

A novel WD40 protein, BnSWD1, is involved in salt stress in *Brassica napus*

Sanghun Lee · Junhee Lee · Kyung-Hee Paek · Suk-Yoon Kwon · Hye Sun Cho · Shin Je Kim · Jeong Mee Park

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Abstract Genes that are expressed early in specific response to high salinity conditions were isolated from rapeseed plant (*Brassica napus* L.) using an mRNA differential display method. Five PCR fragments (DD1–5) were isolated that were induced by, but showed different response kinetics to, 200 mM NaCl. Nucleotide sequence analysis and homology search revealed that the deduced amino sequences of three of the five cDNA fragments showed considerable similarity to those of β -mannosidase (DD1), tomato Pti-6 proteins (DD5), and the tobacco harpin-induced protein hin1 (DD4), respectively. In contrast, the remaining clones, DD3 and DD2, did not correspond to any substantial existing annotation. Using the DD3 fragment as a probe, we isolated a full-length cDNA clone from the cDNA library, which we termed *BnSWD1* (*Brassica napus* salt responsive WD40 1). The predicted amino-acid sequence of BnSWD1 contains eight WD40 repeats and is conserved in all eukaryotes. Notably, the *BnSWD1* gene is expressed at high levels under salt-stress conditions. Furthermore, we found that *BnSWD1* was upregulated after treatment with abscisic acid, salicylic acid, and methyl jasmonate. Our study suggests that BnSWD1, which is a novel WD40 repeat-containing

protein, has a function in salt-stress responses in plants, possibly via abscisic acid-dependent and/or -independent signaling pathways.

Keywords *Brassica napus* · mRNA differential display · *Brassica napus* salt responsive WD40 1 gene · Salt stress · Abscisic acid

Introduction

Plants encounter many adverse environmental challenges during growth and development. Among them, insufficient water is one of the most deleterious factors that affect plant growth. Excess salt or insufficient water often cause dehydration, which can denature many proteins or disrupt membranes. Another consequence of dehydration is ion displacement, which deprives cellular enzymes of the inorganic cofactors required for optimal activity (Garcia et al. 1997).

Many different types of genes are induced in plants exposed to these stresses, and the expression patterns of salt-stress-inducible genes are complex. In general, the products of salt-stress-inducible genes can be classified into two groups: those that directly protect plant cells against environmental stresses and those that regulate gene expression and signal transduction during the stress response (Shinozaki and Yamaguchi-Shinozaki 1997). The first group includes proteins that are likely to function by protecting cells from dehydration, such as the enzymes required for the biosynthesis of various osmoprotectants (Verbruggen et al. 1993; Ishitani et al. 1995; Garcia et al. 1997), late-embryogenesis-abundant (LEA) proteins (Naot et al. 1995), antifreeze proteins (Shinozaki and Yamaguchi-Shinozaki 1997), chaperones, and detoxification

S. Lee · J. Lee · S.-Y. Kwon · H. S. Cho · J. M. Park (✉)
Plant Systems Engineering Research Center,
Korea Research Institute of Bioscience and Biotechnology,
P.O. Box 115, Yusong, Daejeon 305-600, Republic of Korea
e-mail: jmpark@kribb.re.kr

K.-H. Paek
School of Life Sciences and Biotechnology, Korea University,
Seoul 136-701, Republic of Korea

S. J. Kim
FnP Co., Ltd, Jeungpyeong 368-811, Republic of Korea

enzymes (Zhu et al. 2009), The second group includes transcription factors (Urao et al. 1993; Liu et al. 1998), protein kinases and phosphatases (Toroser and Huber 1997), and enzymes involved in phosphoinositide metabolism (Shinozaki and Yamaguchi-Shinozaki 1997).

Most salt-stress-inducible genes also respond to treatment with exogenous abscisic acid (ABA). It appears that dehydration triggers the production of ABA, which in turn induces various genes. However, several genes that are induced by salt stress are not responsive to exogenous ABA treatment, such as a peroxidase, PR1, PR10, and osmotin (PR5), which are typical components of plant defense responses to wounding and pathogen attack (Zhu et al. 1995; Ingram and Bartels 1996). These findings suggest that ABA and other stress-related hormones, such as salicylic acid (SA), ethylene, and methyl jasmonic acid (MeJA), may interact with one another during the regulation of stress-signaling and plant stress tolerance. Conventional genetic screens have been performed successfully based on stress-related phenotypes (Xiong and Zhu 2001, 2002); however, there remains a need to identify additional signaling components to understand abiotic stress-signaling networks.

The WD40 motif (also known as the Trp-Asp motif) is present in many eukaryotic proteins (van der Voorn and Ploegh 1992). These WD40 proteins may play key roles in signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, ribosomal RNA biogenesis, and, especially, chromatin modification and transcription (Neer et al. 1994; Smith et al. 1999). Several genes that encode different WD40 proteins, including the putative G- β subunit, COP1 (constitutive photomorphogenesis protein 1), the cell-differentiation regulation protein, TTG1 (TRANSPARENT TESTA GLABRA1), and the retinoblastoma binding protein, have been isolated from plants (Ma 1994; Ach et al. 1997; Walker et al. 1999). The COP1 protein from *Arabidopsis* comprises three domains and acts as part of a protein complex that negatively regulates gene transcription. The WD40 region of COP1 is essential for the function of this protein as a negative regulator of photomorphogenesis (McNellis et al. 1994). The interaction with other proteins in the complex probably takes place via the WD40 repeat domain.

mRNA differential display analysis is one of the most sensitive methods for the detection of mRNAs that are expressed differentially in various biological systems (Liang and Pardee 1992). This PCR-based method is considered to be simple and rapid, and its sensitivity is greater than that of other conventional differential screening procedures. Moreover, the patterns of the amplified cDNA products of different mRNA samples derived from this method can be compared side by side, which permits the

rapid identification of differentially expressed cDNAs (Goormachtig et al. 1995).

In this paper, rapeseed plant (*Brassica napus* L.) was used to investigate plant responses to osmotic stress. *Brassica napus* is a model system that is suitable for the study of these aspects of the plant stress response because of its natural saline habitat and relatively short life span. Five discrete PCR fragments were isolated from salt-treated *Brassica napus* seedlings using mRNA differential display analysis. The DNA fragments were subjected to nucleotide sequence determination and northern blot analysis. The results of these analyses revealed the specific expression of the corresponding genes in response to high salinity conditions. One of the clones encoded eight WD40-motif repeats, and the predicted protein sequence exhibited no similarity to any known protein. The corresponding gene, which was termed *BnSWD1*, was induced by treatment with salt, SA, or MeJA. Expression analysis suggested the involvement of the *BnSWD1* gene in salt stress and that the BnSWD1 protein may be a new component of the plant stress-signaling pathway.

Materials and methods

Plant materials and abiotic stress treatments

Seeds of rapeseed plant (*Brassica napus* L.) were purchased locally. Seeds were surface sterilized and grown in vitro in 1 \times Murashige and Skoog (MS) liquid medium (Sigma, St. Louis, MO, USA) for 4 days at 25°C with a 16 h light/8 h dark photoperiod. The culture medium was replaced with 1 \times MS liquid medium supplemented with 200 mM NaCl (salt stress) or without NaCl (control). The culture flasks were placed in a shaking incubator at 150g. For treatment with ABA, SA, ethephon (ET), and MeJA, plants were grown on MS agar plates in a growth chamber under a 16 h light/8 h dark photoperiod. Plants were treated and examined at 3 weeks after seed germination. Detached leaves were placed in Petri dishes filled with 100 μ M ABA, 2 mM SA, 1 mM ET, or 100 μ M MeJA for various periods, and were then frozen in liquid nitrogen for further analyses.

mRNA differential display

Differential display analysis was performed as described by Liang and Pardee (1992). Four-day-old *Brassica napus* seedlings were treated with 200 mM NaCl for 0, 0.5, 1, or 3 h, and total RNA was extracted from the treated seedlings using the method described by Ausubel et al. (1989). After DNase I (Promega, Madison, WI, USA) treatment, 2 μ g of each total RNA sample was used for reverse

transcription and subsequent PCR. Reaction products were separated on 6% sequencing gels, dried, and visualized using autoradiography. Bands of interests were excised from the dried gel and reamplified. Adequate amounts of the reamplified fragments were obtained in one or two rounds of reamplification and were purified from agarose gels. The purified fragments were subcloned into the pT7-Blue vector (Novagen, Madison, WI, USA), sequenced, and used as probes for RNA gel blot analysis.

RNA gel blot analysis

Total RNA was isolated from *Brassica napus* seedlings as described by Ausubel et al. (1989). Twenty micrograms of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde and transferred onto a Nytran membrane (Amersham, Little Chalfont, Buckinghamshire, UK). Hybridization was carried out using a ^{32}P -labeled DNA probe according to a published protocol (Church and Gilbert 1984). After hybridization, the membranes were washed with $2\times$ SSC at room temperature for 10 min twice, $0.1\times$ SSC containing 0.1% SDS at room temperature for 10 min, and at 65°C for 5 min. The membranes were dried and exposed to X-ray film or directly visualized using a BAS-2500 phosphorimager (Fuji Photo film; Minato-ku, Tokyo, Japan).

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from leaf tissues using the TRI reagent, according to the manufacturer's instructions (MRC, Cincinnati, OH, USA). Total RNA was treated with 1 U of RNase-free DNase (Promega) for 10 min at 37°C and purified using the TRI reagent. The first-strand cDNA was synthesized using 1 μg of DNase-treated RNA, an oligo dT primer (Amersham), and Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen, Carlsbad, CA, USA) according to standard protocols. All PCR reactions were performed in a total volume of 20 μl in AccuPower PCR PreMix (Bioneer, Deajeon, Korea). The primers used for the RT-PCR analysis were as follows: *BnSWD1*, 5'-CCTTGTGGTGCCTGGACGC C-3' and 5'-TGCTCCTGCATTACGCCCCG-3'; *BnD22* (*Brassica napus* drought-induced protein; GenBank accession number X65637; Downing et al. 1992), 5'-CACTTGGCATCAC CCAGACAC-3' and 5'-CCCTATGCTTTACACTCTTA AACC-3'; *BnP5CSI* (*Brassica napus* delta 1-pyrroline-5-carboxylate synthetase 1; GenBank accession number AF314811), 5'-GCCCTCCAAGTGACCCTA AC-3' and 5'-TTCCAACACGCAGTCTCTAA-3'; *BnActin* (*Brassica napus* actin; GenBank accession number AF111812), 5'-TCTTCCTCACGCTATCCTCCG-3' and 5'-TTAGCC GTCTCCAGCTCTTGC-3'; and *BnPR1* (*Brassica napus*

PR1; GenBank accession number U70666), 5'-ATGAAAGTCACTAACTGTTCTCGAC-3' and 5'-GCCAGTAAACTAGGTAACGGATAA-3'. The PCR using *BnActin*-specific primers was used to ensure that an equal amount of RNA was used for all samples.

To obtain the full-length cDNA of *BnSWD1*, 5'-RACE (rapid amplification of 5' complementary DNA ends) PCR was performed using the SMART RACE cDNA amplification kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. The 5' end of *BnSWD1* cDNA (0.7 kb) was amplified from mRNA that was purified from salt-treated leaves using the gene-specific primer 5'-CCCATGGGATGGTGATGACTGCA CA-3'.

Results and discussion

Isolation of salt-induced cDNA fragments using mRNA differential display

To isolate new components of the salt-stress response pathways of *Brassica napus*, genes that are differentially regulated by salt stress were isolated using the mRNA differential display method. Forty-eight partial cDNA fragments exhibiting a differential expression pattern were initially identified by polyacrylamide gel electrophoresis using 40 different combinations of primer pairs [oligo dT(11)VC, VG, and 20 random decamers]. Fragments in the gel were excised and subjected to reamplification. The expression pattern of each amplified fragment was examined using a preliminary RNA gel blot analysis. As shown in Fig. 1, the results of these experiments confirmed that the expression of 5 out of the 48 genes was induced by salt treatment. However, the exact time of induction and the extent of expression of each gene varied considerably.

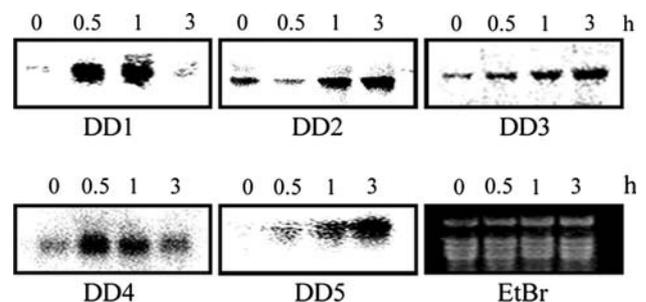


Fig. 1 Expression patterns of the PCR fragments isolated during salt-stress treatments. Twenty micrograms of total RNA was electrophoresed and gel blotted. The blots were hybridized with ^{32}P -labeled DNA fragments derived from differential display experiments (DD1, DD2, DD3, DD4, and DD5). Ethidium bromide (EtBr) staining confirmed equal loading of RNA samples

Table 1 Summary of the salt-stress-induced cDNA fragments of *Brassica napus* identified using mRNA differential display

Clone no.	cDNA (bp)	Induction time (h) ^a	Sequence homology ^b	Accession no. ^c	
DD1	489	0.5	Contains similarity to Bos beta-mannosidase	AC000106	<i>Arabidopsis</i>
DD2	275	1	Unknown		
DD3	253	1	Unknown		
DD4	266	0.5	hin1	Y07563	Tobacco
DD5	393	0.5	Pti6	U89297	Tomato

^a Based on RNA gel blot analysis that was performed using samples harvested at the time, after salt treatment

^b According to the NCBI BlastX search results

^c GenBank/EMBL/SwissProt accession number

These partial cDNAs were subcloned and sequenced. Homology searches revealed that the deduced amino-acid sequences of three of the five cDNA fragments exhibited significant sequence similarity to known proteins in the databases (Table 1). However, the remaining two clones did not show any significant sequence similarity.

The predicted amino-acid sequence of one of the clones isolated, DD1, showed homology to β -mannosidase of *Arabidopsis thaliana*. Several reports suggest that enzymes of sugar metabolism are critical for drought tolerance in plants (Ingram and Bartels 1996). It has been demonstrated that certain sugars, such as trehalose and sucrose, may be central to the protection against water deficit in plants (Ingram and Bartels 1996). It has also been reported that the activity of enzymes involved in glycolysis and gluconeogenesis increases significantly during drought tolerance. For example, the activity of enolase, which is a glycolytic enzyme, increases by more than fourfold in *Mesembryanthemum crystallinum* during salt stress (Forsthoefel et al. 1995). As β -mannosidase catalyzes the degradation of oligosaccharides, it may be involved either in producing small sugar units that act directly as osmoprotectants or in providing raw materials that are needed to synthesize effective osmoprotectant sugars, such as trehalose (Ingram and Bartels 1996). The second cDNA fragment, DD5, shared significant amino acid sequence homology with tomato Pti6. Sequence analysis indicated that the protein has an EREBP/AP2 DNA-binding motif, which is present in the ethylene-response-element-binding proteins (EREBPs) and in the APETALA2 protein. Recently, several EREBP/AP2 transcription factors were isolated that bind a C-repeat/dehydration-responsive element (CRT/DRE) sequence, which is essential for dehydration- and cold-stress-inducible gene expression (Stockinger et al. 1997; Liu et al. 1998; Park et al. 2001).

The third clone, DD4, showed significant amino acid sequence similarity with hin1 and NDR1. *hin1* is a tobacco gene that is highly induced by a bacterial protein, harpin. Induction of *hin1* by *Pseudomonas syringae* pv. *syringae*

Fig. 2 a Comparison of the predicted amino-acid sequence of BnSWD1 with other homologous proteins. The asterisk indicates a putative nuclear localization signal. Dots indicate conserved amino acids in all sequences and dashes were introduced to optimize the alignment. The WD40 motifs are numbered. **b** Schematic structure of BnSWD1. The black box indicates the six consensus WD40 repeats and the gray box includes the two non-canonical WD40 repeats. **c** Phylogenetic relationship of plant BnSWD1-like proteins. The phylogenetic tree was generated by the MEGA 4.0 software using the neighbor-joining method. The bootstrap values from 100 replicates are indicated at each branch. The plant species and GenBank (or TIGR Transcript Assemblies) accession numbers for sequences were: *Brassica napus* BnSWD1 (GU111248), *Arabidopsis thaliana* AtWD1 and AtWD2 (NP_566246 and AAF27015), *Ricinus communis* RcWD1 and RcWD2 (XP_002512270 and XP_002271491), *Populus trichocarpa* PtWD1 and PtWD2 (XP_002328525 and XP_002319610), *Vitis vinifera* VvWD1 and VvWD2 (XP_002271491 and CAO42978), *Oryza sativa* OsWD1 and OsWD48 (NP_001066238 and ABA95885), and *Physcomitrella patens* PpWD1 and PpWD2 (XP_001764540 and XP_001766371)

61 (*Pss61*) is dependent on functional bacterial *hrp* genes, which are involved in the assembly of the harpin secretion pathway (Gopalan et al. 1996).

Sequence analysis of BnSWD1, a member of the WD40 repeat-containing protein family

We found a significant increase in the expression of the DD3 cDNA fragment at an early time after salt induction. This cDNA fragment contained two putative WD40-repeat motifs, which are found in a number of eukaryotic proteins involved in signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, ribosomal RNA biogenesis, and chromatin modification and transcription (Neer et al. 1994; Smith et al. 1999). To identify new regulators of salt stress, the full-length cDNA of DD3 was isolated from a *Brassica napus* cDNA library. The library was prepared from mRNA isolated from *Brassica napus* seedlings that had been salt-stressed for 1 or 3 h. Approximately 2.5×10^5 phages were screened by plaque hybridization using a ³²P-labeled DD3 cDNA fragment as a probe. Among the three clones identified, one was fully

a

	#1	#2	
BnSWD1	MHRVASSGNSGGSVNRNREKLLTYVLNDANDSK--HCAGVNCILDVLKSSVSS-DRSYLFTGSRDGTLLKRWAFDEDAFCSATFESHVDVWNDAALAGEST		100
AtWD1G.A.....T.....T.....I.....N.Q.....T.....		
AtWD2G.A.....T.....T.LQ.....I.....N.Q.....T.....		
RcWD1G.A.NTSS.P.....R.....D.K.....S.....A.....D.GSD.....LA.SAT.....V.V.D.....		
PtWD1G.A.NTNN.P.....R.....S.D.T.....I.....K.....T.N.GCD.....IS.SAT.....V.D.....		
VvWD1G.A.NTNN.T.P.....R.....D.T.....I.....A.W.PLAPD.G.D.....ELT.EVAT.....I.....V.DN.....		
PtWD2G.A.NTNN.P.....R.....S.D.T.....I.....K.....T.D.G.D.....LS.SAT.....V.DR.....		
VvWD2G.A.NTNN.T.P.....R.....D.T.....I.....A.W.PLAPD.G.D.....ELT.EVAT.....I.....V.DN.....		
OsWD1G.A.NTA.S.P.....RF.....DNK.....I.....SY.NA.T.G.TSD.....EPKNGVASF.....IIV.QN.....		
OsWD48G.A.NTA.S.P.....RF.....DNK.....I.....SY.NA.T.G.TSD.....EPKNGVASF.....IIV.QN.....		
PpWD1G.T.NTAN.T.P.....RRI.....R.S.....S.I.SITGPNLKGDDNGEH.SA.....ELGGNEAV.G.....VVVT.DD.....		
PpWD2S.T.NTAN.T.P.....RRF.....V.G.F.....S.I.ITG.NG.KDQGYE.S.....VLGA.EAV.G.....VVVT.D.....		
#3			
BnSWD1	LVSCSDTTVTKWDSLSDGACTRTRFRQHTDVVTCILAVAAKNNNVVASSGGLGGEVFIWDIGAALSPTVKPN-DAMEESSTNGL---GNSQVPTSLRNVGSS		200
AtWD1G.....V.....L.....S.....E.....N.D.S.....ANG.....T.....		
AtWD2G.....V.....L.....S.....E.....N.D.S.....ANG.....T.....		
RcWD1I.....L.NC.....T.K.L.S.....A.E.S.I.....LE.A.S.S.P.DDCS.VNGSS.L.M.....TIS.....		
PtWD1AL.NC.....T.K.L.S.....A.E.S.I.....VE.T.S.SG.....DDYL.VNGST.L.M.....TIS.....		
VvWD1L.NC.....T.L.S.....A.E.S.....V.....LE.T.S.SS.....DCS.INGS.L.I.....TIS.....		
PtWD2L.A.NC.....T.K.L.S.....A.E.S.....VE.T.S.SG.....DDCS.VNGSA.L.I.....TIS.....		
VvWD2L.NC.....T.L.S.....A.E.S.....V.....LE.T.S.SS.....DC.....		
OsWD1L.V.NC.....L.S.....I.ASE.S.I.....LDSS.A.A.SV.K.DEAP.....NSGPAL.T.C.N.....		
OsWD48L.V.NC.....L.S.....I.ASE.S.I.....LDSS.A.A.SV.K.DEAP.....NSGPAL.T.C.N.....		
PpWD1L.KPF.E.....S.....S.A.R.HS.I.....R.....LE.MV.A.AHN.N.DGTYE.G.....PAL-CD.AIT.N.....		
PpWD2L.KPY.E.....S.....S.A.R.HS.T.....R.....LE.IV.ARAHNE.N.DGNHE.....S---D.SIT.N.....		
#4			
BnSWD1	NNISVQSSP-SHGAYPTIAKHKESVYALAMNDAGTMLVSGGTEKVLRVWDFRSGSKTMKLRGHTDNVVRVLLDSTGRFCFLSGSSDSMIRLWDLGQORC		300
AtWD1T.....T.....T.....T.....T.....T.....		
AtWD2T.....T.....T.....T.....T.....T.....		
RcWD1S.MHN.Q.....FV.IA.....S.SI.....V.....T.A.....I.A.....Y.....M.....		
PtWD1S.HTQ.....V.IG.....S.R.....I.....V.....T.A.....I.A.....Y.....M.....		
VvWD1S.LHPT.P.....V.VA.....S.L.....V.I.....T.....I.A.....Y.....M.....		
PtWD2S.AHT.Q.....V.VG.....S.R.....V.....T.A.....I.A.....Y.....M.....		
VvWD2S.L.....V.I.....T.....I.A.....Y.....M.....		
OsWD1S.LASTNGQ.....S.IT.....D.....S.T.NT.....V.....T.K.....I.A.....P.....Y.....		
OsWD48S.LASTNGQ.....S.IT.....D.....S.T.NT.....V.....T.K.....I.A.....P.....Y.....		
PpWD1L.VTAAFAQNL.Q.....LP.....T.S.I.....AV.....T.Q.K.....A.....P.....L.....		
PpWD2L.VTAAFAQNL.Q.....LP.....T.S.I.....AV.....T.Q.K.....A.....P.....L.....		
#5			
BnSWD1	LHTYAVHTDSVVALACTPSPHVSYSGGRDQSLYLTDLATRESVLMCTKEHPIQALQ-DDSIWVATIDSSVERWPAEVQSPKVFQGGSFGLAGNLSFN		400
AtWD1N.S.....C.....L.....N.....T.....KT.....		
AtWD2N.S.....C.....L.....N.....T.....KT.....		
RcWD1M.S.....S.A.S.....N.....L.L.....L.H.G.....T.....D.....GHN.Q.....S.....		
PtWD1V.S.....S.T.S.....L.....L.L.K.....L.H.....H.AHN.Q.....E.....S.....		
VvWD1V.S.....S.T.S.....L.....L.L.....L.H.G.A.T.....R.....GRN.Q.....S.....		
PtWD2V.S.....S.T.S.....L.....L.L.....L.H.....H.AHN.Q.....E.....S.....		
VvWD2V.S.....S.T.S.....L.....L.L.....L.H.G.A.T.....R.....GRN.Q.....S.....		
OsWD1I.S.....S.S.....V.....SN.....L.N.....L.S.....M.....T.....NG.K.AHE.L.L.K.S.....S.....		
OsWD48I.S.....S.S.....V.....SN.....L.N.....L.S.....M.....T.....NG.K.AHE.L.L.K.S.....S.....		
PpWD1I.S.....F.S.....S.....S.V.....I.L.K.E.....VLR.....G.OWL.A.T.LHK.RERATS.TL.AS.V.S.P.A.....		
PpWD2I.S.....F.S.....S.....S.V.....I.L.K.E.....VLR.....G.OWL.A.T.LHK.RERATS.TL.AS.V.S.P.A.....		
#6			
BnSWD1	RARVSEGLNPPPAYKEPLMTIPGSHPIVQHEILNKRQILTKDAGDSVKLWIDITKGVVVEDFGKISFEEKKEELFEMVSIPSWFTVDRGLCLSHLET		500
AtWD1A.....S.I.V.T.....AG.....R.....Y.....V.....		
AtWD2V.MLTFTC.Q-A.....S.I.V.T.....AG.....R.....Y.....V.....		
RcWD1ST.I.V.TL.G.TPA.....R.HV.TVG.....E.R.....Y.V.....Q.Y.....A.....S.V.D.....		
PtWD1SI.V.VH.TL.TSA.....R.HV.TAG.....E.R.I.I.Y.V.....R.Q.....A.....S.V.D.....		
VvWD1A.....ST.V.VH.SF.TPG.....R.HV.TAG.....E.R.I.I.Y.QV.....A.....S.V.D.....		
PtWD2A.....ST.V.VH.SF.TPG.....R.HV.TAG.....E.R.I.I.Y.V.....Q.....A.....S.V.D.....		
VvWD2A.....ST.V.VH.SF.TPG.....R.HV.TAG.....E.R.I.I.Y.QV.....A.....S.V.D.....		
OsWD1A.....SA.V.IH.SSI.VPA.T.....R.HV.TAG.....E.R.A.I.....V.D.K.....A.M.A.....V.D.....		
OsWD48A.....SA.V.IH.SSI.VPA.T.....R.HV.TAG.....E.R.A.I.....V.D.K.....A.M.A.....V.D.....		
PpWD1ANID.SA.V.L.SQ.TSV.TAG.....A.DR.HV.TAGI.....R.E.R.A.I.Y.VN.....EKA.K.V.A.M.....SM.V.D.....		
PpWD2ANID.SA.V.L.TQ.TSV.AAG.....A.DR.HV.AGI.....R.E.R.A.SD.Y.VN.....EK.....M.A.....M.....SM.V.D.....		

BnSWD1	PQCFSAEYMSADLVKVSGRPEDDKINLGRETLKGLLGHWMKAKKHKPKQ-ALTSQDVLVSKDTK-KNLNASKSEDS-SAGNDP-----VYPFFEPSIS		600
AtWD1L.....V.A.....T.....S.T.E.....AS.....S.V.....		
AtWD2L.....V.A.....T.....S.T.E.....AS.....S.V.....		
RcWD1H.....NIA.K.....V.A.....A.L.RRQR.LGS.VSAN.....PG.ITPRS.AH.RV.VDG.TE.SM.....STV.....		
PtWD1NIV.K.....V.A.....A.L.RRQR.LGS.SAN.....G.IAPRS.AH.RVDV.DGG.E.SM.....STV.....		
VvWD1NII.K.....V.A.....S.LT.R.QRFGS.SVNV.E.A.G.EISTRSITH.RM.IDGN.E.SM.....STV.....		
PtWD2NIA.K.....V.A.....A.L.RR.RLGS.P.TSAN.....G.FAHR.S.GH.RV.VDGG.E.SK.....STV.....		
VvWD2NII.K.....V.A.....S.LT.R.QRFGS.SVNV.E.A.G.EISTRSITH.RM.IDGN.E.SM.....STV.....		
OsWD1I.AV.N.A.AQ.L.AQ.....R.V.S.....QRSGSH.G.SN.TSTG.VSL.PHPR.VDDG.E.HASQ.....ML.S.....STV.....		
OsWD48I.AV.N.A.AQ.L.AQ.....R.V.S.....QRSGSH.G.SN.TSTG.VSL.PHPR.VDDG.E.HASQ.....ML.S.....STV.....		
PpWD1AV.H.P.ASEL.L.I.Q.....R.A.LTHRUV.VAP.STPK.....SPGLSSGVGE.GSNRRITLDDSQHE.NSQQTR.L.S.....STQ.....		
PpWD2V.A.H.P.AS.EL.L.I.Q.....R.AN.LTRRA.AAGVT.IPN.ASPIP.SGPGHEGP.RRSALDDNLHE.NNQQTR.F.S.Q.STQA.....		

BnSWD1	PPSIITEGSGGGPWRKITEFTGTEDEKDFPLWCLDAVLNRRNPPRENTKLSFFLHPCGNSVQVVTGLKLSAPRILRVHKVTNVYVVKMVLDSPLDSL		700
AtWD1S.....N.....		
AtWD2S.....N.....		
RcWD1V.DLD.....W.....C.....I.Q.....S.C.Y.....T.I.L.Q.....I.V.....I.L.....K.....G.VN.....		
PtWD1VV.....DLD.S.....W.....C.....C.Y.....AF.I.L.Q.....I.V.....L.....K.....NVN.....		
VvWD1DLD.....DLD.S.....W.....C.....C.Y.QA.....T.I.L.Q.....I.V.....L.....K.....M.VN.....		
PtWD2VV.....DLD.S.....W.....C.....C.Y.....AF.I.L.Q.....I.V.....L.....K.....NVN.....		
VvWD2DLD.....DLD.S.....W.....C.....C.Y.QA.....T.I.L.Q.....I.V.....L.....K.....M.VN.....		
OsWD1S.....R.DLD.S--I.W.IV.C.EH.F.K.....CG.Y.A.PAPNI.Q.....I.A.....R.G.N.....		
OsWD48S.....R.DLD.S--I.W.IV.C.EH.F.K.....CG.Y.A.PAPNI.Q.....I.A.....R.G.N.....		
PpWD1A.LN.....EL.S.I.C.HG.ISQ.....A.C.Y.Y.QTIT.L.Q.....I.L.K.L.LL.ER.EENG.....		
PpWD2A.DLN.....L.G.I.C.HE.SQ.....S.C.Y.Y.QTIT.L.Q.....I.IK.....S.LN.ER.EENM.....		

BnSWD1	IDGASVSGG-----PQQLFAGYGLLTAGSK-PWQKLRPSIEILCNSQ-----VLSPEWSLATVTRTFVWVKKPEDLILNRYAVAK		754 a.a
AtWD1P.S.N.....QS.L.....DM.....AY.....I.R.....(753 a.a)		
AtWD2P.S.N.....QS.L.....S.ASQTSRDP.TIALFL.....DM.....AY.....I.R.....(743 a.a)		
RcWD1P.TF.P.LG.GG.QS.HSAV.D.SYRP.L.....DM.....KAYI.....Q.....V.....VQGR.....(764 a.a)		
PtWD1P.TFAP.LG.G.HL.HSVV.D.SFRP.V.....K.....DM.....AYI.....L.QGR.....(761 a.a)		
VvWD1P.TFAP.LP.GGQSQHSVAVG.D.SFRS.L.....K.....DM.....AYI.....V.....VQGR.....(765 a.a)		
PtWD2P.TFAP.LG.G.PL.HSVV.D.SFRS.L.....K.....DM.....AYI.....V.....VQGR.....(745 a.a)		
VvWD2P.TFAP.LP-----AVG.D.SFRS-----SCAITS-----Q.....DM.....AYI.....V.....VQGR.....(705 a.a)		
OsWD1A.STFGM.LT.S.GQS.FS.LDSSSRL.L.....K.V.....S.V.....-A.M.....AY.....K.VQSR.....(752 a.a)		
OsWD48A.STFGM.LT.S.GQS.FS.LDSSSRL.L.....K.V.....S.AS.....-FGTRNE.GHSAGLCLLEEARGFDS-----S.....(745 a.a)		
PpWD1S.ANAPA.....QAGGNANGK.NNVFRP.....SWP.GTK.IV.....D.....P.M.....AY.....Y.H.TVPSL.....(766 a.a)		
PpWD2PETNASA.QLVSSQAGNGKGGK.SNVFRP.....GWP.DSK.VV.....D.....AS.L.....AYI.....Y.H.IVPSR.....(767 a.a)		

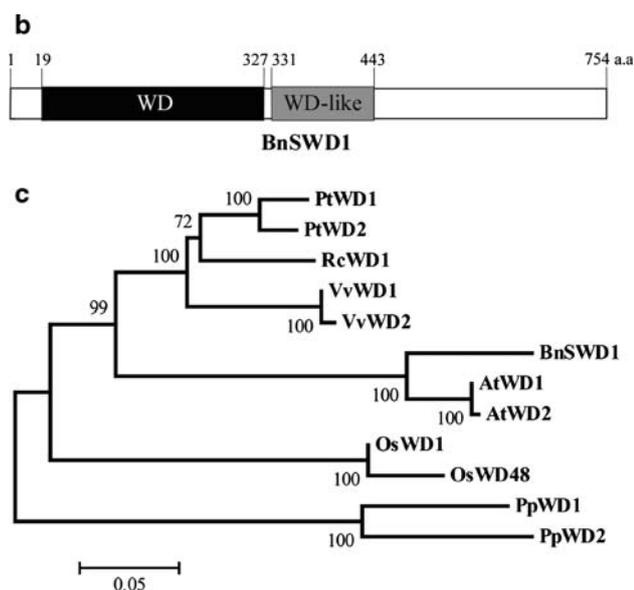


Fig. 2 continued

sequenced and termed *BnSWD1*. This cDNA was 2536 bp in length and contained an open reading frame of 754 amino acids, which corresponds to a predicted polypeptide of 82.5 kDa (Fig. 2a).

Analysis of the deduced amino acid sequence of *BnSWD1* using SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) revealed the presence of eight putative WD40-repeat motifs (which corresponded to 443 amino acids) at its N-terminal region. This motif can be further separated into two regions, based on its preservation of canonical WD40 motif sequences. The first region (Fig. 2b, black box) contained six consensus WD40 repeats and the second region (Fig. 2b, gray box) included two noncanonical WD40 repeats. In addition, the *BnSWD1* protein contained two putative nuclear localization signals (Fig. 2a).

A BLASTX analysis revealed the existence of *BnSWD1* highly conserved homologous proteins in plants. We also found significant *BnSWD1* homology in all other eukaryotes. The overall identity and similarity among the plant *BnSWD1*-like proteins varied between 56 and 90% and 72 and 95%, respectively (Fig. 2a, c). Although there are no studies on the function of *BnSWD1*-like proteins in plants, a few recent reports described a novel function for one of the *BnSWD1* homologs in mammals, WDR48 [also known as a USP1 (ubiquitin-specific protease) associated factor 1 (UAF1)], which controls the deubiquitinating enzyme activity of USP1 via WD40 motif-mediated protein–protein interactions (Park et al. 2002, 2003; Cohn et al. 2007, 2009; Cote-Martin et al. 2008). Disruption of ubiquitination by deubiquitinating enzymes (DUBs) has been implicated in many aspects of plant cellular functions

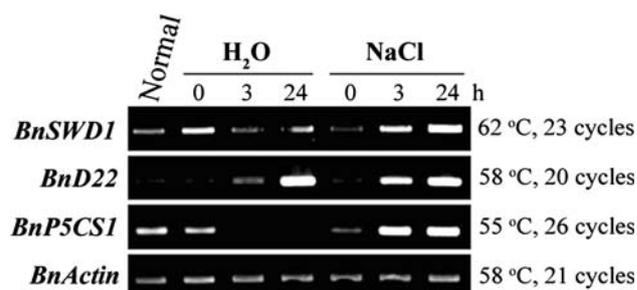


Fig. 3 Expression patterns of the *BnSWD1* gene after NaCl treatment. *Brassica napus* seedlings grown for 4 days in MS agar plates were treated with 200 mM NaCl for the indicated periods. Plants treated with water were used as negative controls. One microgram of total RNA was used for cDNA synthesis and PCR was then performed using the primers described in “Materials and methods”. The expression of *BnD22* and *BnP5CS1* in salt-treated plants was also monitored as a positive control

(Love et al. 2007; Bonnet et al. 2008); however, the physiological roles of most DUBs and the mechanism of regulation of protein deubiquitination in plants are poorly understood.

Expression of *BnSWD1* during salt stress

We next examined the temporal expression pattern of the *BnSWD1* gene in response to salt stress using an RT-PCR analysis. As shown in Fig. 3, the expression of the *BnSWD1* gene was induced rapidly within 3 h after salt treatment and the transcript level increased steadily up to 24 h (Fig. 3). This expression pattern was consistent with that of the partial cDNA fragment. The induction of the *BnSWD1* mRNA was hardly detected in control plants that were treated with water. We also monitored the expression pattern of the marker genes *Brassica napus* *BnD22* drought-induced protein (*BnD22*; Downing et al. 1992) and delta 1-pyrroline-5-carboxylate synthetase (*BnP5CS1*; Xue et al. 2009), which were used as positive controls for the salt stress. The *BnD22* protein, which is a member of the Kunitz-type proteinase inhibitor family, is a 22-kDa protein that is induced by progressive or rapid water stress and salinity (Downing et al. 1992; Reviron et al. 1992). Expression of the proline biosynthetic gene *BnP5CS1* is also rapidly upregulated under treatment with NaCl and ABA (Xue et al. 2009). Induction of the expression of both marker genes began 3 h after treatment with 200 mM NaCl and increased to high levels at 24 h. The expression of *BnD22* was increased under non-salt-stressed conditions, whereas that of *BnP5CS1* was suppressed (Fig. 3). This result partly reflected the effects of mechanical stress caused by detachment. However, we were able to assess whether the induction of *BnD22* was due to salt or wounding stress, as the induction of *BnD22* in response to salt was relatively faster and stronger than that of the

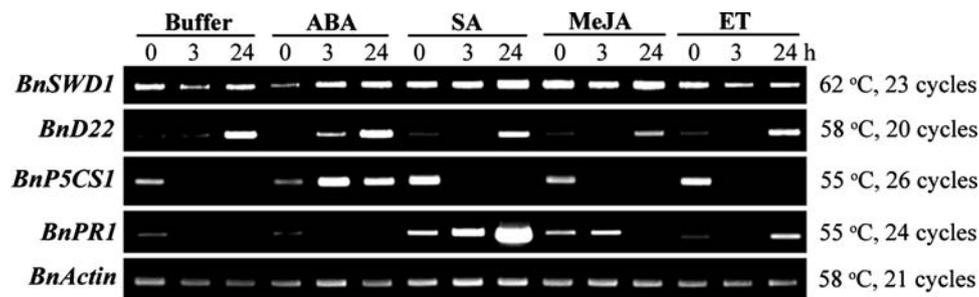


Fig. 4 Semi-quantitative RT-PCR analyses of the expression profiles of *BnSWDI* in rapeseed plants treated with various chemicals. Total RNA was extracted from rapeseed plants treated with buffer [0.1% (v/v) ethanol solution; negative control] or treated with 100 μ M ABA, 2 mM SA, 1 mM ET, or 100 μ M MeJA at the indicated time points.

The reverse transcription and PCR procedures were performed as described in “Materials and methods”. The expression of *BnPRI* (SA or ET) and *BnD22* and *BnP5CSI* (ABA) were monitored as positive or negative controls of each chemical treatment

control. Importantly, these results indicated that *BnSWDI* expression was specifically induced as part of the rapeseed defense mechanism against salt stress.

Expression of *BnSWDI* in response to stress-related chemicals

To analyze the effects of other stimuli on the expression of *BnSWDI*, plant hormones, which included ABA, ethylene, MeJA, and SA, were administered to rapeseed plants. As determined by semi-quantitative RT-PCR (Fig. 4), the steady-state mRNA levels corresponding to *BnSWDI* increased significantly within 3 h for ABA, SA, and MeJA treatment compared with the treatment-with-buffer control. Compared with these three hormones, the addition of 1 mM ET did not activate the expression of *BnSWDI*. On the other hand, the induction of the positive control genes *BnD22* and *BnP5CSI* was only detected after treatment with ABA, as observed previously (Downing et al. 1992; Xue et al. 2009). In addition, we monitored the expression patterns of *BnPRI* as a positive control for treatment with SA and ET (Potlakayala et al. 2007). Expression of the *BnPRI* gene was detected within 24 h after the administration of ET or SA, with different induction patterns (Fig. 4). These results imply that the induction of *BnSWDI* expression after salt stress is dependent on ABA, SA, and MeJA accumulation.

In plants, chemicals such as ABA, SA, ET, and MeJA are important inducers of defense-related genes that act against biotic and abiotic stresses (Reymond and Farmer 1998; Mauch-Mani and Mauch 2005). There are several reports on genes that respond to both abiotic and biotic stresses. For example, lipid transfer protein (*Ltp*) genes have been reported to not only respond to environmental stresses, such as cold and salt stress (Molina et al. 1996), but also to be induced by bacteria or fungal pathogens (Molina and Olmedo 1997). Osmotin and osmotin-like

proteins are another example of genes that respond to both abiotic and biotic stresses. These observations suggest that *BnSWDI* is among the plant genes that are dually responsive to both types of stress. Further studies will be necessary to clarify the roles of the novel plant WD40 protein, *BnSWDI*, in the signal transduction pathways that mediate the responses of plants to various environmental cues.

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References

- Ach RA, Taranto P, Grissem W (1997) A conserved family of WD-40 proteins binds to the retinoblastoma protein in both plants and animals. *Plant Cell* 9:1595–1606
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989) *Current protocol in molecular biology*. Greene/Wiley, New York
- Bonnet J, Romier C, Tora L, Devys D (2008) Zinc-finger UBPs: regulators of deubiquitylation. *Trends Biochem Sci* 33:369–375
- Church GM, Gilbert W (1984) Genomic sequencing. *Proc Natl Acad Sci USA* 81:1991–1995
- Cohn MA, Kowal P, Yang K, Haas W, Huang TT, Gygi SP, D’Andrea AD (2007) A UAF1-containing multisubunit protein complex regulates the Fanconi anemia pathway. *Mol Cell* 28:786–797
- Cohn MA, Kee Y, Haas W, Gygi SP, D’Andrea AD (2009) UAF1 is a subunit of multiple deubiquitinating enzyme complexes. *J Biol Chem* 284:5343–5351
- Cote-Martin A, Moody C, Fradet-Turcotte A, D’Abramo CM, Lehoux M, Joubert S, Poirier GG, Coulombe B, Laimins LA, Archambault J (2008) Human papillomavirus E1 helicase interacts with the WD repeat protein p80 to promote maintenance of the viral genome in keratinocytes. *J Virol* 82:1271–1283
- Downing WL, Mauxion F, Fauvarque MO, Reviron MP, de Vienne D, Vartanian N, Giraudat J (1992) A *Brassica napus* transcript encoding a protein related to the Kunitz protease inhibitor family

- accumulates upon water stress in leaves, not in seeds. *Plant J* 2:685–693
- Forsthoefel NR, Cushman MAF, Cushman JC (1995) Posttranscriptional and posttranslational control of enolase expression in the facultative crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Plant Physiol* 108:1185–1195
- Garcia AB, Engler J, Iyer S, Gerats T, Van Montagu M, Caplan AB (1997) Effects of Osmoprotectants upon NaCl Stress in Rice. *Plant Physiol* 115:159–169
- Goormachtig S, Valerio-Lepiniex M, Szczyglowski K, Van Montagu M, Holsters M, de Bruijn FJ (1995) Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. *Mol Plant Microbe Interact* 8:816–824
- Gopalan S, Wei W, He SY (1996) hrp gene-dependent induction of hin1: a plant gene activated rapidly by both harpins and the avrPto gene-mediated signal. *Plant J* 10:591–600
- Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:377–403
- Ishitani M, Nakamura T, Han SY, Takabe T (1995) Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol Biol* 27:307–315
- Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971
- Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factor, 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* 10:1391–1406
- Love KR, Catic A, Schlieker C, Ploegh HL (2007) Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat Chem Biol* 3:697–705
- Ma H (1994) GTP-binding proteins in plants: new members of an old family. *Plant Mol Biol* 26:1611–1636
- Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. *Curr Opin Plant Biol* 8:409–414
- McNellis TW, von Arnim AG, Araki T, Komeda Y, Miséra S, Deng X-W (1994) Genetic and molecular analysis of an allelic series of cop1 mutants suggests functional roles for the multiple protein domains. *Plant Cell* 6:487–500
- Molina A, Olmedo FG (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J* 12:669–675
- Molina A, Diaz I, Vasil IK, Carbonero P (1996) Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens. *Mol Gen Genet* 252:162–168
- Naot D, Hayyim GB, Eshdat Y, Holland D (1995) Drought, heat and salt stress induce the expression of a citrus homologue of an atypical late-embryogenesis Lea5 gene. *Plant Mol Biol* 27:619–622
- Neer EJ, Schmidt CJ, Nambudripad R, Smith TF (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature* 371:297–300
- Park JM, Park CJ, Lee SB, Ham BK, Shin R, Paek KH (2001) Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* 13:1035–1046
- Park J, Lee BS, Choi JK, Means RE, Choe J, Jung JU (2002) Herpesviral protein targets a cellular WD repeat endosomal protein to down regulate T lymphocyte receptor expression. *Immunity* 17:221–233
- Park J, Cho NH, Choi JK, Feng P, Choe J, Jung JU (2003) Distinct roles of cellular Lck and p80 proteins in herpesvirus saimiri Tip function on lipid rafts. *J Virol* 77:9041–9051
- Potlakayala SD, Reed DW, Covello PS, Fobert PR (2007) Systemic acquired resistance in canola is linked with pathogenesis-related gene expression and requires salicylic Acid. *Phytopathol* 97:794–802
- Reviron MP, Vartanian N, Sallantin M, Huet JC, Pernollet JC, de Vienne D (1992) Characterization of a novel protein induced by progressive or rapid drought and salinity in *Brassica napus* leaves. *Plant Physiol* 100:1486–1493
- Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr Opin Plant Biol* 1:404–411
- Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. *Plant Physiol* 115:327–334
- Smith TF, Gaitatzes C, Saxena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. *Trends Biochem Sci* 24:181–185
- Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc Natl Acad Sci USA* 94:1035–1040
- Toroser D, Huber SC (1997) Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach leaves. *Plant Physiol* 114:947–955
- Urao T, Shinozaki KY, Urao S, Shinozaki K (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* 5:1529–1539
- van der Voorn L, Ploegh HL (1992) The WD-40 repeat. *FEBS Lett* 307:131–134
- Verbruggen N, Villarreal R, Montagu MV (1993) Osmoregulation of a pyroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol* 103:771–781
- Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC (1999) The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* 11:1337–1350
- Xiong L, Zhu JK (2001) Abiotic stress signal transduction in plants: molecular and genetic perspectives. *Physiol Plant* 112:152–166
- Xiong L, Zhu JK (2002) Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell Environ* 25:131–139
- Xue X, Liu A, Hua X (2009) Proline accumulation and transcriptional regulation of proline biosynthesis and degradation in *Brassica napus*. *BMB Rep* 42:28–34
- Zhu B, Chen TH, Li PH (1995) Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants. *Plant Physiol* 108:929–937
- Zhu JQ, Zhang JT, Tang RJ, Lv QD, Wang QQ, Yang L, Zhang HX (2009) Molecular characterization of ThIPK2, an inositol polyphosphate kinase gene homolog from *Thellungiella halophila*, and its heterologous expression to improve abiotic stress tolerance in *Brassica napus*. *Physiol Plant* 136:407–425