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SHORT COMMUNICATION

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

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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 1gene · Salt stress · Abscisic acid

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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

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× 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× MS liquid medium supplemented with 200 mM NaCl (salt stress) or without NaCl (control). The culture flasks were placed in a shaking incubator at 150*g*. 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 μM ABA, 2 mM SA, 1 mM ET, or 100 μM 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 μg 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× SSC at room temperature for 10 min twice, 0.1× 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′-TGCTCCTGCATTCACGCCCG-3′; *BnD22* (*Brassica napus* drought-induced protein; GenBank accession number **X65637**; Downing et al. 1992), 5′-CACTTGGCATCAC CCAGACAC-3′ and 5′-CCCTATGCTTTACACTCTTA AACC-3′; *BnP5CS1* (*Brassica napus* delta 1-pyrroline-5-carboxylate synthetase 1; GenBank accession number **AF314811**), 5′-GCCCTCCAAGTGACCCTA AC-3′ and 5′-TTCCAACACGCAGTCCTCTAA-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′-AT GAAAGTCACTAACTGTTCTCGAC-3′ and 5′-GCCAGT AAACTAGGTAACGGATAA-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.

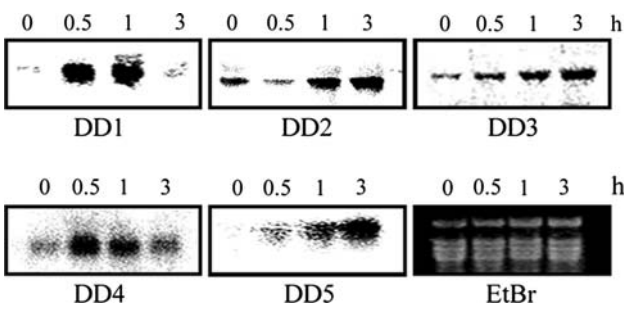


Fig. 1 Expression patterns of the PCR fragments isolated during salt-stress treatments. Twenty micrograms of total RNA was electrophoresed and gel blotted. The blots were hybridized with ^{32}P-labeled DNA fragments derived from differential display experiments (*DD1, DD2, DD3, DD4*, and *DD5*). Ethidium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

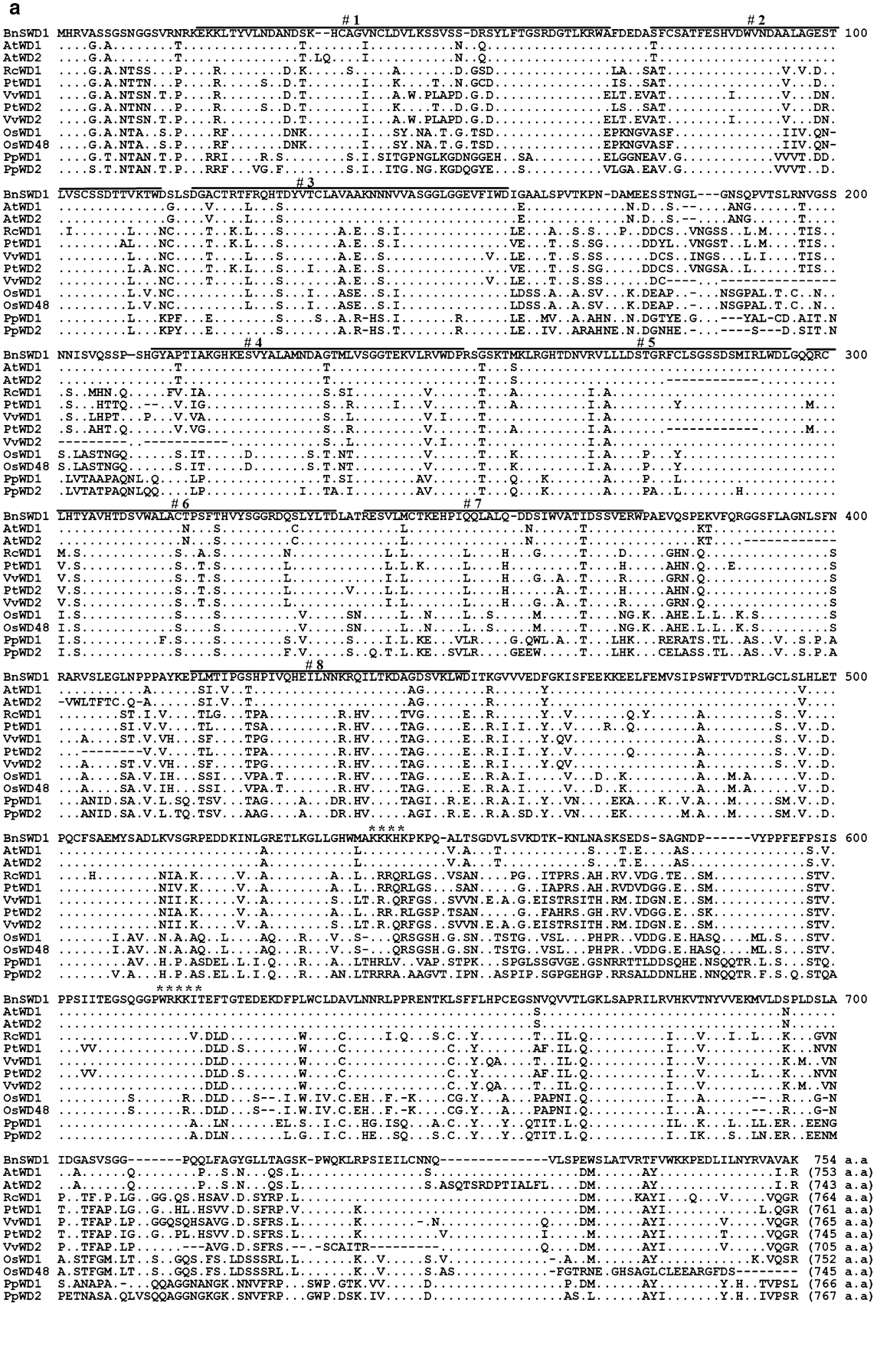
The predicted amino-acid sequence of one of the clones isolated, DD1, showed homology to β-mannosidase of *Arabidopsis thaliana.* Several reports suggest that enzymes of sugar metabolism are critical for drought tolerance in plants (Ingram and Bartels 1996). It has been demonstrated that certain sugars, 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* 61 (*Pss*61) is dependent on functional bacterial *hrp* genes, which are involved in the assembly of the harpin secretion pathway (Gopalan et al. 1996).

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)

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 ^{32}P-labeled DD3 cDNA fragment as a probe. Among the three clones identified, one was fully


a
#1
#2
#3
#4
#5
#6
#7
#8
BnSWD1 MHRVASSGSNGGSVRNRKEKKLTYVLNDANDSK--HCAGVNCLDVLKSSVSS-DRSYLFTGSRDGTLKRWAFDEDASFCSATFESHVDWVNDAALAGEST 100
AtWD1 ....G.A.....T.....T.....I.....N..Q.....T.....
AtWD2 ....G.A.....T.....T.LQ....I.....N..Q.....T.....
RcWD1 ....G.A.NTSS...P....R.....D.K.....S....A.....D.GSD.....LA..SAT.....V.V.D..
PtWD1 ....G.A.NTTN...P....R.....S..D.T.....I...K....T..N.GCD.....IS..SAT.....V...D..
VvWD1 ....G.A.NTSN.T.P....R.....D.T.....I...A.W.PLAPD.G.D.....ELT.EVAT.....I.....V...DN.
PtWD2 ....G.A.NTNN...P....R.....S..D.T.....I...K....T..D.G.D.....LS..SAT.....V...DR.
VvWD2 ....G.A.NTSN.T.P....R.....D.T.....I...A.W.PLAPD.G.D.....ELT.EVAT.....I.....V...DN.
OsWD1 ....G.A.NTA..S.P....RF.....DNK.....I...SY.NA.T.G.TSD.....EPKNGVASF.....IIV.QN-
OsWD48 ....G.A.NTA..S.P....RF.....DNK.....I...SY.NA.T.G.TSD.....EPKNGVASF.....IIV.QN-
PpWD1 ....G.T.NTAN.T.P...RRI....R.S.....S.I.SITGPNGLKGDNGGEH..SA.....ELGGNEAV.G.....VVVT.DD.
PpWD2 ....S.T.NTAN.T.P...RRF...VG.F.....S.I..ITG.NG.KGDQGYE...S.....VLGA.EAV.G.....VVVT..D.
BnSWD1 LVSCSSDTTVKTWDSLSDGACTRTFRQHTDYVTCLAVAAKNNNVVASGGLGGEVFIWDIGAALSPVTKPN-DAMEESSTNGL---GNSQPVTSLRNVGSS 200
AtWD1 .....G....V....L...S.....E.....N.D..S.--....ANG.....T....
AtWD2 .....G....V....L...S.....E.....N.D..S.--....ANG.....T....
RcWD1 .I.....L...NC....T..K.L...S.....A.E..S.I.....LE...A..S.S...P..DDCS..VNGSS..L.M....TIS..
PtWD1 .....AL...NC....T..K.L...S.....A.E..S.I.....VE...T..S.SG.....DDYL..VNGST..L.M....TIS..
VvWD1 .....L...NC....T....L...S.....A.E..S.....V..LE...T..S.SS.....DCS..INGS...L.I....TIS..
PtWD2 .....L.A.NC....T..K.L...S...I...A.E..S.....VE...T..S.SG.....DDCS..VNGSA..L.....TIS..
VvWD2 .....L...NC....T....L...S.....A.E..S.....V..LE...T..S.SS.....DC-----..----------
OsWD1 .....L.V.NC.....L...S...I...ASE..S.I.....LDSS.A..A.SV...K.DEAP..-...NSGPAL.T.C..N..
OsWD48 .....L.V.NC.....L...S...I...ASE..S.I.....LDSS.A..A.SV...K.DEAP..-...NSGPAL.T.C..N..
PpWD1 .....L...KPF...E.....S....S..A.R-HS.I.....R.....LE..MV..A.AHN..N.DGTYE.G...---YAL-CD.AIT.N
PpWD2 .....L...KPY...E.....S.....A.R-HS.T.....R.....LE..IV..ARAHNE.N.DGNHE.-...----S---D.SIT.N
BnSWD1 NNISVQSSP-SHGYAPTIAKGHKESVYALAMNDAGTMLVSGGTEKVLRVWDPRSGSKTMKLRGHTDNVRVLLLDSTGRFCLSGSSDSMIRLWDLGQQRC 300
AtWD1 .....T.....T.....T...S.....
AtWD2 .....T.....T.....T...S.....----------.....
RcWD1 .S..MHN.Q.....FV.IA.....S.SI.....V.....T...A.....I.A.....
PtWD1 .S...HTTQ..-...V.IG.....S..R.....I...V.....T...A.....I.A.....Y.....M...
VvWD1 .S..LHPT...P...V.VA.....S..L.....V.I...T.....I.A.....
PtWD2 .S..AHT.Q.....V.VG.....S..R.....V.....T...A.....I.A.....----------.....M...
VvWD2 ---------.----------.....S..L.....V.I...T.....I.A.....
OsWD1 S.LASTNGQ.....S.IT.....D.....S.T.NT.....V.....T...K.....I.A....P...Y.....
OsWD48 S.LASTNGQ.....S.IT.....D.....S.T.NT.....V.....T...K.....I.A....P...Y.....
PpWD1 .LVTAAPAQNL.Q....LP.....T.SI.....AV.....T...Q...K.....A....P...L.....
PpWD2 .LVTATPAQNLQQ....LP.....I..TA.I.....AV.....T...Q...K.....A....PA..L.....H.....
BnSWD1 LHTYAVHTDSVWALACTPSFTHVYSGGRDQSLYLTDLATRESVLMCTKEHPIQQLALQ-DDSIWVATIDSSVERWPAEVQSPEKVFQRGGSFLAGNLSFN 400
AtWD1 .....N...S.....C.....L.....N.....T.....KT.....
AtWD2 .....N...S.....C.....L.....N.....T.....KT...----------
RcWD1 M.S.....S..A.S.....N.....L.L.....L....H...G.....T...D.....GHN.Q.....S
PtWD1 V.S.....S..T.S.....L.....L.L.K.....L....H.....T....H.....AHN.Q...E.....S
VvWD1 V.S.....S..T.S.....L.....I.L.....L....H...G..A..T....R.....GRN.Q.....S
PtWD2 V.S.....S..T.S.....L.....V....L.L.....L....H.....A..T....H.....AHN.Q.....S
VvWD2 V.S.....S..T.S.....L.....I.L.....L....H...G..A..T....R.....GRN.Q.....S
OsWD1 I.S.....S....S.....V.....SN.....L..N....L..S.....M.....T....NG.K..AHE.L.L..K.S.....S
OsWD48 I.S.....S....S.....V.....SN.....L..N....L..S.....M.....T....NG.K..AHE.L.L..K.S.....S
PpWD1 I.S.....F.S....S.....S.V.....S....I.L.KE...VLR....G.QWL.A..T...LHK...RERATS.TL..AS..V..S.P.A
PpWD2 I.S.....S....S.....F.V.....S..Q.T.L.KE..SVLR....GEEW.....T...LHK...CELASS.TL..AS..V..S.P.A
BnSWD1 RARVSLEGLNPPPAYKEPLMTIPGSHPIVQHEILNNKRQILTKDAGDSVKLWDITKGVVVEDFGKISFEEKKEELFEMVSIPSWFTVDTRLGCLSLHLET 500
AtWD1 .....A.....SI.V..T.....AG.....R.....Y.....V....
AtWD2 -VWLTFTC.Q-A.....SI.V..T.....AG.....R.....Y.....V....
RcWD1 .....ST.I.V....TLG...TPA.....R.HV....TVG.....E..R.....Y..V.....Q.Y.....A.....S..V....
PtWD1 .....SI.V.V....TL....TSA.....R.HV....TAG.....E..R.I.I..Y..V....R..Q.....A.....S..V..D.
VvWD1 ...A....ST.V.VH...SF....TPG.....R.HV....TAG.....E..R.I....Y.QV.....A.....S..V..D.
PtWD2 ...-------V.V....TL....TPA.....R.HV....TAG.....E..R.I.I..Y..V.....Q.....A.....S..V..D.
VvWD2 ...A....ST.V.VH...SF....TPG.....R.HV....TAG.....E..R.I....Y.QV.....A.....S..V..D.
OsWD1 ...A....SA.V.IH...SSI...VPA.T.....R.HV....TAG.....E..R.A.I.....V...D..K.....A...M.A.....V..D.
OsWD48 ...A....SA.V.IH...SSI...VPA.T.....R.HV....TAG.....E..R.A.I.....V...D..K.....A...M.A.....V..D.
PpWD1 ...ANID.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.
PpWD2 ...ANID.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 PQCFSAEMYSADLKVSGRPEDDKINLGRETLKGLLGHWMAKKKHKPKPQ-ALTSGDVLSVKDTK-KNLNASKSEDS-SAGNDP------VYPPFEFPSIS 600
AtWD1 .....A.....L.....V.A...T.....S...T.E....AS.....S.V.
AtWD2 .....A.....L.....V.A...T.....S...T.E....AS.....S.V.
RcWD1 ....H.....NIA.K.....V..A.....A..L..RRQRLGS..VSAN....PG..ITPRS.AH.RV.VDG.TE..SM.....STV.
PtWD1 .....NIV.K.....V..A.....A..L..RRQRLGS...SAN.....G..IAPRS.AH.RVDVDGG.E..SM.....STV.
VvWD1 .....NII.K.....V..A.....S..LT.R.QRFGS..SVVN.E.A.G.EISTRSITH.RM.IDGN.E..SM.....STV.
PtWD2 .....NIA.K.....V..A.....A..L..RR.RLGSP.TSAN.....G..FAHRS.GH.RV.VDGG.E..SK.....STV.
VvWD2 .....NII.K.....V..A.....S..LT.R.QRFGS..SVVN.E.A.G.EISTRSITH.RM.IDGN.E..SM.....STV.
OsWD1 .....I.AV..N.A.AQ..L....AQ...R...V..S-...QRSGSH.G.SN..TSTG..VSL...PHPR..VDDG.E.HASQ....ML.S...STV.
OsWD48 .....I.AV..N.A.AQ..L....AQ...R...V..S-...QRSGSH.G.SN..TSTG..VSL...PHPR..VDDG.E.HASQ....ML.S...STV.
PpWD1 .....AV..H.P.ASDEL.L.I.Q...R...A..LTHRLV..VAP.STPK...SPGLSSGVGE.GSNRRTTLDDSQHE.NSQQTR.L.S...STQ.
PpWD2 .....V.A...H.P.AS.EL.L.I.Q...R...AN.LTRRRA.AAGVT.IPN..ASPIP.SGPGEHGP.RRSALDDNLHE.NNQQTR.F.S.Q.STQA
BnSWD1 PPSIITEGSQGGPWRKKITEFTGTEDEKDFPLWCLDAVLNNRLPPRENTKLSFFLHPCEGSNVQVVTLGKLSAPRILRVHKVTNYVVEKMVLDSPLDSLA 700
AtWD1 .....S.....N.....
AtWD2 .....S.....N.....
RcWD1 .....V.DLD.....W....C.....I.Q...S.C..Y.....T..IL.Q.....I...V...I..L...K...GVN
PtWD1 .....DLD.S.....W....C.....C..Y.....AF.IL.Q.....I...V.....K...NVN
VvWD1 .....DLD.....W....C.....C..Y.QA....T..IL.Q.....I...V.....K.M..VN
PtWD2 .....DLD.S.....W....C.....C..Y.....AF.IL.Q.....I...V.....L...K...NVN
VvWD2 .....DLD.....W....C.....C..Y.QA....T..IL.Q.....I...V.....K.M..VN
OsWD1 .....S.....R..DLD...S--.I.W.IV.C.EH..F.-K....CG.Y...A....PAPNI.Q.....I...A.....--..R...G-N
OsWD48 .....S.....R..DLD...S--.I.W.IV.C.EH..F.-K....CG.Y...A....PAPNI.Q.....I...A.....--..R...G-N
PpWD1 .....A..LN.....EL.S..I.C..HG.ISQ...A.C..Y...Y..QTIT.L.Q.....IL..K...L..LL.ER..EENG
PpWD2 .....A.DLN.....L.G..I.C..HE..SQ...S.C..Y...Y..QTIT.L.Q.....I..IK...S..LN.ER..EENM
BnSWD1 IDGASVSGG-------PQQLFAGYGLLTAGSK-PWQKLRPSIEILCNNQ----------VLSPEWSLATVRTFVWKKPEDLILNYRVAVAK 754 a.a
AtWD1 ..A.....Q.....P..S.N...QS.L.....S.....DM.....AY.....I.R (753 a.a)
AtWD2 ..A.....Q.....P..S.N...QS.L.....S.ASQTSRDPTIALFL....DM.....AY.....I.R (743 a.a)
RcWD1 P..TF.P.LG..GG.QS.HSAV.D.SYRP.L.....DM.....KAYI...Q...V.....VQGR (764 a.a)
PtWD1 T..TFAP.LG..G..HL.HSVV.D.SFRP.V.....K.....DM.....AYI.....L.QGR (761 a.a)
VvWD1 P..TFAP.LP..GGQSQHSAVG.D.SFRS.L.....K.....-.N.....Q....DM.....AYI.....V.....VQGR (765 a.a)
PtWD2 T..TFAP.IG..G..PL.HSVV.D.SFRS.L.....K.....V.....I.....M.....AYI.....T.....VQGR (745 a.a)
VvWD2 P..TFAP.LP.....---AVG.D.SFRS.--.-SCAITR---------.....Q....DM.....AYI.....V.....VQGR (705 a.a)
OsWD1 A.STFGM.LT..S..GQS.FS.LDSSSRL.L.....K..V.....S.V.....-.A..M.....AY.....K.VQSR (752 a.a)
OsWD48 A.STFGM.LT..S..GQS.FS.LDSSSRL.L.....K..V.....S.AS.....-FGTRNE.GHSAGLCLEEARGFDS-------- (745 a.a)
PpWD1 S.ANAPA.-...QQAGGNANGK.NNVFRP...SWP.GTK.VV.....D.....P.DM.....AY.....Y.H..TVPSL (766 a.a)
PpWD2 PETNASA.QLVSQQAGGNGKGK.SNVFRP...GWP.DSK.IV.....D.....AS.L.....AYI.....Y.H..IVPSR (767 a.a)

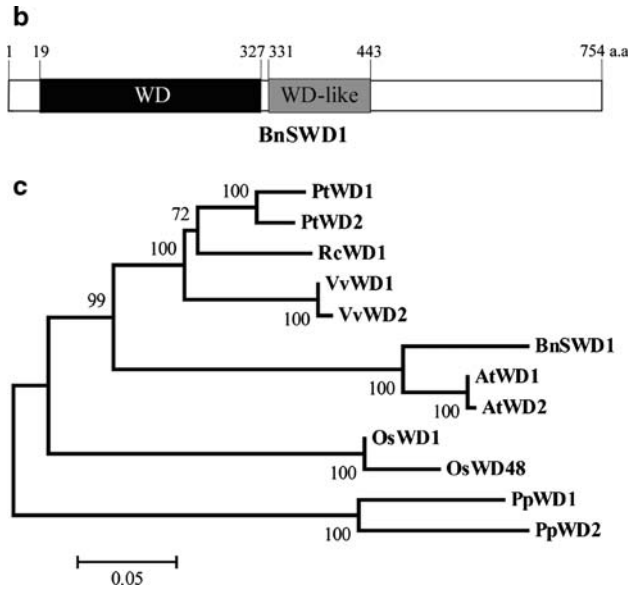


Fig. 2 continued

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

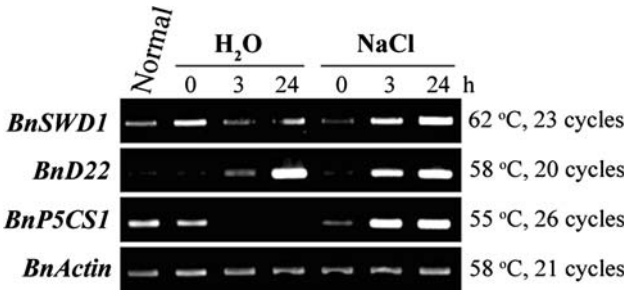


Fig. 3 Expression patterns of the *BnSWD1* gene after NaCl treatment. *Brassica napus* seedlings grown for 4 days in MS agar plates were treated with 200 mM NaCl for the indicated periods. Plants treated with water were used as negative controls. One microgram of total RNA was used for cDNA synthesis 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

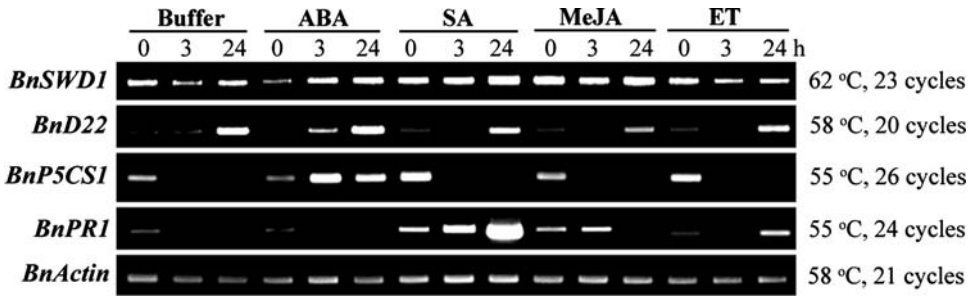


Fig. 4 Semi-quantitative RT-PCR analyses of the expression profiles of *BnSWD1* in rapeseed plants treated with various chemicals. Total RNA was extracted from rapeseed plants treated with buffer [0.1% (v/v) ethanol solution; negative control] or treated with 100 μM ABA, 2 mM SA, 1 mM ET, or 100 μM MeJA at the indicated time points. The reverse transcription and PCR procedures were performed as described in "Materials and methods". The expression of *BnPR1* (SA or ET) and *BnD22* and *BnP5CS1* (ABA) were monitored as positive or negative controls of each chemical treatment

control. Importantly, these results indicated that *BnSWD1* expression was specifically induced as part of the rapeseed defense mechanism against salt stress.

Expression of *BnSWD1* in response to stress-related chemicals

To analyze the effects of other stimuli on the expression of *BnSWD1*, 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 *BnSWD1* 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 *BnSWD1*. On the other hand, the induction of the positive control genes *BnD22* and *BnP5CS1* 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 *BnPR1* as a positive control for treatment with SA and ET (Potlakayala et al. 2007). Expression of the *BnPR1* 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 *BnSWD1* 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 *BnSWD1* 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, BnSWD1, in the signal transduction pathways that mediate the responses of plants to various environmental cues.

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