

Identification and expression analysis of genes in response to high-salinity and drought stresses in *Brassica napus*

Liang Chen[†], Feng Ren[†], Hui Zhong, Weimin Jiang, and Xuebao Li*

Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Sciences, Huazhong Normal University, Wuhan 430079, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel/Fax: +86-27-67862443; E-mail: xbli@mail.ccnu.edu.cn

High salinity and drought are the major abiotic stresses that adversely affect plant growth and agricultural productivity. To investigate genes that are involved in response to abiotic stresses in *Brassica napus*, a comprehensive survey of genes induced by high-salinity and drought stresses was done by macroarray analysis. In total, 536 clones were identified to be putative high-salinity- or drought-responsive genes. Among them, 172 and 288 clones are detected to be putative high-salinity- and drought-inducible genes, whereas 141 and 189 are candidates for high-salinity- and drought-suppressed genes, respectively. The functional classification of these genes are indicated that belonged to gene families encoding metabolic enzymes, regulatory factors, components of signal transduction, hormone responses, some abiotic stresses-related proteins, and other processes related to growth and development of *B. napus*. From the up-regulated candidate genes, some interested genes were further demonstrated to be high-salinity- or/and drought-induced expression by real-time quantitative RT-PCR analysis. The experimental results also revealed that some genes may function in abscisic acid-dependent signaling pathway related to drought or salinity stress. Collectively, the data presented in this study will facilitate the understanding of molecular mechanism of *B. napus* in response to high-salinity and drought stresses, and also provide us the basis of effective genetic engineering strategies for improving stress tolerance of *B. napus*.

Keywords *Brassica napus*; macroarray; gene expression; high-salinity and drought stress

Received: September 22, 2009

Accepted: November 15, 2009

Introduction

High salinity and drought are the major abiotic stresses that adversely affect plant growth and agricultural productivity

[1,2]. To overcome these limitations for improving crop yield, it is important to increase stress tolerance of crops, such as *Brassica napus*. The identification of novel genes, analysis of their expression patterns in response to these stresses, and evaluation of their potential functions in stress adaptation will provide us the basis of effective engineering strategies to improve crop stress tolerance [3,4].

It was demonstrated that one-fifth of irrigated agriculture is adversely affected by soil salinity [1]. Plant requires mineral nutrients absorbed from the soil by roots for its development. Although Na⁺ is a major cation present in the soil, it is not considered an essential mineral for most plants. Excessive Na⁺ disrupts the balance of other minerals such as K⁺, causing osmotic stress that leads to secondary stresses such as oxidative stress and nutritional disorders [5]. In recent years, great effort has been focused on elucidating the molecular basis of plant salt tolerance, and as a result, several important pathways involved in the salt-stress signal transduction have been identified [6]. These pathways include the salt-over-sensitive (SOS) pathway (SOS3–SOS2–SOS1) that regulates ion homeostasis under salt stress and results in Na⁺ efflux and vacuolar compartmentation, the calcium-dependent protein kinase (CDPK) pathway that plays an important role in osmotic stress [7], and the mitogen-activated protein kinase (MAPK) pathway that functions importantly in both abiotic and biotic stresses [8]. One of the effective strategies to improve plant salt tolerance is through overexpression of the genes that are either involved in salt perception and responses and/or shown to be required for normal levels of tolerance [9]. Overexpression of these regulatory genes, such as transcription factors (DREB/CBF) and protein kinases (MAPK, CDPK), can increase plant salt tolerance [10–13].

Drought is also one of the most severe environmental stresses that affect plant development and productivity [14]. Drought limits plant growth and development mainly due to photosynthetic decline, osmotic stress-imposed

constraints on plant processes, and interference with nutrient availability [15]. Therefore, the osmotic stress and the associated oxidative stress appear to be common consequences of exposure to drought and salinity. In recent years, a lot of plant drought-inducible genes have been identified. Transgenic plants have demonstrably enhanced their tolerance to abiotic stresses by introducing several stress-inducible genes [16–18]. Previous studies indicated that the genes encoding late embryogenesis abundant (LEA) proteins are useful for improving plant drought and salt tolerance [18–20]. The regulatory factors (such as transcription factors, protein kinases, and enzymes involved in the abscisic acid [ABA] biosynthesis) play important roles in improving plant tolerance to drought and other abiotic stresses. A study reported that CBF/DREB1 expression in transgenic plants increases its tolerance to freezing, drought, and salt stresses [21]. An active form of DREB2 is shown to enhance drought tolerance of transgenic *Arabidopsis* [22]. Overexpression of OsDREB1 or AtDREB1 also improves rice tolerance to drought and chilling [23]. *Arabidopsis* ABF3 or AREB2/ABF4 causes ABA hypersensitivity, reduces the transpiration rate, and enhances drought tolerance of transgenic plants [24]. It was observed that an LEA-type gene (*RAB16A*) is induced to enhance plant salt/drought tolerance in the *OsCDPK7*-overexpressing transgenic rice [25]. Previous studies revealed that SRK2C, a number of SNF1-related protein kinase family activated by osmotic/salt stress and ABA treatment, regulates many downstream stress-inducible genes, resulting in an enhanced stress tolerance of transgenic plants [26,27].

Although a lot of abiotic stress-induced genes have been characterized from model species such as *Arabidopsis*, tobacco and rice, little is known in economically important species such as oilseed plants so far. In our study, over 500 genes related to high-salinity and drought stresses were identified from *B. napus* cDNA libraries using macroarray analysis, and the expression patterns of 13 genes were further analyzed under high-salinity and drought stresses. This study provides insights into the regulation network of *B. napus* in response to high-salinity and drought stresses.

Materials and Methods

Plant growth conditions

Seeds of *B. Napus* (cv. Zhongyou 821) were surface sterilized and germinated on plates with half-strength Murashige and Skoog (MS) medium (pH 5.8) containing 0.8% agar under a 16 h light/8 h dark cycle at 25°C for 7 days. The 7-day-old seedlings were transferred onto MS medium containing 150 mM NaCl, 200 mM mannitol, or 100 µM ABA for treatment, respectively.

cDNA library construction

Total RNA was isolated and purified from different tissues of *B. napus* according to the protocol of the RNeasy® plant mini kit (Qiagen, Valencia, USA). Equal amounts of total RNA samples were reversely transcribed into cDNAs using SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, USA) according to manufacturer's instruction. The cDNAs were digested with *Sfi*I and size fractionated by CHROMA SPIN-400 columns (Clontech). Then, the cDNAs were inserted into TriplEx2 vector at the *Sfi*I site. The λ-phages were packaged according to the manufacturer's instruction. The cDNA library was amplified using *Escherichia coli* XL1-Blue as host cells. Titters of the cDNA library were calculated based on the plaques obtained on LB plates.

Macroarray preparation

The excision of pTriplEx2 from recombinant λTriplEx2 was performed using *E. coli* BM25.8 as the host cells. The pTriplEx2 is released as a result of Cre recombinase-mediated site-specific recombination at the *loxP* sites flanking the embedded plasmid. Release of the plasmid occurs automatically when the recombinant phage is transduced into a bacterial host *E. coli* BM25.8 in which Cre recombinase is being expressed. After the excision of pTriplEx2, a total of 3000 colonies randomly selected from the library were cultured overnight on 96-well cell plates. cDNA insertions were then amplified with standard SP6 and T7 primers in 96-well PCR plate with T-gradient PCR instrument (BioRad, Hercules, USA) using *E. coli* cultures as templates. PCR reactions were performed in a total volume of 50 µl (37 µl ddH₂O, 5 µl of 10× PCR buffer with MgCl₂, 4 µl of 2.5 mM dNTP, 1 µl of 10 µM SP6 primer, 1 µl of 10 µM T7 primer, 1 U Taq polymerase and 1 µl *E. coli* culture as template) as follows: 94°C for 5 min, 1 cycle; then with 34 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 3 min; and finally with an extension at 72°C for 10 min. Five microliters PCR products were checked on 1% agarose gel by electrophoresis. The remaining PCR products were quantified by spectrophotometer and the DNA concentration was adjusted identically.

Nylon membranes (Millipore, Bedford, USA) were pre-cut for DNA spotting. PCR products were arrayed on nylon membranes manually. Two hundred cDNA samples were spotted in an array on each membrane. A clone of the *B. napus* actin2 gene (*BnACT2*) was also arrayed as the positive control [28] and the ddH₂O as the negative control. The spotted membranes were denatured with 0.5 M NaOH and 1.5 M NaCl for 5 min, and neutralized with 0.5 M Tris-HCl (pH 7.5) for 5 min, followed by washing with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) for 5 min. The DNA was stabilized on the nylon membranes with UV crosslinker.

Hybridization

Totally 50 µg RNA, extracted from roots of 1-week-old seedlings of *B. napus* treated by high-salinity (150 mM NaCl for 6 h) and drought (200 mM mannitol for 24 h) stresses, respectively, was labeled with [α - 32 P]dCTP [29] through reverse transcription using RNA labeling kit (Promega, Madison, USA). The labeled first-strand cDNAs were used as hybridization probes. Hybridization was performed at 65°C overnight in a solution containing 50 mM sodium phosphate buffer (pH 7.2), 0.4% SDS, 5% dextran sulfate, 5× SSC, 5× Denhart's, 10% denatured salmon sperm DNA, and 2.5 µM EDTA, followed by washing twice at room temperature with 1× SSC and 0.1% SDS for 5 min, and then twice at 65°C with 0.5× SSC and 0.1% SDS for 15 min. Autoradiography was done at -70°C and the resulted profiles were analyzed.

DNA sequencing and analysis

DNA sequencing was carried out on ABI 3730 sequencers by Sunny Biotech. Co., Ltd (Shanghai, China). The clones were sequenced using SP6 primer. Similarities of cDNA sequences were analyzed with BLASTX program of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Quantitative RT-PCR analysis

Total RNA was isolated and purified from different tissues of *B. napus* according to the protocol of the RNeasy® plant mini kit (Qiagen). Expression of the selected genes in roots was analyzed by real-time quantitative reverse transcriptase (RT)-PCR using the fluorescent intercalating dye SYBRGreen with a detection system (Opticon 2, MJ Research, Waltham, USA). A *BnACT2* was used as a standard control in the RT-PCR reactions. Two-step RT-PCR procedure was performed in all experiments using a method described earlier [30]. In brief, total RNAs, predigested with DNase I for removing any contaminated genomic DNA, were reversely transcribed into cDNAs that were used as templates in PCR reactions. Real-time PCR reaction was performed using real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions, with gene-specific primers (**Table 1**). The Ct (cycle threshold), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene. Relative quantity of the target gene expression level was performed using the comparative Ct method. The relative value for the expression level of each target gene was calculated by the equation $Y = 10^{\Delta Ct/3.5} \times 100\%$ (ΔCt is the differences in Ct between the control *BnACT2* products and the target gene products). For the accurate amplification of each specific target gene, we carefully designed the primer sets for each gene based on the sequences corresponding to 3'-untranslated region.

Table 1 Gene-specific primers used in RT-PCR

Clone no.	Primer sequence
101	5'-ACCGTCTACGACTGGCTGTACAG-3' (F) 5'-CATTACAAGAGACAAGAGGACGC-3' (R)
121	5'-GATGTTGCATGACGCAACCAATG-3' (F) 5'-CCAAACACTTCAGACATTACAG-3' (R)
124	5'-GTTGCCCAACATCATTATCAAGC-3' (F) 5'-AGAAGAAAACAGAGCACTGCTCC-3' (R)
149	5'-GATGGATGCAAGTGCGGATCTG-3' (F) 5'-TCACAAACCCCATACACTTTCG-3' (R)
307	5'-ATTGAGTGACAACCTCGTGAAGC-3' (F) 5'-GCCATACATAAAAGAGCCTCTTC-3' (R)
311	5'-AACCATCCAGTCTTCGTCGGAC-3' (F) 5'-AGCTGAGAAAACATCATCAGCAG-3' (R)
526	5'-GCAGTTGCGAGAAGAACTACAAC-3' (F) 5'-TGAAAACATTACACAATAAACACC-3' (R)
575	5'-TAACTCCTCCCTACTCTCAACG-3' (F) 5'-TTGTGAACATAGTACAGACGACC-3' (R)
581	5'-GACCGTGAAAGGACTCCATGAAGCATC-3' (F) 5'-GTCCCGGGTCAACGTCTTACTCTTGG-3' (R)
588	5'-CATCCAGGATGCAGTTGAGTAC-3' (F) 5'-CCCCAACTGAATCAGTAGTACC-3' (R)
648	5'-AGTCACAACAACAGTGGTACTTC-3' (F) 5'-GGATATTTTAAGAATCTAACGTC-3' (R)
691	5'-AATCGAGTGGGAACAGGAGTC-3' (F) 5'-GAGACTCTGAACATCGTAAAG-3' (R)
961	5'-GGAAGACGAGCACAAGAAGG-3' (F) 5'-CATCTACCCCTCCCTCTCTATC-3' (R)
Actin2	5'-GTGTTGTTGGTAGGCCAAGACATCA-3' (F) 5'-CTTGATGTCTCTTACAATTTCCCGC-3' (R)

F, forward; R, reverse.

To achieve optimal amplification, PCR conditions for each primer combination were optimized for annealing temperature, and PCR products were verified by melting curve analysis and confirmed on an agarose gel. Mean values and standard errors (bar) were calculated from three independent experiments with three biological replicates of root materials, and the data were normalized with the relative efficiency of each primer pair.

Results

cDNA isolation and macroarray analysis

A cDNA library of *B. napus* was constructed with mRNAs from different tissues of the plant species. cDNA insertions were amplified from the cDNA library via PCR using SP6 and T7 primers, and sizes of the insertions were 0.5–3 kb in length. A total of 3000 PCR products that represent same numbers of randomly selected cDNA clones were arrayed on 40-nylon membranes. Each four-nylon membranes, which

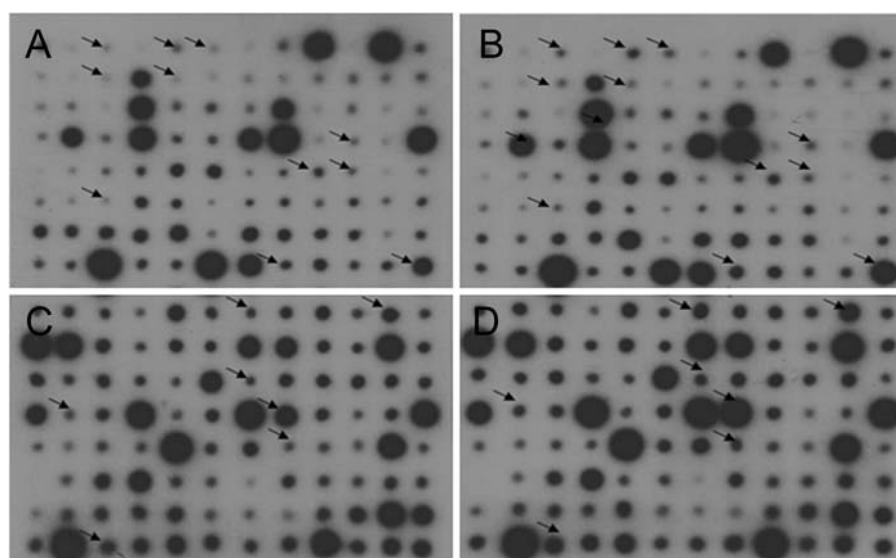


Figure 1 Screening of high-salinity/drought-induced genes with macroarray analysis The PCR products of cDNA clones were blotted on two-nylon membranes (A:B, C:D) at same locations, and the membranes were, respectively, hybridized with cDNA probes from the roots of control (A) and 150 mM NaCl-treated plants (B), or control (C) and 200 mM mannitol-treated plants (D) of *B. napus*. Arrows indicate the representatives of the up-regulated clones (partial data shown).

were spotted with identical cDNA samples, were hybridized with cDNA probes reversely transcribed from mRNAs in roots of high-salinity- or drought-treated plants and controls. The experimental results revealed that the genes on the macroarray showed differential expression profiles in response to various abiotic stresses. In total, 536 clones were identified to be putative high-salinity- or drought-responsive genes, including 310 up-regulated and 226 down-regulated genes under high-salinity or/and drought conditions (**Fig. 1**). Among them, 172 and 288 clones are detected to be putative high-salinity- and drought-inducible genes, whereas 141 and 189 are candidates for high-salinity- and drought-suppressed genes, respectively (**Fig. 2**). Based on the Venn diagram analysis, we analyzed differences and cross-talk of gene expression between high-salinity- and drought-stress responses in *B. napus*. As shown in **Fig. 2**, 18 genes were up-regulated only by high salinity, whereas 138 genes were up-regulated only by drought. Similarly, 37 and 85 genes were down-regulated only by high salinity or drought, respectively. In contrast, 154 genes were induced by both high salinity and drought, whereas 104 genes were suppressed by both high salinity and drought, suggesting the existence of a substantial common regulatory system or a cross-talk between high-salinity and drought stresses.

EST sequencing and gene identification

To identify the high-salinity- and drought-induced genes, these isolated cDNA clones were sequenced for obtaining their ESTs. The insert cDNA sequences were determined by a single DNA sequencing with SP6 primer. The sequence data were analyzed by BLASTX to determine

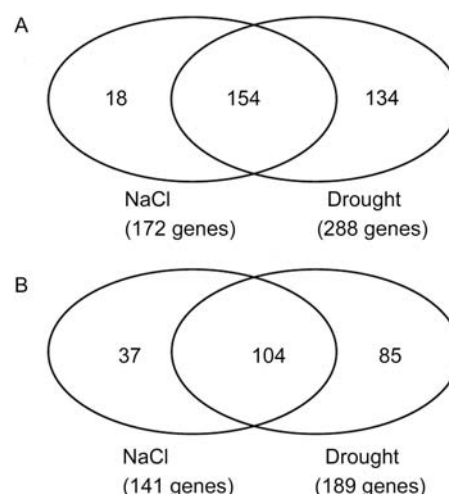


Figure 2 Venn diagrams showing the classification of high-salinity- and drought-responsive genes identified on the basis of macroarray In total, 313 high-salinity-responsive genes (including 172 up-regulated and 141 down-regulated genes), and 477 drought-responsive genes (including 288 up-regulated and 189 down-regulated genes) were identified by cDNA microarray. (A) Intersection of genes that were up-regulated by high-salinity stress with those that were up-regulated by drought stress. (B) Intersection of genes that were down-regulated by high-salinity stress with those that were down-regulated by drought stress.

DNA and protein homologies. The results indicated that amino acid sequences encoded by a portion of these genes share significant similarities to the known proteins (such as metabolic enzymes, regulatory factors, structure proteins, components of signal transduction, hormone response proteins, transporters, or abiotic stress-related proteins) that have been previously described in other species, and the remaining are unknown proteins that have no or very low

Table 2 Numbers of cDNA clones involved in different functional groups up-regulated by drought and/or high-salinity stress

Functional category	Number	Description
Protein kinase	5	MAPK 12, MAPKKK, Botrytis-induced kinase 1, CIPK
Iron homeostasis	4	FeS protein, metallothionein-like protein type 2, metallothionein protein
Transport	2	Plasma membrane aquaporin, ATOEP16-3; P–P bond hydrolysis-driven protein transmembrane transporter
Electron transport system	1	Electron carrier
Hormone	2	Auxin-responsive family protein, auxin-repressed protein
Lipid metabolism	11	Lipid-transfer protein, lipid-binding protein
Pollen-related protein	5	Pollen-coat protein, pollen development-related protein
Photosynthesis	1	Photosystem II 10 kDa polypeptide
Cytoskeleton	1	Profilin
Cell wall protein	2	Glycine-rich protein
Stress	5	Cold-regulated protein, COR25, dehydrin, ERD15, allergen V5/Tpx-1-related family protein, allergen family protein
Metabolic enzymes	31	Peroxidase, carbon–nitrogen hydrolase family protein, glycosyl transferase family, probable MPT-synthase sulfurylase, phosphoric monoester hydrolase, formate dehydrogenase, S-adenosylmethionine synthetase, S-adenosyl-L-homocystein hydrolase, ubiquitin-protein ligase, ATP binding/nucleoside triphosphatase, S-methyltransferase, ubiquitin carboxyl-terminal hydrolase family 1 protein, thioredoxin-dependent peroxidase, putative dTDP-glucose 4-6-dehydratase, O-methyltransferase family 2 protein, ribonucleoside-diphosphate reductase, ubiquinol-cytochrome C reductase complex
Transcriptional factor	2	Transcription factor-like protein, MYC-like protein
Unknown protein	28	Unknown protein
Total clones obtained	100	

identity with the proteins in other plant species (partial data shown in **Table 2**).

From the up-regulated candidate genes, 13 interested genes (GenBank accession no.: GU189578–GU189590, respectively) were selected for further confirming their salinity- or/and drought-induced expression by real-time quantitative RT-PCR, using gene-specific primers (**Table 1**). The experimental results demonstrated that expression levels of all the 13 genes were significantly increased under high-salinity stress (**Fig. 3**) and drought stress (**Fig. 4**). Of the 13 genes, six were induced by both high-salinity and drought stresses, whereas the other 6 genes were induced only by drought stress and 1 gene only by high-salinity stress. On the whole, the results of quantitative RT-PCR are consistent with the data from macroarray analysis. These 13 clones belong to 6 families, including MAPKKK, metallothionein proteins, auxin-responsive or -repressed proteins, lipid transfer protein, stress-related proteins, and metabolic enzyme (**Table 3**).

Expression pattern analysis of the stress-induced genes

To investigate the expression profiles of the salinity- and drought-induced genes, expressions of the identified 13 genes under stresses were analyzed by real-time quantitative

RT-PCR. Under 150 mM NaCl treatment, the transcripts of six high-salinity-inducible genes encoding auxin-repressed protein (101), protein kinase (124), metallothionein (526), glyceraldehyde-3-phosphate dehydrogenase (307), ubiquitin-protein ligase (588), and CIPK (581), respectively, were accumulated at highest levels at 6 h, whereas expression of one gene encoding water stress-induced protein (961) reached its peak value at 12 h. Two genes encoding auxin-repressed protein (101) and metallothionein (526), respectively, displayed similar expression patterns. Their transcripts gradually increased and reached its highest level at 6 h, and then decreased to very low levels under NaCl treatment (**Fig. 3**). However, the other 6 genes encoding MAPKKK (121), metallothionein (149), auxin-responsive protein (311), lipid transfer protein (575), transcription factor-like protein (648), and early responsive to dehydration 15 (ERD15) (691), respectively, were not induced by high-salinity stress (data not shown).

On the other hand, 12 of the selected 13 genes were induced to up-express by treatment with 200 mM mannitol (**Fig. 4**), of which 6 genes were induced by high-salinity stress simultaneously (**Fig. 3**). The transcripts of five genes encoding protein kinase (124), metallothionein (149 and 526), lipid transfer protein (575) and ERD15 (691),

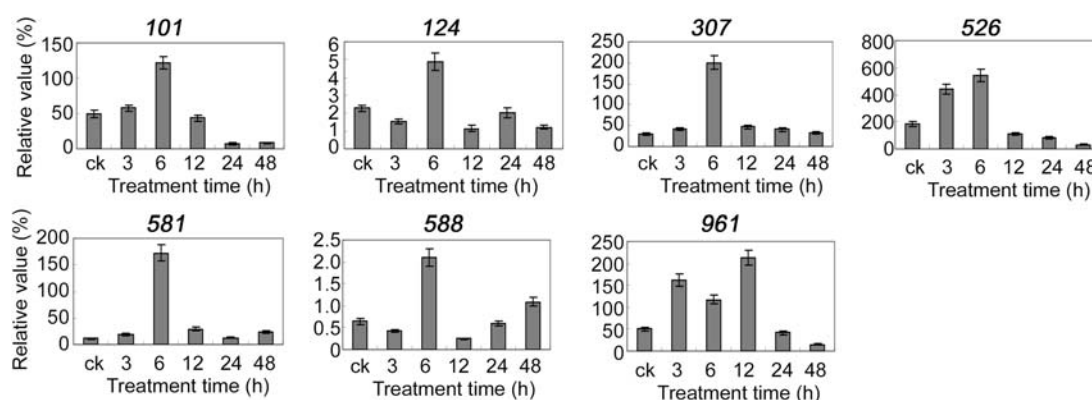


Figure 3 Quantitative RT-PCR analysis of expression of the identified genes in *B. napus* under salt stress Total RNAs were isolated from 7-day-old roots treated with 150 mM NaCl for 3, 6, 12, 24, 48 h respectively. ck, untreated roots (control). Relative value of the expression of the identified genes in *B. napus* roots was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (bar) were shown from three independent experiments.

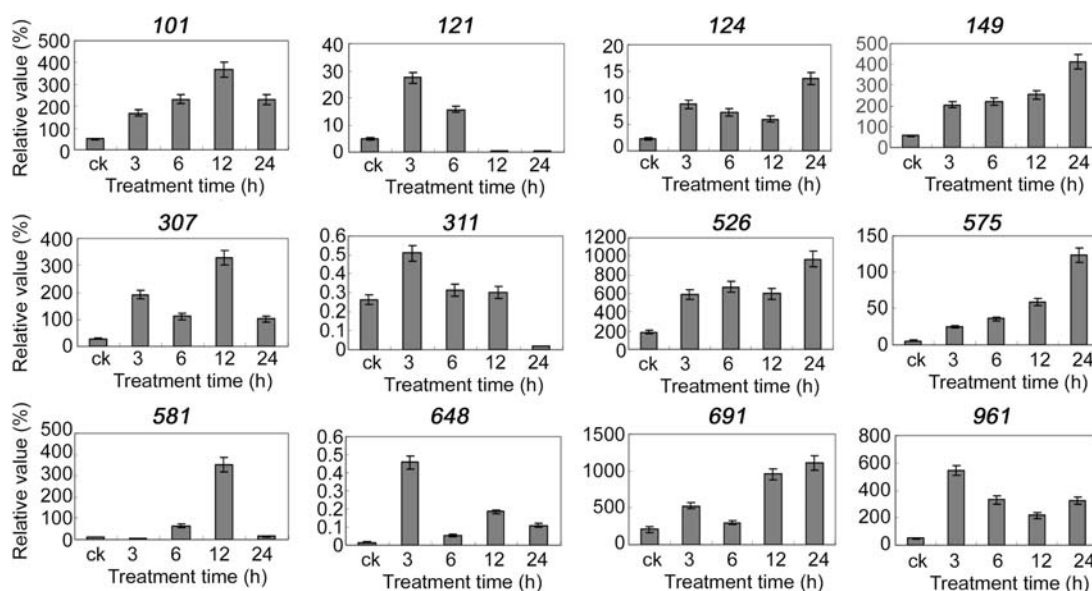


Figure 4 Quantitative RT-PCR analysis of expression of the identified genes in *B. napu* under drought stress Total RNAs were isolated from 7-day-old roots treated with 200 mM mannitol for 3, 6, 12, 24 h respectively. ck, untreated roots (control). Relative value of the expression of the identified genes in *B. napu* roots was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (bar) were shown from three independent experiments.

respectively, were accumulated to their peak values at late stage (24 h) of drought stress, while the other 4 genes encoding MAPKKK (121), auxin-responsive protein (311), transcription factor-like protein (648) and water stress induced protein (961), respectively, reached their maximum expression levels at early stage (3 h) of drought stress. In contrast, the remaining three genes encoding auxin-repressed protein (101), glyceraldehyde-3-phosphate dehydrogenase (307), and CIPK (581), respectively, were induced to express at the highest levels at 12 h after mannitol treatment (Fig. 4).

It is believed that the phytohormone ABA plays an important role in the signaling pathway related to plant

response to abiotic stresses (such as drought, salinity). To investigate whether the identified genes are involved in ABA-dependent signal transduction, expression profiles of the six genes, which are thought to be potentially related to ABA signaling, were analyzed in roots of *B. napus* plants with 100 μ M ABA treatment. As shown in Fig. 5, the transcripts of the gene encoding MAPKKK (121) reached its peak value at 1 h after ABA treatment. Four genes that encode protein kinase (124), metallothionein (149), CIPK (581), and ERD15 (691), respectively, showed a similar expression pattern. The products of all the four genes were accumulated at the highest levels at 6 h. On the contrary, the expression of the gene encoding ubiquitin-protein

Table 3 Summary of ESTs of the 13 induced genes in *B. napus*

Clone no.	Accession no.	Homologous gene	Species	E-value ^a	Homology
101	GU189578	Auxin-repressed protein	<i>Brassica oleracea</i>	2e-57	Id.100%; Po.100%
121	GU189579	Protein kinase family protein	<i>Arabidopsis thaliana</i>	6e-67	Id.95%; Po.97%
124	GU189580	Protein kinase, putative	<i>Arabidopsis thaliana</i>	4e-120	Id.96%; Po.98%
149	GU189581	Metallothionein protein	<i>Pringlea antiscorbutica</i>	2e-23	Id.89%; Po.93%
307	GU189589	Glyceraldehyde-3-phosphate dehydrogenase	<i>Arabidopsis thaliana</i>	8e-164	Id.96%; Po.98%
311	GU189582	Auxin-responsive family protein	<i>Arabidopsis thaliana</i>	1e-56	Id.87%; Po.93%
526	GU189583	Metallothionein protein	<i>Brassica juncea</i>	1e-09	Id.96%; Po.96%
575	GU189584	Lipid transfer protein	<i>B. napus</i>	6e-61	Id.94%; Po.99%
581	GU189585	CIPK	<i>Arabidopsis thaliana</i>	0.0	Id.95%; Po.97%
588	GU189590	Ubiquitin-protein ligase	<i>Arabidopsis thaliana</i>	3e-89	Id.95%; Po.96%
648	GU189586	Transcription factor-like protein	<i>Arabidopsis thaliana</i>	1e-61	Id.61%; Po.69%
691	GU189587	ERD15	<i>Brassica rapa</i>	1e-79	Id.98%; Po.98%
961	GU189588	Water stress-induced protein	<i>Brassica oleracea</i>	8e-12	Id.94%; Po.96%

^aE-value resulted from BLASTx search. Id., identities; Po., positives.

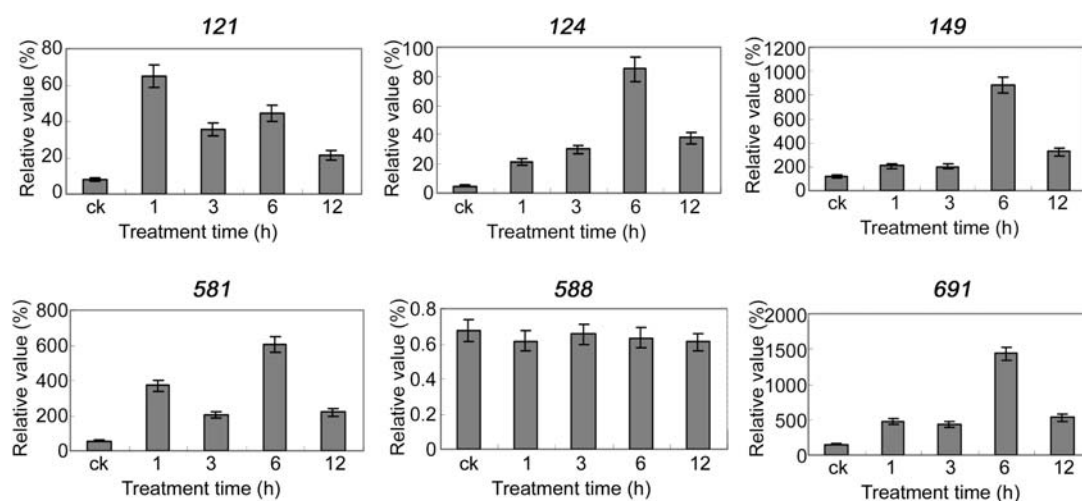


Figure 5 Quantitative RT-PCR analysis of expression of the identified genes in *B. napus* under 100 μM ABA treatment Total RNAs were isolated from 7-day-old roots treated with 100 μM ABA for 1, 3, 6, 12 h respectively. ck, untreated roots (control). Relative value of the expression of the identified genes in *B. napus* roots was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (bar) were shown from three independent experiments.

ligase (588) was not induced by ABA treatment. The above results suggest that the five genes (nos. 121, 124, 149, 581, and 691) may function in the ABA-dependent signaling pathway related to drought or salinity stress.

Moreover, expression patterns of the 13 genes in different tissues/organs of *B. napus* were analyzed by quantitative RT-PCR (Fig. 6). The results revealed that three genes encoding auxin (101), metallothionein (149), and ERD15 (691), respectively, were preferentially expressed in cotyledons, but their mRNAs were detected at relatively low levels in other tissues. One gene encoding auxin-responsive protein (311) was specifically expressed in stems, whereas transcripts of another gene encoding MAPKKK (121) were

detected at high level in stems, at moderate level in cotyledons, but at low or undetectable levels in other tissues. Additionally, two genes encoding ubiquitin-protein ligase (588) and water stress-induced protein (961), respectively, were predominantly expressed in stems, such as the MAPKKK (121) gene. However, their transcripts were also detected at high levels in flowers, unlike the MAPKKK (121) gene. On the other hand, the other four genes encoding protein kinase (124), glyceraldehyde-3-phosphate dehydrogenase (307), lipid transfer protein (575), and CIPK (581), respectively, showed similar expression patterns. Their products were accumulated at highest levels in flowers, but at moderate or low levels in other tissues. One

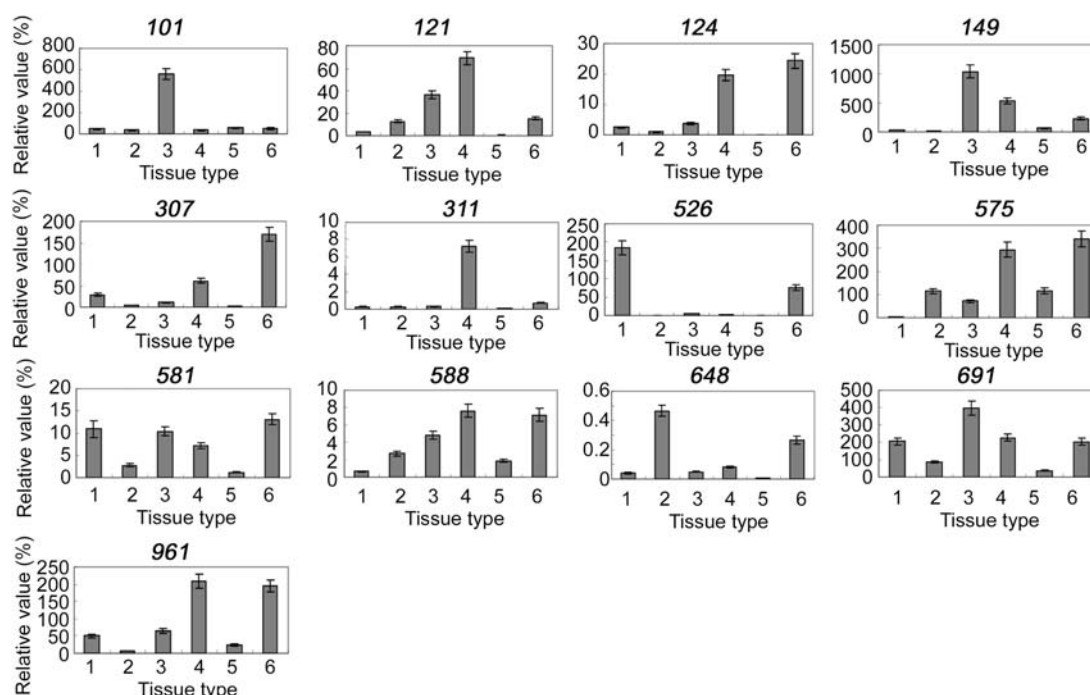


Figure 6 Quantitative RT-PCR analysis of expression of the isolated genes in *B. napus* tissues. Total RNAs were isolated from roots (column 1), hypocotyls (column 2), cotyledons (column 3), stems (column 4), leaves (column 5), and flowers (column 6) of *B. napus* respectively. Relative value of the expression of the identified genes in *B. napus* tissues was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (bar) were shown from three independent experiments.

gene encoding metallothionein (526) was preferentially expressed in roots, but its mRNA was found at very low or undetectable levels in other tissues.

Discussion

In this study, the cDNA library was constructed and the genes were screened using macroarray analysis for identifying high-salinity- or drought-responsive genes in *B. napus*. From 3000 cDNA clones in the macroarray assay, a total of 536 genes were identified, including 310 up-regulated and 226 down-regulated genes, of which 13 stress-induced genes were identified for further study.

Comparative analysis of *B. napus* with *Arabidopsis* is quite useful not only for understanding the genomic similarities across the two species, but also for discovering important genes for genetic engineering of *B. napus*. We compared the identified stress-inducible genes of *B. napus* with those *Arabidopsis* genes that are thought to be involved in plant response or tolerance to environmental stresses. The results demonstrated that there are considerable similarities between the two genomes in response to abiotic stresses. Our analysis revealed that a lot of stress-inducible genes in *B. napus* are homologues of those in *Arabidopsis*. These stress-inducible genes include the genes encoding ERD15, cold-regulated proteins (COR), dehydrins, water stress-induced protein, aldehyde dehydrogenase, transcription factor, lipoxygenase, protein

phosphatase, protein kinase, transporter, and metallothionein-like proteins, etc. All of the genes were found to be up-regulated in response to abiotic stresses in *B. napus*, like those stress-inducible genes in *Arabidopsis*. However, our macroarray data also revealed some differences in the genes responsive to environmental stresses between the two plant species. There are a number of *B. napus* genes that have been reported with similar functions or gene name in *Arabidopsis* but not documented as genes responsive to abiotic stresses in *Arabidopsis*. These included many enzymes, such as formate dehydrogenase, carbon-nitrogen hydrolase, phosphoric monoester hydrolase, *S*-adenosyl-L-homocystein hydrolase, ubiquitin carboxyl-terminal hydrolase family 1 protein, sulfurylase, and a number of proteins with unknown functions (**Table 2**). In addition, among the 13 stress-inducible genes identified in *B. napus*, nine have already been characterized to share similar functions in *Arabidopsis*, but to date, the remaining four are not reported to be salinity- or drought-induced genes in *Arabidopsis*, suggesting that some of the identified genes in *B. napus* are the novel genes responding to abiotic stresses.

The data presented in this study indicated that the most salinity-inducible genes are also drought-induced ones, while five of six drought-inducible genes examined are also responsive to ABA, suggesting that a significant cross-talk may occur between drought and high-salinity stress signaling and between drought and ABA responses

as well. Our results from *B. napus* are consistent with the overlap of expressions of drought- and high-salinity-responsive genes observed in *Arabidopsis* [31,32].

MAPK cascades are three kinase signaling modules that are highly conserved among eukaryotes, which are important mediators of signal transduction in cells [29]. An increasing number of studies have demonstrated that MAPKs play important functions in regulating both stress responses and plant development. Plant MAPKs can be activated by a variety of abiotic and biotic stresses, including salinity, drought, cold, pathogens, and ABA [33–37]. In our study, we identified two genes (nos. 121 and 124) encoding MAPKKs related to abiotic stresses and ABA in *B. napus*. The 124 gene expression was induced by high-salinity, drought stress, and ABA, whereas transcripts of the 121 gene were largely accumulated under drought stress and ABA treatment. The results suggest that the two genes may play important roles in signal transduction related to abiotic stresses and ABA in *B. napus*.

Previous studies revealed that metallothionein-like proteins were involved in response to stresses such as metal ions, heat shock, glucose and sucrose starvation, high levels of sucrose, low temperature, wounding and viral infection [38–42]. By microarray or differential screening cDNA libraries, many metallothionein-like proteins related to dehydration or NaCl stress were identified in plants [4,43]. Similarly, we identified two genes (nos. 149 and 526) encoding metallothionein-like proteins in *B. napus*. One gene (526) was induced by both high-salinity and drought stresses, whereas the other gene (149) was induced by drought stress and ABA treatment, suggesting that the isolated metallothionein-like proteins may function in *B. napus* response to high salinity, drought, and ABA signaling.

As a second messenger in plants, intracellular calcium (Ca^{2+}) levels are modulated in response to various signals including abiotic stresses, light, pathogens, and hormones [44]. Three major families of calcium sensors have been identified in higher plants: CDPK, calmodulin (CaM), and calcineurin B-like (CBL). They achieve function by interacting with their target proteins. CBLs interact with and regulate the activity of a specific group of Ser/Thr protein kinases called CBL-interacting protein kinases (CIPKs) [45]. The CBL–CIPK pathway plays an important role in plant responses to ABA signaling under abiotic stresses. Previous studies revealed that some drought-inducible genes also respond to exogenous ABA treatment, but the others were not affected by ABA, indicating that both ABA-independent and ABA-dependent regulatory systems function in governing expressions of drought-inducible genes [46]. In this study, we identified a CIPK gene (no. 581) which was induced by high salinity, drought and ABA, suggesting that this gene may be involved in

B. napus plants responding to abiotic stresses via ABA signaling pathway. The results also imply that the 581 gene might play an important role in *B. napus* drought tolerance through ABA-dependent regulatory system, but the precise function of this gene still remains to be explored so far.

ABA plays an essential role in plant adaptation to adverse environmental conditions including drought stress [31,47]. Apart from ABA, auxin is also involved in plant adaptation to biotic and abiotic stresses [48]. A number of genes encoding proteins responsive to auxin were identified as drought-down-regulated genes, suggesting that auxin may negatively modulate drought-stress signaling [49]. On the other hand, there are a few exceptions. For example, some auxin-responsive genes (IAA3, At4g00880) were up-regulated by drought [32]. Similarly, our results indicated that one gene encoding auxin-repressed protein (no. 101) in *B. napus* was induced by both high-salinity and drought stress. Another gene (no. 311) encoding auxin-responsive protein was also induced by drought stress. In addition, we identified two stress-related genes that encode ERD15 (no. 691) and water stress-induced protein (no. 961). Collectively, we identified over 500 drought and/or high-salinity stress-inducible genes in *B. napus* by microarray and quantitative RT-PCR analyses. The data generated in this study will facilitate to understand which genes involved in *B. napus* response to abiotic stresses and ABA signaling. Studying the roles of these stress-inducible genes in detail will further understand the molecular mechanism of *B. napus* in response to drought and high-salinity stresses, and also provide us the basis of effective genetic engineering strategies for improving stress tolerance of *B. napus*.

Funding

This work was supported by the projects from Ministry of Agriculture of China for transgenic research (Grant No. 2008ZX08009-003, 2009ZX08009-117B), Ministry of Education of China (Grant No. 20070511007) and Natural Sciences Foundation of Hubei Province of China (Grant No. 2007ABA354).

References

- 1 Flowers TJ and Yeo AR. Breeding for salinity resistance in crop plants. Where next? Aust J Plant Physiol 1995, 22: 875–884.
- 2 Yamaguchi-Shinozaki K, Kasuga M, Liu Q, Nakashima K, Sakuma Y, Abe H and Shinwari ZK, *et al.* Biological mechanisms of drought stress response. JIRCAS Working Rep 2002, 23: 1–8.
- 3 Cushman JC and Bohnert HJ. Genomic approaches to plant stress tolerance. Curr Opin Plant Biol 2000, 3: 117–124.
- 4 Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y and Yoshiwara K, *et al.* Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol 2003, 133: 1755–1767.

- 5 Chinnusamy V, Zhu J and Zhu JK. Salt stress signaling and mechanisms of plant salt tolerance. *Genet Eng (N Y)* 2006, 27: 141–177.
- 6 Xiong L, Schumaker KS and Zhu JK. Cell signaling during cold, drought, and salt stress. *Plant Cell* 2002, 14: S165–S183.
- 7 Sanchez-Barrena MJ, Martinez-Ripoll M, Zhu JK and Albert A. The structure of the *Arabidopsis thaliana* SOS3: molecular mechanism of sensing calcium for salt stress response. *J Mol Biol* 2005, 345: 1253–1264.
- 8 Nakagami H, Pitzschke A and Hirt H. Emerging MAP kinase pathways in plant stress signalling. *Trends Plant Sci* 2005, 10: 339–346.
- 9 Yang Q, Chen ZZ, Zhou XF, Yin HB, Li X, Xin XF and Hong XH, *et al.* Overexpression of SOS (Salt Overly Sensitive) genes increases salt tolerance in transgenic arabidopsis. *Mol Plant* 2009, 2: 22–31.
- 10 Liu J and Zhu JK. A calcium sensor homolog required for plant salt tolerance. *Science* 1998, 280: 1943–1945.
- 11 Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K and Shinozaki K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* 1999, 17: 287–291.
- 12 Kovtun Y, Chiu WL, Tena G and Sheen J. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc Natl Acad Sci* 2000, 97: 2940–2945.
- 13 Moon H, Lee B, Choi G, Shin D, Prasad T, Lee O and Kwak SS, *et al.* NDP kinase 2 interacts with two oxidative stress activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc Natl Acad Sci USA* 2003, 100: 358–363.
- 14 Valliyodan B and Nguyen H. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curr Opin Plant Biol* 2006, 9: 1–7.
- 15 Chinnusamy V, Schumaker K and Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J Exp Bot* 2004, 55: 225–236.
- 16 Zhang JZ, Creelman RA and Zhu JK. From laboratory to field. Using information from arabidopsis to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 2004, 135: 615–621.
- 17 Bartels D and Sunkars R. Drought and salt tolerance in plants. *Crit Rev Plant Sci* 2005, 24: 23–58.
- 18 Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K and Shinozaki K. Engineering drought tolerance in plants: discovering and tailoring genes unlock the future. *Curr Opin Biotechnol* 2006, 17: 113–122.
- 19 Xu D, Duan X, Wang B, Hong B, Ho T and Wu R. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol* 1996, 110: 249–257.
- 20 Brini F, Hanin M, Lumberras V, Amara I, Khoudi H, Hassairi A and Pagès M, *et al.* Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. *Plant Cell Rep* 2007, 26: 2017–2026.
- 21 Liu Q, Sakuma Y, Abe H, Kasuga M, Miura S, Yamaguchi-Shinozaki K and Shinozaki K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 1998, 10: 1491–1506.
- 22 Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K and Yamaguchi-Shinozaki K. Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought responsive gene expression. *Plant Cell* 2006, 18: 1292–1309.
- 23 Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M and Shinozaki K, *et al.* Functional analysis of rice DREB1/CBF-type transcription factors involved in cold responsive gene expression in transgenic rice. *Plant Cell Physiol* 2006, 47: 141–153.
- 24 Kang JY, Choi HI, Im MY and Kim SY. *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* 2002, 14: 343–357.
- 25 Saijo Y, Hata S, Kyojuka J, Shimamoto K and Izui K. Overexpression of a single Ca^{2+} -dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J* 2000, 23: 319–327.
- 26 Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K and Shinozaki K. SRK2C. A SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 2004, 101: 17306–17311.
- 27 Shinozaki K and Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. *J Exp Bot* 2007, 58: 221–227.
- 28 Xu BB, Li JN, Zhang XK, Wang R, Xie LL and Chai YR. Cloning and molecular characterization of a functional flavonoid 30-hydroxylase gene from *Brassica napus*. *J Plant Physiol* 2007, 164: 350–363.
- 29 Hirt H. Multiple roles of MAP kinases in plant signal transduction. *Trends Plant Sci* 1997, 2: 11–15.
- 30 Li XB, Fan XP, Wang XL, Cai L and Yang WC. The cotton ACTIN1 gene is functionally expressed in fibers and participates in fiber elongation. *Plant Cell* 2005, 17: 859–875.
- 31 Shinozaki K and Yamaguchi-Shinozaki K. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol* 2000, 3: 217–223.
- 32 Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y and Kamiya A, *et al.* Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J* 2002, 31: 279–292.
- 33 Munnik T, Ligterink W, Meskiene I, Calderini O, Beyerly J, Musgrave A and Hirt H. Distinct osmo-sensing protein kinase pathways are involved in signaling moderate and severe hyper-osmotic stress. *Plant J* 1999, 20: 381–388.
- 34 Mikolajczyk M, Awotunde OS, Muszynska G, Klessig DF and Dobrowolska G. Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. *Plant Cell* 2000, 12: 165–178.
- 35 Jonak C, Kiegl S, Ligterink W, Baker PJ, Huskisson NS and Hirt H. Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc Natl Acad Sci USA* 1996, 93: 11274–11279.
- 36 Suzuki K and Shinshi H. Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. *Plant Cell* 1995, 7: 639–647.
- 37 Xiong LZ and Yang Y. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* 2003, 15: 745–759.
- 38 Hsieh HM, Liu WK and Huang PC. A novel stress-inducible metallothionein-like gene from rice. *Plant Mol Biol* 1995, 28: 381–389.
- 39 Chevalier C, Bourgeois E, Pradet A and Raymond P. Molecular cloning and characterization of six cDNAs expressed during glucose starvation in excised maize (*Zea mays* L.) root tips. *Plant Mol Biol* 1995, 28: 473–485.
- 40 Chatthai M, Kaukinen KH, Tranbarger TJ, Gupta PK and Misra S. The isolation of a novel metallothionein-related cDNA expressed in somatic and zygotic embryos of douglas-fir: regulation by ABA, osmoticum, and metal ions. *Plant Mol Biol* 1997, 34: 243–254.
- 41 Reid SJ and Ross GS. Up-regulation of two cDNA clones encoding metallothionein-like proteins in apple fruit during cool storage. *Physiol Plant* 1997, 100: 183–189.
- 42 Choi D, Kim HM, Yun HK, Park JA, Kim WT and Bok SH. Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L and its induction by wounding and tobacco mosaic virus infection. *Plant Physiol* 1996, 112: 353–359.

- 43 Talame V, Ozturk NZ, Bohnert HJ and Tuberosa R. Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. *J Exp Bot* 2007, 58: 229–240.
- 44 Knight H and Knight MR. Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci* 2001, 6: 262–267.
- 45 Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S and Gruissem W. Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell Suppl* 2002, 14: S389–S400.
- 46 Yamaguchi-Shinozaki K and Shinozaki K. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci* 2005, 10: 88–94.
- 47 Riera M, Valon C, Fenzi F, Giraudat J and Leung J. The genetics of adaptive responses to drought stress: abscisic acid dependent and abscisic acid-independent signalling components. *Physiol Plant* 2005, 123: 111–119.
- 48 Park JE, Park JY, Kim YS, Staswick PE, Jeon J, Yun J and Kim SY, *et al.* GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. *J Biol Chem* 2007, 282: 10036–10046.
- 49 Huang DQ, Wu WR, Abrams SR and Cutler AJ. The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *J Exp Bot* 2008, 59: 2991–3007.