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Research article

Characterization and expression analysis of dirigent family genes related to stresses in *Brassica*

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ABSTRACT

The dirigent (*DIR*) genes are playing a vital role in enhancing stress resistance in different crop plants. In this study, we collected 29 *DIR* like genes, two from a *Brassica rapa* cv. Osome full length cDNA library and 27 from the *B. rapa* database designated as *B. rapa* Dirigent (*BrDIR*) like genes. Sequence analysis and a comparison study of these genes confirmed that seven were dirigent and the remaining 22 were dirigent like genes. Expression analysis revealed an organ specific expression of these genes. *BrDIR2* showed differential responses after *Fusarium oxysporum* f.sp. *conglutinans* infection in cabbage. Four *Brassica oleracea* dirigent like genes highly homologous to *BrDIR2* also showed similar responses in cabbage plants infected with this fungus. Moreover, several *BrDIR* like genes showed significant responses after water, ABA and cold stress treatments in Chinese cabbage. Under water stress, most responsive genes showed the highest expression at 24 h, at which time the acid soluble lignin content of samples under the same stress condition were also highest, indicating a possible relationship between *BrDIR* like genes and lignin content. Taken together, our results indicate a protective role of *BrDIR* genes against biotic and abiotic stresses in *Brassica*.

1. Introduction

Biotic and abiotic stresses are major concerns for *Brassica* crops, which comprise an important and diverse group of crops grown worldwide. Inducible biochemical reactions create protective physiological conditions to limit pathogen growth and invasion in the host tissues. Inducible plant defense responses include the synthesis of signals such as salicylic acid, ethylene and jasmonates, which regulate gene expression and the production of defense molecules such as reactive oxygen species, phenylpropanoids, phytoalexins and Pathogenesis-related (PR) [1]. Plants respond to biotic and abiotic challenges by activating a variety of genes, including the (*DIR*) gene. *DIR* is an important disease resistance responsive gene (*DRRG*) that plays a potential role in enhancing stress resistance in different crop plants [2].

DIR proteins are believed to mediate the free radical coupling of monolignol plant phenols in plants to yield lignans and lignins [3,4] and some *DIR* proteins have been shown to be involved in the plant disease-resistance response [5]. These proteins do not appear to have an oxidative radical forming activity of their own in the absence of oxidative enzymes such as peroxidase and oxidase. *DIR* proteins also show no significant level of similarity or identity to any other protein of known function [6,7].

Forsythia suspensa DIR proteins have been shown to direct the stereospecific coupling of E-coniferyl alcohol to produce lignan (+)-pinoresinol. Lignan has antifungal properties, and appears to primarily be involved in plant defense, with its formation being either under constitutive or inducible control [8,9]. Lignin is an important compound that is mainly deposited in terminally differentiated cells of supportive and water-conducting tissues and is primarily associated with mechanical support and allowing plants to stand, with water transport in the xylem vases and with defense against pests and microorganisms [10,11]. Wang et al. [12] proposed that when the *DIR* gene was expressed constitutively in canola, increased resistance was observed against a broad range of

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Table 1
Homology analysis of *BrDIR* like genes.

Name	Subfamily	Top matched clones	Identity (%)	Top homologous sp.	Functions	References
BrDIR1	DIR-g	AK353489	89	*T. halophila*	DRRP[a]	[16,17]
BrDIR2	DIR-a	NM_118500	92	*A. thaliana*	LBP[b], DR[c]	[18]
BrDIR3	DIR-g	XM_002880957	89	*A. lyrata* subsp. *lyrata*	DRRP	Unpublished
BrDIR4	DIR-a	XM_002863706	83	〃	Unknown	Unpublished
BrDIR5	DIR-a	XM_002872510	83	〃	DRRP	Unpublished
BrDIR6	DIR-g	NM_117432	90	*A. thaliana*	Unknown	[18]
BrDIR7	〃	XM_002870327	91	*A. lyrata* subsp. *lyrata*	DRRP	Unpublished
BrDIR8	〃	NM_127681	84	*A. thaliana*	DR	[19]
BrDIR9	〃	NM_113307	92	〃	Unknown	[20]
BrDIR10	DIR-a	XM_002887871	92	*A. lyrata* subsp. *lyrata*	DRRP	Unpublished
BrDIR11	DIR-g	AK117592.1	73	*A. thaliana*	Unknown	Unpublished
BrDIR12	〃	NM_113307	90	〃	Unknown	[20]
BrDIR13	〃	NM_115381	85	〃	DR	[20]
BrDIR14	〃	AK117592	84	〃	Unknown	Unpublished
BrDIR15	DIR-a	NM_118500	90	〃	LBP, DR	[18]
BrDIR16	DIR-g	NM_118500	82	〃	LBP, DR	[18]
BrDIR17	〃	XM_002868810	83	*A. lyrata* subsp. *lyrata*	Unknown	Unpublished
BrDIR18	DIR-a	NM_118500	91	*A. thaliana*	LBP, DR	[18]
BrDIR19	DIR-g	XM_002886660	84	*A. lyrata* subsp. *lyrata*	Unknown	Unpublished
BrDIR20	〃	AK353489	85	*T. halophila*	DRRP	[16,17]
BrDIR21	〃	AK353489	89	〃	DRRP	[16,17]
BrDIR22	〃	AK353489	87	〃	DRRP	[16,17]
BrDIR23	DIR-a	XM_002880310	87	*A. lyrata* subsp. *lyrata*	Unknown	Unpublished
BrDIR24	DIR-g	AK353489	86	*T. halophila*	DRR	[16,17]
BrDIR25	〃	AK353489	87	〃	DRR	[16,17]
BrDIR26	〃	DQ245667	88	*Zea mays*	Unknown	[21]
BrDIR27	〃	NM_112213	79	*A. thaliana*	LBP, DR	[20]
BrDIR28	〃	XM_002882790	83	*A. lyrata* subsp. *lyrata*	DRRP	Unpublished
BrDIR29	〃	NM_112212	82	*A. thaliana*	DRRP	[20]

[a] Disease resistance responsive family protein.
[b] Lignan biosynthetic process.
[c] Defense response.

Table 2
List of 29 *BrDIR* like genes identified in *Brassica rapa* and their sequence characteristics (bp, base pair; aa, amino acids; kDa, kilo Dalton).

Name	Accession number	ORF (bp)	Chromosome number	Protein						
				Length (aa)	Domain		N-Glyc (Asn) position	Mol.wt (kDa)	pI	Instability index
					Dirigent start–end	Transmembrane start–end				
BrDIR1	Bra014354[a] EU186317 [b]	567	A08	188	44–187	–	60, 72, 93, 128	20.77	9.55	18.68
BrDIR2	Bra019277[a] EU186318 [b]	564	A03	187	39–183	13–30	59, 123	21.42	6.82	23.62
BrDIR3	Bra035680	1239	A04	412	258–412	–	193	39.74	5.05	30.12
BrDIR4	Bra035323	573	Scaffold000103	190	36–179	5–27	88	20.94	6.09	48.66
BrDIR5	Bra035243	537	A08	178	30–174	–	50, 114	20.25	6.81	34.36
BrDIR6	Bra034872	738	A08	245	105–245	7–26	–	25.47	5.02	28.52
BrDIR7	Bra032718	735	A04	244	104–244	7–29	–	25.40	5.03	29.80
BrDIR8	Bra031162	585	A09	194	44–193	13–32	75, 134, 188	21.45	7.02	28.72
BrDIR 9	Bra028366	735	A01	244	104–244	7–29	–	25.39	4.87	30.54
BrDIR10	Bra027681	549	A09	182	34–178	7–24	54, 118	20.73	8.41	24.24
BrDIR11	Bra024546	558	A09	185	41–185	5–22	70	20.36	7.86	25.34
BrDIR12	Bra015015	738	A07	245	105–245	7–26	–	25.56	4.89	31.52
BrDIR13	Bra014768	939	A04	312	150–309	–	–	33.07	4.84	35.83
BrDIR14	Bra014587	561	A04	186	40–186	–	69, 170	20.03	9.56	21.05
BrDIR15	Bra013723	561	A01	186	38–182	–	58, 122	21.27	6.81	26.20
BrDIR16	Bra013109	567	A03	188	39–184	12–34	60, 124	21.41	6.28	24.35
BrDIR17	Bra011895	534	A01	177	24–176	–	40, 50, 115	19.23	9.63	23.76
BrDIR18	Bra010542	561	A08	186	39–183	12–29	59, 123	21.20	7.68	28.69
BrDIR19	Bra038929	558	Scaffold000155	185	38–184	–	62, 66, 125	20.62	8.97	27.45
BrDIR20	Bra037986	567	A06	188	44–187	–	60, 95, 130	21.06	9.92	26.08
BrDIR21	Bra037987	567	A06	188	44–187	–	60, 72, 93, 128	20.87	9.57	19.02
BrDIR22	Bra037985	561	A06	186	41–185	4–26	57, 91, 126	20.57	9.65	19.25
BrDIR23	Bra031161	561	A09	186	40–186	–	56, 181	20.21	6.29	23.02
BrDIR24	Bra030878	570	A08	189	44–188	–	72, 94, 129	20.81	9.68	17.06
BrDIR25	Bra030876	570	A08	189	44–188	–	60, 72, 94, 129	21.00	9.46	23.08
BrDIR26	Bra027410	567	A05	188	44–187	4–26	60, 72, 93, 128	20.57	9.75	30.10
BrDIR27	Bra021497	558	A01	185	41–184	–	57, 69, 90, 125	20.27	8.67	24.82
BrDIR28	Bra021496	555	A01	184	41–183	4–22	57, 89, 124	20.53	9.85	30.26
BrDIR29	Bra021495	561	A01	186	40–185	–	56, 91	20.59	9.57	0.41

[a] *Brassica* database (http://brassicadb.org/brad/index.php) accession number.
[b] NCBI accession number.

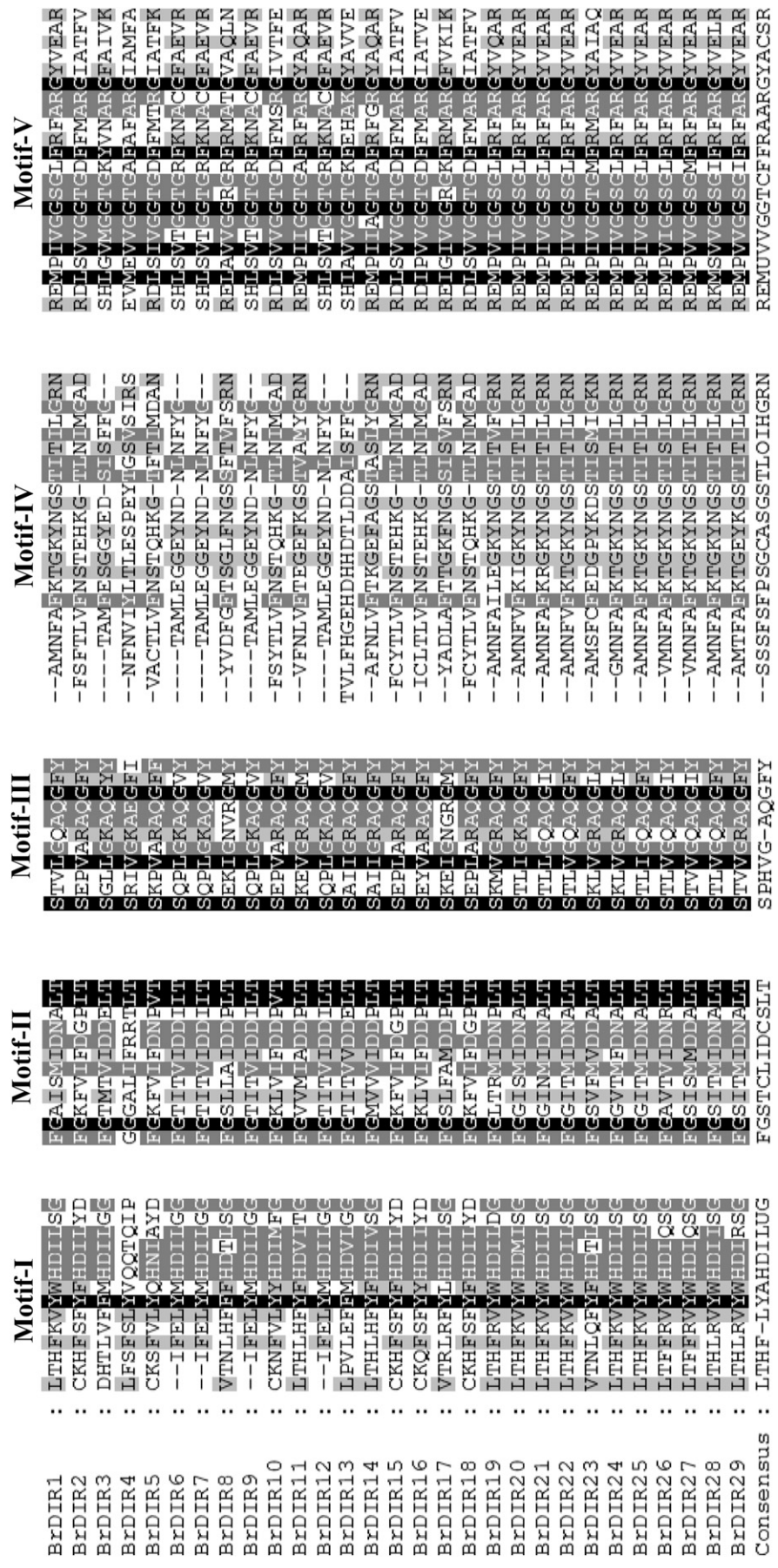

Fig. 1. Conserved five characteristic motifs (I–V) of dirigent proteins in *BrDIR* protein sequences.

fungal pathogens including *Rhizoctonia solani* and *Leptosphaeria maculans*. *Boea hygrometrica* (*BhDIR*) has been found to be expressed in response to various abiotic stresses including dehydration, $CaCl_2$, ABA, H_2O_2 and EGTA, and may be involved in response to various stresses and play a common role in abiotic stress response [13].

In this study, we characterized 29 *BrDIR* like genes based on sequence analysis, a comparison study, and their expression in different organs and after biotic and abiotic stress treatments. We also discussed their association with biotic and abiotic stress resistance in *Brassica*.

2. Results and discussion

2.1. Identification and sequence analysis

We previously functionally characterized the EST sequences of our full length cDNA library of *Brassica rapa* cv. Osome based on gene ontology [14,15] and obtained two dirigent genes. Sequence analysis, these genes were designated *B. rapa* Dirigent (*BrDIR*) like *1* and *2*. The sequence data of *BrDIR* like *1* and *2* were deposited in GenBank under accession numbers EU186317 and EU186318, respectively. Additional dirigent like genes were collected from the *Brassica* database. The results of analysis of these genes are summarized in Table 1. The 29 *BrDIR* like genes have protein lengths ranging from 177 to 412 and predicted pI values ranging from 4.84 to 9.92. The predicted molecular masses range from 19.23 to 39.74 kDa and they all have DIR protein domains.

N-glycosylation sites (Asn), which have been found in *FiDIR1* genes, are a feature of secreted proteins and the first and the best characterized DIR protein [22]. Amino acids of *BrDIR* like genes were predicted using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), and the results revealed the presence of Asn site, indicating that *BrDIR* like genes are likely to be secreted as protein Table 2. Dirigent proteins are also well characterized by five distinct motifs conserved in their amino acid sequences [2,23]. Our 29 *BrDIR* like genes also showed five well conserved motifs in their amino acid sequences (Fig. 1). Pairwise sequence similarities among the amino acids of the 29 *BrDIR* like genes are shown in Table 4S, Supplementary data.

Sequences were also characterized by cellular localization. Burlat et al. [3] and Donaldson et al. [24] first identified *DIR* genes and described their cellular localization and exclusive domain of lignification on *Forthysia intermedia* in plant organs. Ralph et al. [2] also used to identify the subcellular localization by using the TargetP 1.1 server [25] and PSORT [26] subcellular localization software. Hence, we also predicted that all 29 *BrDIR* like genes are targeted to the secretory pathway, either through the default pathway for extracellular release or possible final localization in the endoplasmic reticulum membrane, microbody or vacuole Table 3S, Supplementary data. These results are well characterized and supported as *BrDIR* like genes to extend our further analysis.

2.2. Organ specific expression analysis

For organ specific expression analysis, cDNA templates were prepared from the mRNA isolated from roots, stems, leaves and flower buds of Chinese cabbage and gene specific primers were used for semi quantitative RT-PCR (Table 1S, Supplementary data). We observed the expression of these genes in an organ specific

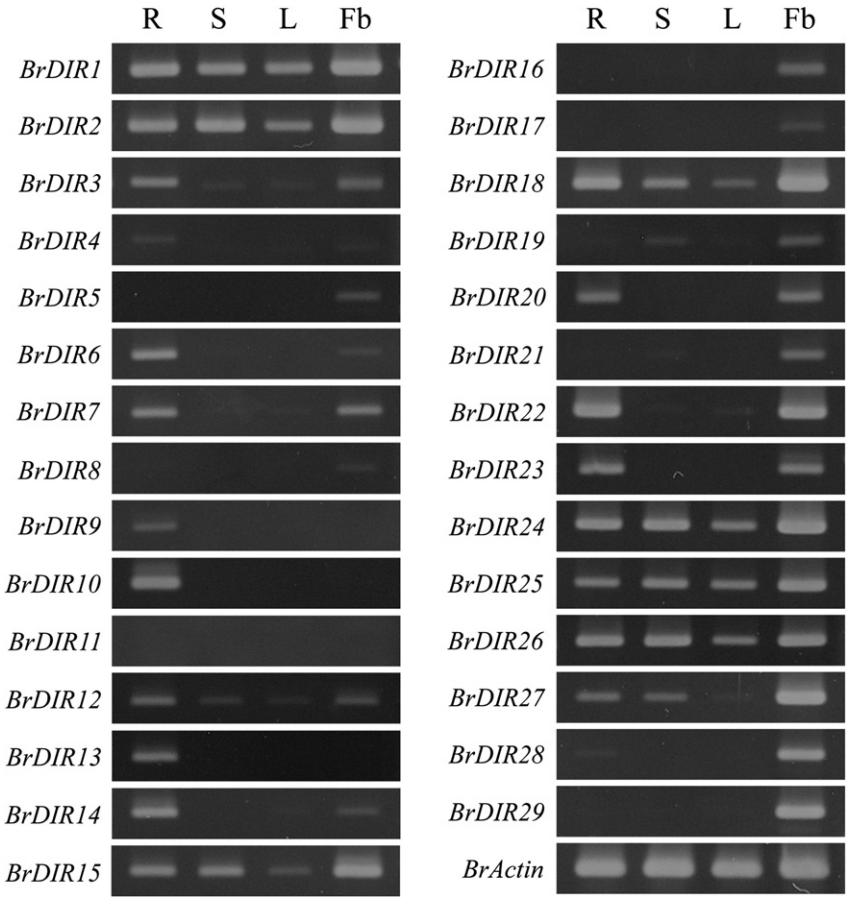

Fig. 2. Analysis of organ specific expression pattern of 29 *BrDIR* like genes by RT-PCR, cDNA isolated from, R: Root, S: Stem, L: Leaves, FB: Flower buds.

pattern. Among these genes, *BrDIR* like *1*, *2*, *3*, *12*, *15*, *18*, *24*, *25*, *26* and *27* showed the highest expression in all organs. *BrDIR* like *6*, *7*, *14*, *20*, *22*, *23* and *28* were only expressed in roots and flower buds, while *BrDIR* like *19* showed expression in stems and flower buds, *BrDIR* like *4*, *9*, *10* and *13* were only expressed in roots and *BrDIR* like *5*, *8*, *16*, *17*, *21* and *29* were only expressed in flower buds. It should be noted that the expression of most genes was comparatively higher in roots and flower buds than stems and leaves. *BrDIR* like *11* did not show any expression during 40 cycles of PCR, indicating that it may be a pseudo gene (Fig. 2). In previous studies, expression of the *DIR* genes was observed in the roots, stems and petioles of *F. intermedia* [3]. Conversely, Wu et al. [13] predicted the role of DIR protein in lignin biosynthesis in *B. hygrometrica* and roots, stems and flower buds were found to participate most in lignin biosynthesis. These organs also share characteristics that make them particularly prone to other stresses and protection of these organs against stress attack is critical; accordingly, production of antifungal molecules could be part of a local defense strategy [27]. Furthermore, Rogers and Campbell [28] found that in lignification occurring during the course of normal tissue development, various abiotic and biotic stresses can trigger the production of lignin at specific sites. *BrDIR* like genes were expressed in an organ specific manner, suggesting possible roles of these genes in specific organs through lignin formation and participation in developmental processes.

2.3. Expression analysis of after stress treatment

A review of the available literature revealed that the expression of *DIR* and *DIR* like genes is induced by biotic stress agents such as *R.*

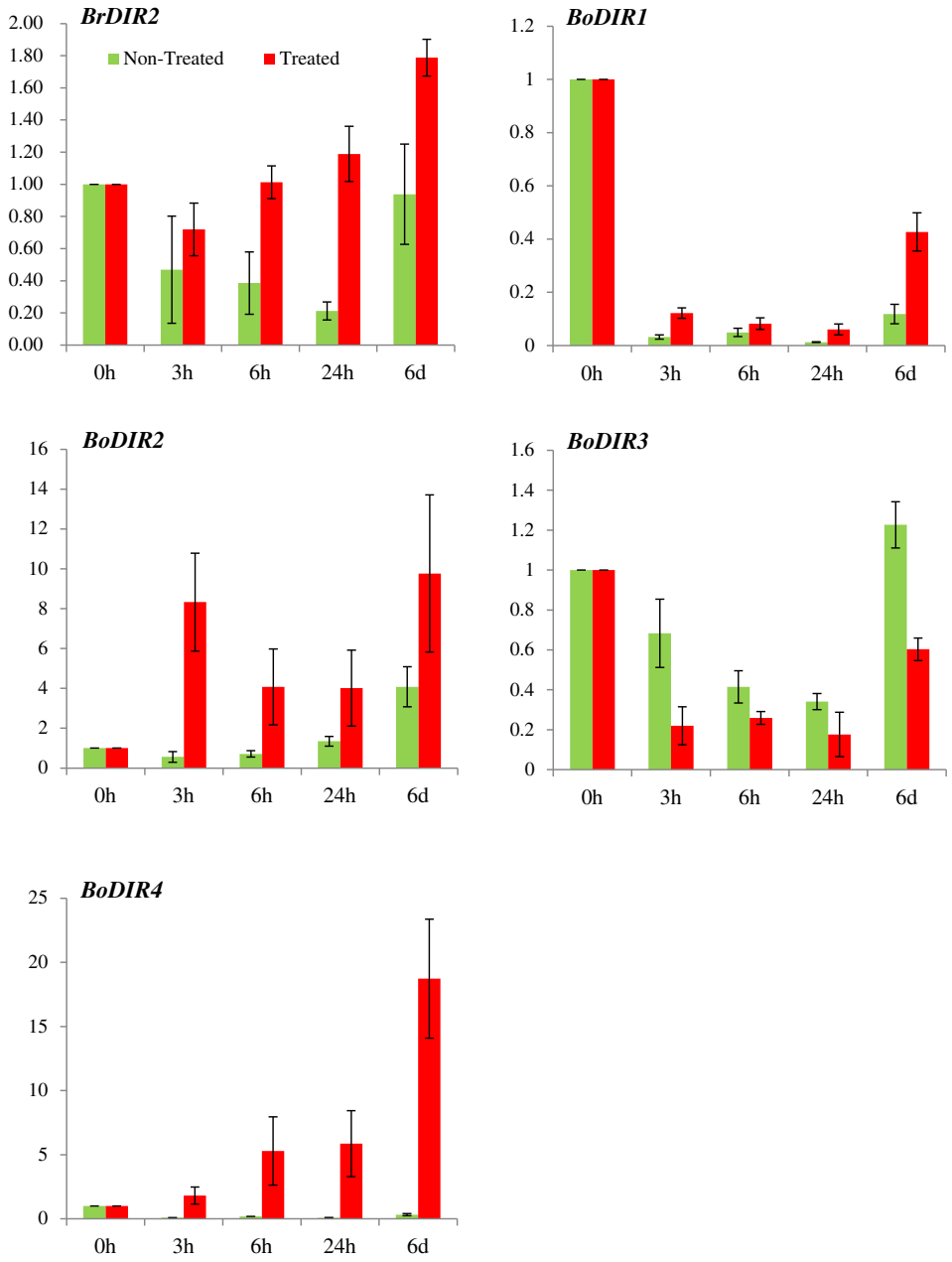

Fig. 3. Real time PCR expression analysis of *BrDIR2* and *BoDIR1* to *4* after treated with *Fusarium oxysporum* f. sp. *conglutinans* in cabbage (*Brassica oleracea*). The *error bars* represent the standard error of the means of three independent replicates.

solani, *L. maculans* and *Verticillium dahliae* [12,29,30]. To explore such responses, we attempted to investigate the expression of 29 *BrDIR* like genes in Chinese cabbage after *Fusarium oxysporum* f.sp. *conglutinans* infection at various times. The results revealed that the genes were not differentially expressed. However, *BrDIR* like *2* showed differential expression in cabbage (Fig. 3). Specifically, the expression gradually increased from 3 h to 6 d post infection and was five fold higher at 24 h and two fold higher at 6 d after infection when compared to non-infected plants. Other genes might be responsive to other fungi in cabbage and Chinese cabbage. Since this *B. rapa* gene responded in cabbage plants, we investigated the expression of *Brassica oleracea* genes, which are highly homologous to *B. rapa*, and identified four ESTs (BOLC06620, BOLC07339, BOLS23847 and BOLS24786) of *B. oleracea*. These four EST sequences have also been found to be dirigent like genes based on their functional annotation; therefore, they were designated as *B. oleracea* DIR like (*BoDIR*) 1 to 4. Among these *B. oleracea* dirigent sequences, *BoDIR2* showed the highest identity (91%) with *BrDIR2* and showed a similar pattern of expression in *F. oxysporum* f.sp. *conglutinans* infected cabbage plants. The remaining three *BoDIR* genes also showed a particular pattern of differential expression in cabbage (Fig. 3). The other *BrDIR* genes may be responsive to other biotic stresses, indicating a likely defense related role against biotic stress in *Brassica*.

DIR and *DIR* like genes are not only inducible by biotic stresses, but also by abiotic stresses including $CaCl_2$, H_2O_2, SA, ABA, drought and low temperature [13,31] suggesting multiple roles of *DIR* genes in plants. Therefore, this study was extended to include expression analysis of 29 *BrDIR* like genes after cold, ABA and water stress treatments. Among these genes, *BrDIR* like *7*, *10*, *12*, *14*, *15*, *18*, *20*, *22*, *23*, *25*, *27* and *29* were highly expressed in response to cold; while *BrDIR* like 2, *3*, 6, *7*, *9*, *10*, *15*, *18*, *20*, *23*, *28* and *29* were expressed following ABA stress treatments (Fig. 4). Wu et al. [13] observed similar responses of *BhDIR1* genes to cold and ABA stress in *B. hygrometrica*. Similarly, *BrDIR* like *2*, *6*, *7*, *9*, *10*, *13*, *14*, *15*, *18*, *22* and *23* responded to water stress treatments, and the expression of all of these genes except *BrDIR* like *13* and *22* was many times higher at 24 h than 0 h. *BrDIR* like *13* and *22* were early inducible with water stress and showed the greatest expression after treatment for 30 min (Fig. 5). Interestingly, this water stress responsive *BrDIR* like genes all showed higher expression in roots. Kotula et al. [32] and Shunsaku et al. [33] detected higher lignin content in cell walls of rice roots under deoxygenated conditions than aerated conditions. Abiko et al. [34] proposed early formation of lignin in the roots of *Zea nicaraguensis* under water logging conditions. These observations suggest possible roles of *BrDIR* like genes in lignin formation under stress conditions and in defense activities.

2.4. Change in acid soluble lignin content analysis in stress condition

In a previous study, the acid-soluble lignin content in dehydrated leaves of *B. hygrometrica* was found to decrease significantly from 0 to 72 h [13]. In this study, we analyzed the acid soluble lignin contents at various times (0, 24, 72 h) after water stress treatments and found that lignin contents were two fold higher at 24 h and about double at 72 h than at 0 h (Fig. 6). The water stress responsive genes also showed the highest level of expression at 24 h. Taken together, these results indicate that *BrDIR* like genes may participate in the coupling of monolignols and consequently increased the lignin content of *Brassica*. Our findings also suggest that *BrDIR* like genes play roles in lignin biosynthesis and therefore participate in the defense activities of *Brassica*.

2.5. Phylogeny of the plant DIR family including BrDIR like genes

To validate our analysis of *BrDIR* like genes, we conducted a comprehensive search of GenBank and collected 65 *DIR* and *DIR* like genes of other plant species [23]. The collected genes included known and putative *DIR* from 34 gymnosperms and 31 angiosperms. Alignments were then conducted using the Dialign software [35], which is capable of identifying local similarities among divergent sequences, after which they were manually adjusted to define a conserved sequence of ca. 153 amino acids [2,23]. Ralph et al. [23] suggested that the *DIR* gene family consisted of six distinct subfamilies, DIR-a, DIR-b/d, DIR-c, DIR-e, DIR-f and DIR-g. In the

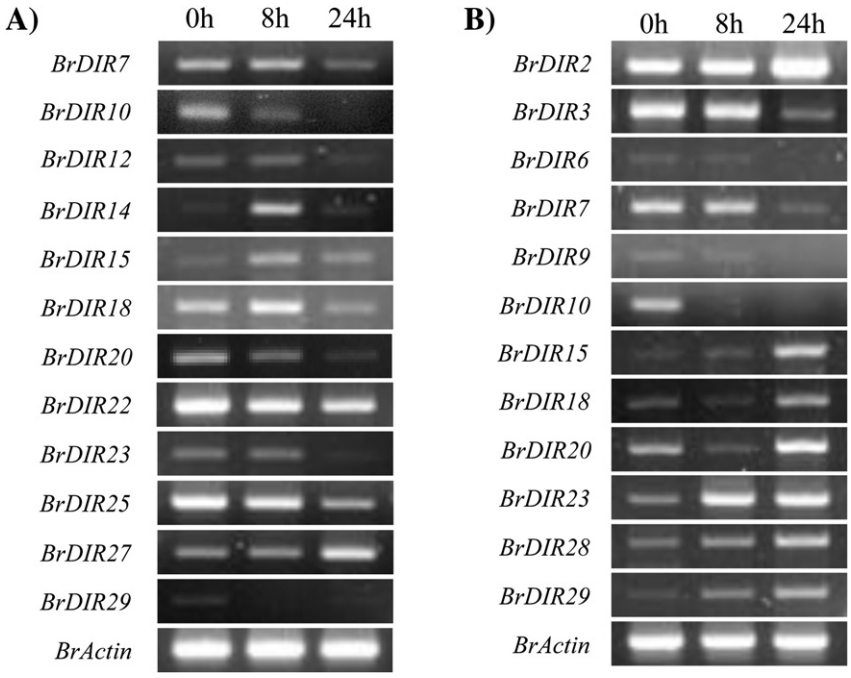

Fig. 4. RT-PCR expression analysis of *BrDIR* like genes after application of (A) cold and (B) ABA stresses treatments in Chinese cabbage.

phylogenetic tree, 29 *BrDIR* like genes were clearly separated into two distinct groups, seven in the DIR-a group and 22 in the DIR-g group (Fig. 7). A previous study demonstrated that only members of the DIR-a family had the ability to direct *in vitro* stereoselective formation of lignans, and members of this group are known as dirigent genes [23]. Conversely, as the biochemical functions of the members of DIR-b/d, DIR-c, DIR-f, and DIR-g subfamilies are not known, these genes are referred to as dirigent like genes [2,23]. Therefore, our *BrDIR2*, *4*, *5*, *10*, *15*, *18* and *23* are dirigent genes (Fig. 1S, Supplementary data) and the remaining 22 *BrDIR* genes are dirigent like genes (Fig. 2S, Supplementary data). The finding that *BrDIR* and *BrDIR* like genes only belong to subfamilies DIR-a and DIR-g suggests that they share a common *DIR* ancestor [2,13,23].

3. Conclusion

DIR genes are very important in enhancing biotic and abiotic stress resistance in *Brassica*. In this study we identified 29 *BrDIR* like genes 2 from our *B. rapa* cv. Osome full length cDNA library and 27 from the *B. rapa* database. Sequence analysis of these genes revealed well connected five characteristics DIR motifs (I–V) and well conserved DIR domains. Comparison study of these genes with other reported DIR genes showed high homology and grouped into two distinct subfamilies such as, DIR-a and DIR-g. In the expression analysis, an organ specific expression of these genes was observed and *BrDIR2* gene showed responses after infection with *F. oxysporum* f.sp. *conglutinans* in cabbage. Highly homologous four *B. oleracea* dirigent like genes of *BrDIR2*, also showed similar responses in this fungus infected cabbage plants. On the other hand, responsive expression of 11 *BrDIRs* in water, 12 *BrDIRs* in ABA and 12 *BrDIRs* in cold stress treatments was observed. Acid soluble lignin content was also quantified in water stressed Chinese cabbage plants and found a probable relation of *BrDIR* genes with lignin biosynthesis. Overall, the results presented herein indicate that *BrDIR* genes of two subfamilies may be involved in resistance of *Brassica* against biotic and abiotic stresses.

4. Materials and methods

4.1. Plant materials

Chinese cabbage (*B. rapa* 'SUN-3061') plants were grown in the Department of Horticulture, Sunchon National University, Korea. Fresh roots, stems, leaves and flower buds of Chinese cabbage were

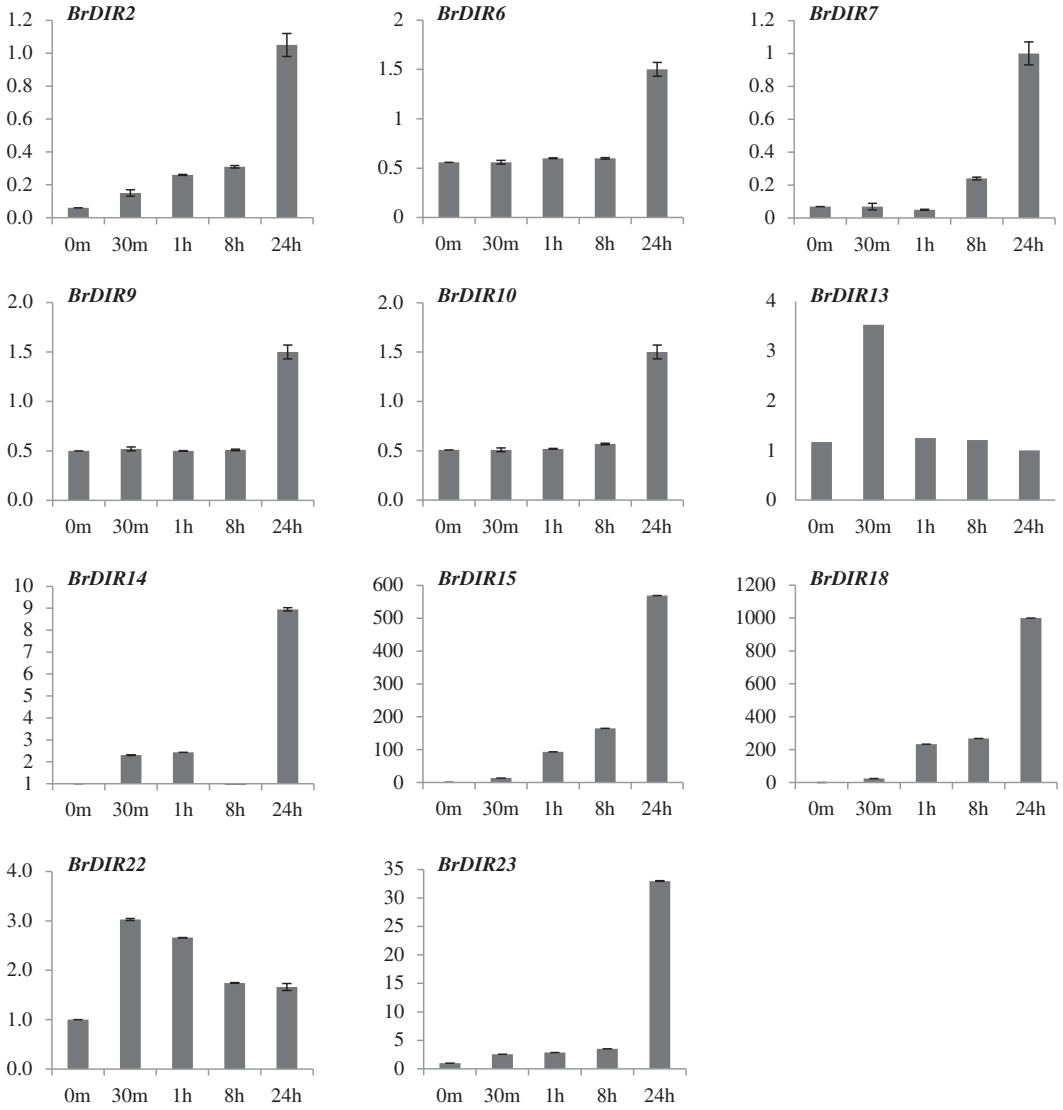

Fig. 5. Real time PCR expression analysis of *BrDIR* like genes after water stress treatment (0–24 h) in Chinese cabbage. The *error bars* represent the standard error of the means of three independent replicates.

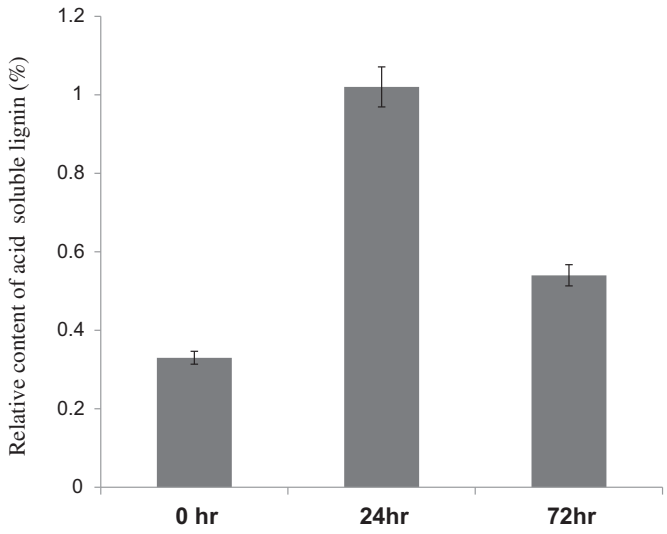

Fig. 6. Acid soluble lignin content in *B. rapa* (whole) plants after water stress treatment at certain time courses (0–72 h).

harvested, frozen immediately in liquid nitrogen, and stored at −80 °C for RNA isolation.

4.2. Sequence and phylogenic analysis

Domain analysis was performed with SMART (http://smart.embl-heidelberg.de/). Conserved motifs were identified using the Consensus tool (www.bork.embl-heidelberg.de/). Predictions for pI and molecular mass were made using the entire ORF and the pI/Mw tool at Expasy (www.expasy.org/tools/pi_tool.html). The subcellular localization of *BrDIR* like genes was predicted using the TargetP v1.01 [25] and PSORT [26] software programs. Since transit peptides are not well conserved, these were truncated prior to phylogenetic analysis using SignalP 3.0 with a neural network model [[36], www.cbs.dtu.dk/services/SignalP/]. All plant *DIR* and *DIR* like sequence were aligned using Dialign (threshold = 0) [[35], http://bioweb.pasteur.fr/seqanal/interfaces/dialign2-simple.html]. Multiple sequence alignment was manually adjusted prior to maximum likelihood analysis. SEQBOOT of the Phylip v3.62 package [37] was used to generate 75 bootstrap replicates for construction of a phylogenetic tree using the neighbor joining algorithm in Mega5

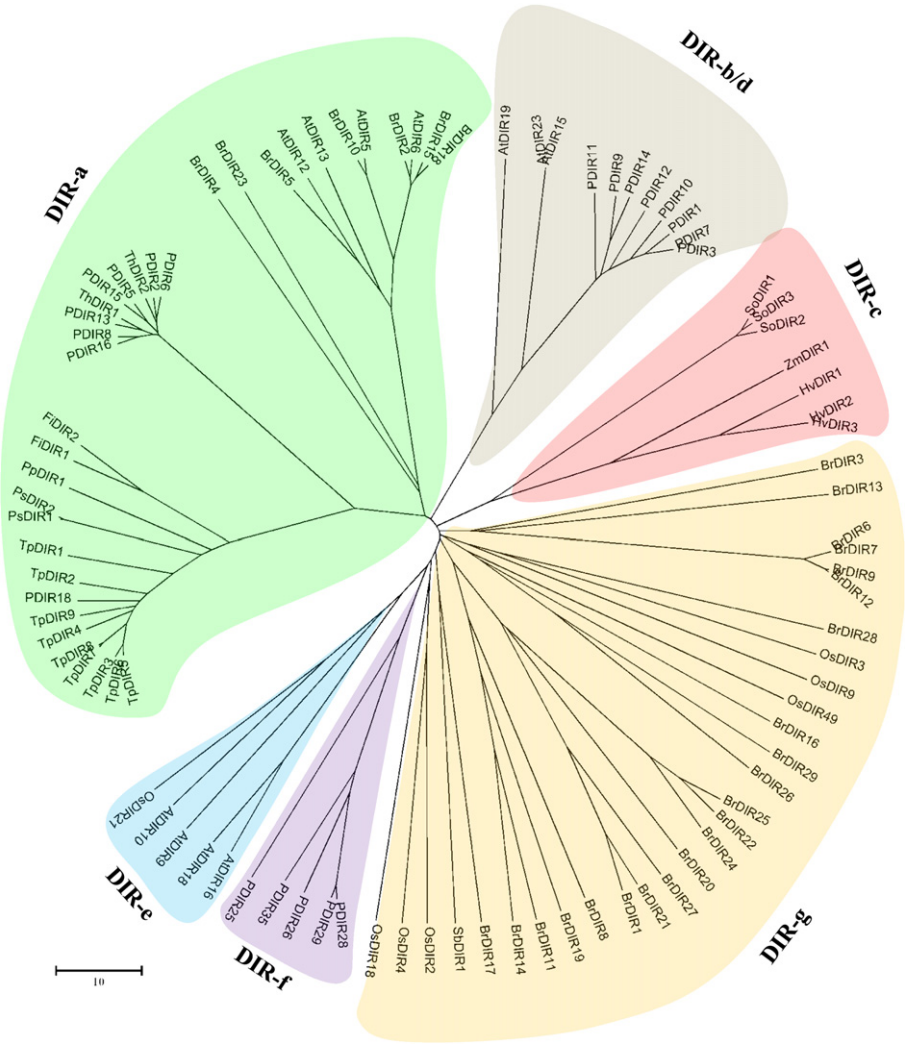

Fig. 7. Phylogenetic tree constructed with *BrDIR* like proteins and *DIR* and *DIR* like proteins of other plant species. Amino acids of 94 *DIR* or *DIR* like proteins were analyzed by maximum likelihood using Mega5. Bootstrap values are only provided for nodes with greater than 50% support. *DIR* nomenclature is as follows: Br, *Brassica rapa*, P, *Picea glauca*, *P. sitchensis* or *P. glaucaengelmannii*; Th, *Tsuga heterophylla*; Tp, *Thuja plicata*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*; So, *Saccharum officinarum*; Ps, *Pisum sativum*; Fi, *Forsythia inter-media*; Pp, *Podophyllum peltatum*.

estimated parameters [38]. Amino acid alignments of all identified *DIR* and *DIR* like family genes were conducted using ClustalW [www.ebi.ac.uk/clustalw/] and Boxshade [http://bioweb.pasteur.fr/seqanal-/interfaces/boxshade.html].

4.3. Biotic stress treatments

The root-dip inoculation (RDI) method was employed for inoculation of cabbage and Chinese cabbage with *F. oxysporum* f.sp. *conglutinans*. Briefly, the fungus was grown on potato dextrose agar (PDA) at 24 °C until conidia appeared. Conidia were then collected from the Petri dish by immersion in sterilized distilled water and removal of the sticky spores from the agar media with sterilized brushes. Suspended conidia were collected with sterilized pipettes and filtered through two layers of sterile Miracloth. The conidia concentrations were measured using a haemocytometer and adjusted to 10^6 conidia ml^{-1} using sterilized distilled water. Three week old seedlings were removed from the soil and immersed in the conidial suspension. While the seedlings were submerged in the suspension, their roots were slightly trimmed using scissors to allow the spores to directly enter the vascular system. After immersion in the conidial suspension for 1 min, the plants were returned to the soil. Control plants were inoculated with sterile water. Seedlings were not watered for 24 h post-inoculation to prevent the inoculums from being washed from the root zone, but were watered with sterile every 48 h following this period. Samples were collected from infected and mock-treated plants at 0, 3, 6, and 24 h, and on days 6, 8, and 11. Upon collection, samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation. The local (fourth) and systemic (fifth) leaves were harvested as samples in all cases.

4.4. Abiotic stress treatments

Chinese cabbage (*B. rapa* 'SUN-3061') seeds were aseptically grown on MS agar medium in a culture room under a 16 h light photoperiod at 25 °C. After three weeks of growth, the seedlings were transferred to fresh liquid MSH (half-strength MS medium without sucrose) medium containing 100 mM abscisic acid (ABA) for 24 h. To induce cold stress, the seedlings were maintained at 4 °C for 24 h. The samples were then subjected to all stress treatments for 0 (wild type), 8 and 24 h. For water stress, plants were transferred to a dish containing water for 0 min, 30 min or 1, 4, 8, or 24 h and then frozen immediately in liquid nitrogen and stored at −80 °C for RNA isolation.

4.5. RNA extraction

Total RNA was extracted from roots, stems, leaves and flower buds of frozen samples using an Rneasy mini kit (Qiagen, USA). RNA was treated with RNase-free DNase (Promega, USA) to remove genomic DNA contaminants. The cDNA was synthesized using the Superscript® III First-Strand synthesis kit (Invitrogen, USA) according to its instructions.

4.6. Expression analysis

RT-PCR was performed using an AMV one step RT-PCR kit (Takara, Japan). Specific primers for all genes were used for RT-PCR and actin primers of *Brassica* were used as a control Table 1S, Supplementary data. The PCR reaction was performed using 50 ng of cDNA from the roots, leaves, stems and flower buds as templates. Briefly, 20 pmol of each primer, 150 μM of each dNTP, 1.2 U of *Taq* polymerase, 1× *Taq* polymerase buffer, and double-distilled H_2O to a total volume of 20 μl were added to 0.5 ml PCR tubes and mixed. The samples were then subjected to initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min.

Real-time PCR (qPCR) was performed using 1 μl of cDNA in a 25 μl reaction volume with iTaq™ SYBR® Green Super-mix with ROX (California, USA). Specific primers for the genes were used to conduct real-time PCR Table 2S, Supplementary data. The thermal cycler conditions were as follows: 10 min at 95 °C, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s. The fluorescent product was detected in the last step of each cycle. Amplification, detection, and data analysis were carried out using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia).

4.7. Measurement of acid-soluble lignin content after water stress treatment

The acid-soluble lignin content was measured using the Klason method [13,39]. After water stress treatment, samples (whole plants) were collected at various times (0, 24, 72 h). The samples were then dried in an oven at 80 °C for 24 h, ground and extracted for 8 h with alcohol. The powder was the air-dried and 12 ml of 72% H_2SO_4 was added per 0.8 g powder. This mixture was then hydrated at 20 °C for 2 h (shaking once every 10 min). Subsequently, 448 ml water was added to the mixture and it was sterilized by heating to 121 °C for 1 h. The mixture was then filtered after cooling and rinsed with water (90 °C) several times to remove the H_2SO_4. Finally, the powder was dried in an oven at 80 °C overnight and weighed. The relative content of acid-soluble lignin was calculated as the percentage of weight of the powder to the dry weight of the plant material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.02.030.

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