770	Putative RING zinc finger protein	11	At1g31340	Putative polyubiquitin	3.3
At1g10560	Putative zinc-binding protein containing RING finger	10.8			
At3g13550	Putative ubiquitin-conjugating enzyme	9.5			
At3g01050	Unknown protein containing ubiquitin signature	6			
At5g07270	Putative protein, RING finger signature	4.2			
At3g53000	Putative protein containing F-box	4.1			
At1g20810	Hypothetical protein containing RING zinc finger	3.9			
At1g76900	Tubby-like protein containing F-box	3.8			
At3g59940	Putative protein containing F-box	3.6			
At2g39720	Unknown protein containing RING-H2 finger	3.5			
At3g07360	Hypothetical protein containing Ubox	3.2			
At5g53300	Ubiquitin-conjugating enzyme E2-17 kD 10	3			

mediated by the ubiquitin-proteasome system is a key regulatory component of many cellular processes including cell cycle control, transcription, and receptor desensitization (Ciehanover, 1998; Kirschner, 1999). Recently, Lopez-Molina et al. demonstrated that the bZIP factor ABI5 is rapidly degraded in the absence, but not in the presence, of ABA (Lopez-Molina et al., 2001). This degradation can be blocked by inhibitors of the 26S proteasome suggesting that the ubiquitin-proteasome system may play a role in ABA signaling. Table 2 summarizes the regulation of 25 genes coding for proteins putatively involved in regulated proteolysis. Three quarters of these genes are upregulated, eight of them more than tenfold. ABA might trigger the controlled degradation of a variety of cellular regulatory proteins via the ubiquitin pathway. The gene products include hypothetical proteins with RING finger motifs, F-boxes, or U-boxes, which may interact with target proteins of the ubiquitin-proteasome pathway (del Pozo and Estelle, 2000; Azevedo et al., 2001). The ABA-mediated repression of other genes encoding proteins related to the pathway suggests that ABA can also block protein degradation as shown for ABI5. (4) Kinases and phosphatases: protein phosphorylation/dephosphorylation events represent key steps in signal transduction. Twenty-nine and 31 protein kinase genes were induced and repressed following ABA treatment. By contrast, protein phosphatase genes were almost exclusively upregulated and they predominantly encode type 2C protein phosphatases including *ABI1*, *ABI2* and *AtPP2C*. All three gene products have been suggested to act in the ABA signaling network, thus correlating ABA-induced expression of a gene with its function in the signaling pathway (Leung et al., 1994; Meyer et al., 1994; Sheen, 1998). We found that the transcriptional upregulation of *ABI1* by ABA was detected 1 hour after treatment. This early ABA response was unaffected in *abi1-1*, indicating that the mutation does not block its own early ABA-responsive expression (Fig. 5A). However, ABA induction of *ABI2* was abolished in *abi1-1* (Fig. 5A). ABI2 may therefore act downstream of ABI1, thus explaining why an *abi1-1/abi2-1* double mutant is not more resistant to ABA than the single mutants (Finkelstein and Somerville, 1990). It has recently

been suggested that in guard cells the *abi2-1* mutation disrupts early ABA signaling downstream of the *abi1-1* mutation (Murata et al., 2001).

In the presence of ABA, dephosphorylation events may activate or inhibit transcription factors. However, target proteins for dephosphorylation have not yet been identified. Alternatively, ABI1-mediated dephosphorylation could be involved in determining the cytosolic Ca^{2+} concentration thereby controlling transcription. In fact, it has been reported that the *abi1-1* mutation reduced the ABA-induced elevation in cytosolic $[Ca^{2+}]$ in *Arabidopsis* guard cells (Allen et al., 1999). In animals cells, the nature of $[Ca^{2+}]$ oscillations determines gene expression (Dolmetsch et al., 1998; Li et al., 1998). It is possible that ABA-induced $[Ca^{2+}]$ oscillations also optimize the efficiency and specificity of gene expression in guard cells.

Dissecting the regulation of gene expression in response to drought and ABA

In spite of the number of experimental parameters that affect gene expression data, our results correspond well with those obtained by full-length cDNA microarray experiments on drought-induced gene expression. Using an array with 1300 spotted cDNAs, Seki et al. identified 14 known and 30 new drought-induced genes (Seki et al., 2001). Comparing our results with these data we determined a two-thirds correspondence (Table 3). Large differences in abundance ratios seen in array and MPSS data may be explained by a combination of two factors. First, the treatments of plant samples used in both experiments are very different. Second, these differences may reflect the higher dynamic range of MPSS compared to arrays. The correlation between the ratios from the MPSS and the array experiment is significant for genes that are highly expressed in the absence of ABA [e.g. above 50 ppm (r=0.79, P<0.001)]. However, the correlation is not significant for genes with expression levels below 50 ppm in the absence of ABA (r=0.26, P=0.472). For these lowly expressed genes the ratios from MPSS are significantly higher than those from microarray experiments (Mann-Whitney U test

Table 3. Comparison of drought- and ABA-responsive genes

GenBank ID	Gene	Ratio (SD) microarray	Induction (MPSS)	Abundance (ppm) 0 μM ABA
D13044	Rd29A	6.4 (2.8)	58.3	6
U01377	COR15a	5.0 (1.1)	5.8	191
X55053	KIN2	5.8 (1.2)	6.2	1226
D17714	ERD10	6.0 (2.3)	18.4	31
X51474	KIN1	2.9 (0.9)	24.0	0
AB004872	RD17/ COR47	6.4 (1.6)	6.6	24
AB039924	RD20	5.1 (2.0)	18.0	49
AB039929	ERD7	3.8 (1.5)	4.1	15
AB039928	ERD4	2.6 (0.8)	1.5	81
AB039927	ERD3	2.6 (0.5)	0.8	46
D17715	ERD14	3.5 (1.2)	1.8	656
D13042	RD19A	2.8 (0.3)	2.5	491
D01113	RD22	2.2 (0.2)	2.7	760
D32138	P5CS	2.8 (0.9)	22.3	3
AB044404	Cold acclimation protein	6.2 (0.8)	17.7	244
X91919	LEA 76 type 1 protein	2.9 (0.8)	1799.0	0
AF121355	Peroxiredoxin TPX1	2.2 (0.6)	n.e.	-
AF057137	TIP2	2.0 (0.5)	1.2	4864
M80567	LTP1	2.7 (0.3)	1.3	46
AB050576	Rice glyoxalase I homolog	2.3 (0.7)	n.d.	-
T45998	Putative protein (At5g61820)	2.1 (0.4)	4.9	175
AB050549	Putative aquaporin	2.4 (0.6)	1.3	767
AF034255	Reversibly glycosylated polypeptide-3	2.1 (0.5)	n.e.	-
AB050542	Nodulin-like, putative protein	2.9 (1.3)	3.2	182
X58107	Enolase	2.0 (0.6)	3.1	410
AB050562	Heat shock protein dnaJ homolog	2.8 (0.3)	n.e.	-
AB050563	LEA protein SAG21 homolog	3.2 (1.4)	0.9	127
H37392	GSH-dep. dehydroascorbate reductase 1	3.0 (1.2)	n.d.	-
D14442	Ascorbate peroxidase	2.6 (0.8)	n.e.	-
AB015098	Abscisic acid-induced HVA22 homolog	3.7 (1.1)	n.e.	-
AB044405	Putative cysteine proteinase inhibitor B	2.2 (0.4)	1.1	163
X80342	DC 1.2 homolog	2.0 (1.0)	1.8	237
AB050543	Major latex protein type 1 homolog	2.4 (0.6)	1.6	1991
AB050565	Brassica napus jasmonate-inducible protein homolog	2.4 (0.3)	1.4	528
AB050566	Brassica napus beta-glucosidase homolog	2.3 (0.8)	2.0	1921
AC006403	Hypothetical protein (At2g24420)	2.7 (0.7)	n.e.	-
M80567	Aquaporin homolog (At2g39010)	2.0 (0.3)	1.3	767
X94248	Ferritin	2.8 (0.9)	1.7	391
AF104330	Glycine-rich protein 3 short isoform (GRP3S)	2.6 (0.5)	n.d.	-
Z35474	(GIF1) mRNA for thioredoxin	2.3 (0.5)	0.9	769
AB050572	Putative photosystem I reaction center subunit II precursor	2.1 (0.3)	1.0	3715
U43147	Catalase 3, CAT3	2.4 (0.7)	0.9	6260
AB050573	Cysteine proteinase like protein	3.0 (0.9)	3.7	57

Abbreviations: n.d., no data; n.e., not expressed.

P=0.025). This may indicate that ratios from array experiments may be underestimated for lowly expressed genes because of high background.

The majority of drought-responsive genes may therefore require ABA as a mediator of the drought response. Genes that are inducible by drought but not by ABA are targets of an ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 2000). This pathway has been postulated because genes that are not responsive to exogenous ABA are still induced by dehydration in ABA-deficient mutants (Ingram and Bartels, 1996). Different cis-acting promoter elements, ABREs (ABA-response elements), have been identified through the functional dissection of promoter regions of ABA-responsive genes (Busk and Pagès, 1998; Leung and Giraudat, 1998; Seki et al., 2001; Shinozaki and Yamaguchi-Shinozaki, 2000). However, ABRE-like motifs are not involved in the regulation of all known ABA-responsive genes (Iwasaki et al., 1995). The large number of new ABA-responsive genes described in this study provides a basis for the identification of additional cis-acting elements and a better understanding of the contribution of different cis-acting elements to ABA-dependent gene expression. This could also help to dissect the different signaling pathways involved in stress responses.

What is the role of the *abi1-1* mutation in gene expression?

The ABA-insensitive mutant *abi1-1* is affected in many different ABA-dependent processes during early development as well as in the adult plant. The impairment of ABA-responsive expression of few genes in *abi1-1* has been previously reported (e.g. Gilmour and Thomashow, 1990; Leung et al., 1997; Leung and Giraudat, 1998). Using MPSS we have extended this analysis to the genomic scale and shown that the *abi1-1* mutation results in a dramatic impairment of ABA-responsive transcription (supplementary data; Fig. 4). A possible explanation for this impairment could be that the mutation changes the localization of the protein within the cell.

However, the subcellular localization of both the WT and the mutant phosphatase protein is identical and therefore cannot account for the observed differences (Fig. 5B). Assuming that ABI1 acts in the ABA pathway and that the ABA signal is transduced in a single linear pathway, all ABA-responsive genes should be deregulated in the *abi1-1* mutant. Nonetheless, our results show that some genes continue to be regulated by ABA as in WT plants. This indicates the existence of a separate ABA pathway that still operates in *abi1-1*. Future investigations of the transcriptional control of genes that are unaffected by the *abi1-1* mutation will help to identify signaling components of this second pathway. The genomic-scale expression profiling also revealed that a large number of ABA-responsive genes are expressed in WT but not in *abi1-1* even in the absence of exogenous ABA (c.f. Strizhov et al., 1997). Thus, these genes may respond to low levels of endogenous ABA through high affinity ABA-responsive elements. Furthermore, this observation suggests that endogenous ABA concentrations may play an important role in normal cellular processes. A detailed analysis of signature sequences that differ between WT and *abi1-1* may shed light on these processes which may not necessarily be related to stress responses (S.H., M.M., S.V.T. and N.-H.C., unpublished).

Supplementary data

Two separate Excel-files, 'induced.xls' and 'repressed.xls', provide the 660 induced and the 694 repressed genes, respectively. Besides the respective signature sequence, the MIPS-ID and the gene, the tables provide the induction/repression factor, the normalized abundances in parts per million for WT as well as *abi1-1*, and the putative functional category of the gene product. If the abundance in one condition was 0 ppm it was considered 1 ppm to determine the factor of change. Column J contains a code that is described in Materials and Methods.

References

Allen, G. J., Kuchitsu, K., Chu, S. P., Murata, Y. and Schroeder, J. I. (1999). Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell* **11**, 1785-1798.

Audic, S. and Claverie, J. M. (1997). The significance of digital gene expression profiles. *Genome Res.* **7**, 986-995.

Ausubel, F. M., Brent, R., Kinston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. and Struhl, K. (1994). In *Current Protocols in Molecular Biology*. New York: John Wiley.

Azevedo, C., Santos-Rosa, M. and Shirasu, K. (2001). The U-box protein family in plants. *Trends Plant Sci.* **6**, 354-358.

Barakat, A., Szick-Miranda, K., Chang, I. F., Guyot, R., Blanc, G., Cooke, R., Delseny, M. and Bailey-Serres, J. (2001). The organization of cytoplasmic ribosomal protein genes in the Arabidopsis genome. *Plant Physiol.* **127**, 398-415.

Bray, E. A. (1997). Plant responses to water deficit. *Trends Plant Sci.* **2**, 48-54.

Brenner, S., Williams, S. R., Vermaas, E. H., Storck, T., Moon, K.,

McCollum, Mao, J., Luo, S., Kirchner, J. J., Eletr, S. et al. (2000a). In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs. *Proc. Natl. Acad. Sci. USA* **97**, 1665-1670.

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D. H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M. et al. (2000b). Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat. Biotechnol.* **18**, 630-634.

Busk, P. K. and Pages, M. (1998). Regulation of abscisic acid-induced transcription. *Plant Mol. Biol.* **37**, 425-435.

Ciehanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151-7160.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996). A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. *Science* **273**, 1239-1241.

De Bruxelles, G. L., Peacock, W. J., Dennis, E. S. and Dolferus, R. (1996). Abscisic acid induces the alcohol dehydrogenase gene in Arabidopsis. *Plant Physiol.* **111**, 381-391.

del Pozo, J. C. and Estelle, M. (2000). F-box proteins and protein degradation: an emerging theme in cellular regulation. *Plant Mol. Biol.* **44**, 123-128.

Desprez, T., Amselem, J., Caboche, M. and Höfte, H. (1998). Differential gene expression in Arabidopsis monitored using cDNA arrays. *Plant J.* **14**, 643-652.

Dolmetsch, R. E., Xu, K. and Lewis, R. S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392**, 933-936.

Finkelstein, R. R. and Lynch, T. J. (2000). The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. *Plant Cell* **12**, 599-609.

Finkelstein, R. R. and Somerville, C. R. (1990). Three classes of abscisic acid (ABA)-insensitive mutations of Arabidopsis define genes that control overlapping subsets of ABA responses. *Plant Physiol.* **94**, 1172-1179.

Gilmour, S. J. and Thomashow, M. F. (1990). Cold acclimation and cold-regulated gene expression in ABA mutants of Arabidopsis thaliana. *Plant Mol. Biol.* **17**, 1233-1240.

Gosti, F., Bertauche, N., Vartanian, N. and Giraudat, J. (1995). Abscisic acid-dependent and -independent regulation of gene expression by progressive drought in Arabidopsis thaliana. *Mol. Gen. Genet.* **246**, 10-18.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A. A., Vartanian, N. and Giraudat, J. (1999). ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* **11**, 1897-1910.

Hugouvieux, V., Kwak, J. M. and Schroeder, J. I. (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. *Cell* **106**, 477-487.

Ingram, J. and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377-403.

Iwasaki, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1995). Identification of a cis-regulatory region of a gene in Arabidopsis thaliana whose induction by dehydration is mediated by abscisic acid and requires protein synthesis. *Mol. Gen. Genet.* **247**, 391-398.

Kirschner, M. (1999). Intracellular proteolysis. *Trends Cell Biol.* **9**, M42-M45.

Koornneef, M., Reuling, G. and Karssen, C. M. (1984). The isolation and characterization of abscisic acid-insensitive mutatnts of *Arabidopsis thaliana*. *Physiol. Plant.* **61**, 377-383.

Kost, B., Spielhofer, P. and Chua, N. H. (1999). A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J.* **16**, 393-401.

Leung, J. and Giraudat, J. (1998). Abscisic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 199-222.

Leung, J., Bouvier-Durand, M., Morris, P. C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994). Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science* **264**, 1448-1452.

Leung, J., Merlot, S. and Giraudat, J. (1997). The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* **9**, 759-771.

Li, W., Llopis, J., Whitney, M., Zlokarnik, G. and Tsien, R. Y. (1998). Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature* **392**, 936-941.

Lopez-Molina, L. and Chua, N. H. (2000). A null mutation in a bZIP factor

confers ABA-insensitivity in Arabidopsis thaliana. *Plant Cell Physiol.* **41**, 541-547.

Lopez-Molina, L., Mongrand, S. and Chua, N. H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **98**, 4782-4787.

Lu, C. and Fedoroff, N. (2000). A mutation in the Arabidopsis HYL1 gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* **12**, 2351-2365.

Maguire, B. A. and Zimmermann, R. A. (2001). The ribosome in focus. *Cell* **104**, 813-816.

McAinsh, M. R., Gray, J. E., Hetherington, A. M., Leckie, C. P. and Ng, C. (2000). Ca^{2+} signalling in stomatal guard cells. *Biochem. Soc. Trans.* **28**, 476-481.

Meyer, K., Leube, M. P. and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. *Science* **264**, 1452-1455.

Murata, Y., Pei, Z. M., Mori, I. C. and Schroeder, J. I. (2001). Abscisic acid activation of plasma membrane Ca(2+) channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell* **13**, 2513-2523.

Naora, H. (1999). Involvement of ribosomal proteins in regulating cell growth and apoptosis: translational modulation or recruitment for extraribosomal activity? *Immunol. Cell Biol.* **77**, 197-205.

Ramanjulu, S. and Bartels, D. (2002). Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environment* **25**, 141-151.

Sanchez, J.-P. and Chua, N.-H. (2001). Arabidopsis PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell* **13**, 1143-1154.

Schaffer, R., Landgraf, J., Accerbi, M., Simon, V. V., Larson, M. and Wisman, E. (2001). Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. *Plant Cell* **13**, 113-123.

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C. and Manners, J. M. (2001). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc. Natl. Acad. Sci. USA* **97**, 11655-11660.

Schroeder, J. I., Kwak, J. M. and Allen, G. J. (2001). Guard cell abscisic acid signalling and engineering drought hardiness in plants. *Nature* **410**, 327-330.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001). Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13**, 61-72.

Seo, M., Peeters, A. J. M., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J. A. D., Koornneef, M., Kamiya, Y. and Koshiba, T. (2000). The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. *Proc. Natl. Acad. Sci. USA* **97**, 12908-12913.

Sheen, J. (1998). Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci. USA* **95**, 975-980.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* **3**, 217-223.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1996). Molecular responses to drought and cold stress. *Curr. Opin. Biotechnol.* **7**, 161-167.

Söderman, E. M., Brocard, I. M., Lynch, T. J. and Finkelstein, R. R. (2000). Regulation and function of the Arabidopsis ABA-insensitive 4 gene in seed and abscisic acid response signaling networks. *Plant Physiol.* **124**, 1752-1765.

Steber, C. M. and McCourt, P. (2001). A role for brassinosteroids in germination in Arabidopsis. *Plant Physiol.* **125**, 763-769.

Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C. and Szabados, L. L. (1997). Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. *Plant J.* **12**, 557-569.

Szick-Miranda, K. and Bailey-Serres, J. (2001). Regulated heterogeneity in 12-kDa P-protein phosphorylation and composition of ribosomes in maize (Zea mays L.). *J. Biol. Chem.* **276**, 10921-10928.

Taylor, I. B., Burbridge, A. and Thompson, A. J. (2000). Control of abscisic acid synthesis. *J. Exp. Bot.* **51**, 1563-1574.

Thomashow, M. F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 571-599.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. USA* **97**, 11632-11637.

Wang, X. Q., Ullah, H., Jones, A. M. and Assmann, S. M. (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. *Science* **292**, 2070-2072.

Wu, Y., Kuzma, J., Marechal, E., Graeff, R., Lee, H. C., Foster, R. and Chua, N. H. (1997). Abscisic acid signaling through cyclic ADP-ribose in plants. *Science* **278**, 126-130.

Xiong, L., Ishitani, M., Lee, H. and Zhu, J. K. (2001). The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. *Plant Cell* **13**, 2063-2083.

Xiong, L., Lee, H., Ishitani, M. and Zhu, J. K. (2002). Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. *J. Biol. Chem.* **277**, 8588-8596.