Expression Characteristics of *LSH* Genes in *Brassica* Suggest their Applicability for Modification of Leaf Morphology and the Use of their Promoter for Transgenesis

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ABSTRACT The functions of DUF640/ALOG (*Arabidopsis LSH1* and *Oryza G1*) domain proteins, which are found in most land plants, have not been well characterized, but some of these proteins regulate inflorescence architecture in rice and specify organ boundaries in *Arabidopsis*. *Arabidopsis* DUF640-domain genes are initially identified as *LIGHT-SENSITIVE HYPOCOTYLS (LSH)* genes. Chinese cabbage leaves have large, white midribs and photosynthetic leaf blades (or lamina). A DUF640 domain gene of *Brassica rapa*, *BrLSH2*, is specifically expressed in the midrib of Chinese cabbage. *Arabidopsis* and rice possess ten *LSH* family genes, but *B. rapa* has 24 *LSH* genes, which can be categorized into two or four groups based on sequence identity. Here, we examined the expression patterns of the *LSHs* in various *Brassica* species and analyzed the promoter sequence of the *BrLSH2* gene. The transcript levels of most *LSH* genes were very high in the midrib but low in the leaf blade. These genes were evenly expressed throughout the petiole region of Korean cabbage and highly expressed in the leaf base region near the stem and in the border area in *B. oleracea*. In addition, *BrLSHs* were expressed in both bundle and mesophyll cells of the midrib. These expression patterns suggest the possible use of these genes to generate leafy vegetables with altered leaf morphology. The *BrLSH2* promoter, which contains auxin- and cytokinin-responsive elements as well as leaf development-related elements, may confer midrib-specific expression, suggesting that this promoter may be useful for the production of midrib-targeted transgenic Chinese cabbage.

Keywords ALOG, DUF640, Leaf boundary, Leaf morphology, Midrib

INTRODUCTION

DUF640 (domain of unknown function 640) domain genes, or ALOG (*Arabidopsis LSH1* and *Oryza G1*) family genes, are found in land plants. DUF640 (InterPro: IPR006936) is also found in plant proteins including resistance protein-like protein. Ten ALOG family genes are present in rice and *Arabidopsis* (Yoshida *et al.* 2009; Cho *et al.* 2011). All members of this family have a highly conserved region and a nuclear localization signal (NLS), KKRK, flanking the C-terminal region of this domain. Although the precise function of the DUF640 motif (or ALOG) is still unclear, the roles of some of these genes have been studied in rice, *Arabidopsis* and tomato. ALOG

genes regulate inflorescence architecture in rice and tomato (Yoshida *et al.* 2009, 2013; MacAlister *et al.* 2012; Yan *et al.* 2013), while they determine organ boundaries and influence light sensitivity in *Arabidopsis* (Zhao *et al.* 2004; Cho and Zambryski 2011; Takeda *et al.* 2011).

Rice *LONG STERILE LEMMA (G1)*, the first identified ALOG gene from monocots, specifies the identity of the sterile lemma by repressing lemma identity via the regulation of downstream target genes (Yoshida *et al.* 2009). Another rice DUF640 domain gene, *TRIANGULAR HULL1/BEAK-SHAPED GRAIN 1 (TH1/BSG1)*, determines grain shape and size by regulating cell division and extension of the lemma and palea (Li *et al.* 2012; Yan *et al.* 2013). *TAWAWAI (TAWI)* encodes an ALOG family protein in

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rice and regulates inflorescence architecture, partly through promoting the expression of *SHORT VEGETATIVE PHASE* (*SVP*)-like genes (Yoshida *et al.* 2013). Tomato *TERMINATING FLOWER* (*TMF*), like rice *TAW1*, affects inflorescence organization (MacAlister *et al.* 2012), possibly by preventing the early expression of orthologous genes of *Arabidopsis* *UNUSUAL FLORAL ORGANS* (*UFO*), *LEAFY* (*LFY*), *APETALLA 1* (*API*) and *SEPALLATA* (*SEP*), which contribute to promoting floral fate (Teo *et al.* 2014).

Arabidopsis contains ten *ALOG* or *LSH* (*LIGHT-SENSITIVE HYPOCOTYLS*) genes. *LSH1* was first identified as *Light-dependent Short Hypocotyl 1* (Zhao *et al.* 2004) and later renamed “Light-Sensitive Hypocotyls”. *LSH1* is expressed in hypocotyls, shoot apices and lateral root primordia in *Arabidopsis*, and its overexpression leads to the hypersensitive response to various light wavelengths, with plants exhibiting short hypocotyls and enlarged cotyledons (Zhao *et al.* 2004). *LSH3* and *LSH4*, known as *ORGAN BOUNDARY 1* (*OBO1*) and *OBO4*, are *Arabidopsis* *ALOG* family genes that are expressed at the boundary of the shoot apical meristem (SAM) and lateral organs (Cho *et al.* 2011; Takeda *et al.* 2011). *LSH3* and *LSH4* are activated by *CUP-SHAPED COTYLDON 1* (*CUC1*) (Takeda *et al.* 2011) and might interact with *UFO*; constitutive expression of *LSH3* and *LSH4* partially mimics the phenotype exhibited by mutations in *UFO* [similar to the interaction between *TMF* and *ANANTHA* (*AN*), an ortholog of *Arabidopsis* *UFO*] (Teo *et al.* 2014). Taken together, these observations suggest that

ALOG family proteins are important regulators that affect inflorescence architecture through mediating the transition status of SAM differentiation due to their roles in maintaining meristem indeterminacy and suppressing floral identity, together with the unique expression patterns of *LSH* and *TMF* genes (Teo *et al.* 2014).

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is one of the most important leafy vegetables in Asian countries including Korea. Compared to other plants, the leaf morphology of Chinese cabbage is quite peculiar, comprising a large midrib with a leaf blade but lacking a petiole when mature (Fig. 1A). The outer leaf of mature Chinese cabbage, i.e., the leaf blade and midrib, can be divided into source and sink tissues, respectively, with respect to photosynthesis. The *B. rapa* *LSH2* gene (Brapa_ESTC024477 for Br300K array = Bra040188 for <http://brassicadb.org/brad/>) is specifically expressed in midribs throughout the day (Fig. 1B). In the current study, we examined the expression patterns of *LSH* family genes in various *Brassica* species. In addition, we discuss the possible application of these genes to biotechnology.

MATERIALS AND METHODS

Plant materials

Seeds of the *Brassica rapa* inbred lines ‘Chiifu’ and ‘Kenshin’ were obtained from Korea *Brassica rapa* Genome

Fig. 1. Leaf morphology of Chinese cabbage (A) and levels of *LIGHT-SENSITIVE HYPOCOTYLS* (*LSH*)2 transcripts measured throughout the day (B). Transcript levels were obtained from *B. rapa* inbred line ‘Chiifu’ using a Br300K microarray (Dong *et al.* 2013). Probe intensity (hybridization) values are shown at the top of the graph.

Resource Bank (KBGRB), and other seeds were purchased from a local market. The plants were grown in a greenhouse at Chungnam National University. Leaf tissues were sampled, frozen in liquid nitrogen and stored at -70°C until use.

RNA isolation and microarray analysis

Total RNA was isolated from midrib and leaf blade tissues using an Easy-BLUE™ Total RNA Extraction Kit (Invitrogen, U.S.A.) and purified using an RNeasy MinElute™ Cleanup Kit (Qiagen, Germany). Microarray experiments were carried out as described by Dong *et al.* (2013); the full results of the analysis have not yet been published. To assess the reproducibility of the microarray analysis, the

experiment was repeated twice with total RNAs from independent cultures and treatments. Normality of Cy3 intensities was tested with the qqline function in R Statistical Software. Data were subsequently normalized with cubic spline normalization using quantiles to adjust signal variations among chips from the robust multi-chip analysis and a median polish algorithm implemented in NimbleScan. Finally, the average value from the perfect match (PM) values of six probes was used to select responsive genes.

Semi-quantitative RT-PCR analysis

The total RNA from each plant sample amounting to 5 µg was combined with random hexamer primers in a Super Script first-strand cDNA synthesis system according to the

Table 1. List of DUF640 superfamily (*LSH: LIGHT-SENSITIVE HYPOCOTYLS*) genes from *Arabidopsis*, *B. rapa* and *B. oleracea*. Numbers 1, 2 and 3 after the gene name indicate the highest to lowest identity to *Arabidopsis* sequences. *Br_Locus* and *Bo_Locus* (Bol) were obtained by BlastN at <http://brassicadb.org/brad/> against the *Arabidopsis* sequence. RNA_Seq Id of *B. oleracea* are unpublished data.

<i>Arabidopsis thaliana</i>		<i>Brassica rapa</i>		<i>Brassica oleracea</i>		
Gene name	<i>At_Locus</i>	Gene name	<i>Br_Locus</i>	Gene name	<i>Bo_Locus</i>	RNA_Seq Id
<i>AtLSH1</i>	AT5G28490	<i>BrLSH1-1</i>	Bra020610	<i>BoLSH1-1</i>	-	Locus_22975
		<i>BrLSH1-2</i>	Bra009997	<i>BoLSH1-2</i>	-	-
		<i>BrLSH1-3</i>	Bra036134	<i>BoLSH1-3</i>	Bol032364	Locus_36030
<i>AtLSH2</i>	AT3G04510	<i>BrLSH2-1</i>	Bra040188	<i>BoLSH2-1</i>	-	Locus_48663
		<i>BrLSH2-2</i>	Bra040137	<i>BoLSH2-2</i>	-	Locus_27811/Locus_52336
		<i>BrLSH2-3</i>	Bra001109	-	-	-
<i>AtLSH3</i>	AT2G31160	<i>BrLSH3-1</i>	Bra018263	<i>BoLSH3-1</i>	Bol008257	Locus_49179
		<i>BrLSH3-2</i>	Bra021707	<i>BoLSH3-2</i>	-	Locus_10104
<i>AtLSH4</i>	AT3G23290	<i>BrLSH4-1</i>	Bra014935	<i>BoLSH4-1</i>	Bol037267	Locus_9116
		<i>BrLSH4-2</i>	Bra023738	<i>BoLSH4-2</i>	Bol028747	Locus_19519
<i>AtLSH5</i>	AT5G58500	<i>BrLSH5-1</i>	Bra020365	<i>BoLSH5-1</i>	Bol015532	Locus_16670
		<i>BrLSH5-2</i>	Bra002642	<i>BoLSH5-2</i>	Bol016895	-
		<i>BrLSH5-3</i>	Bra002644	<i>BoLSH5-3</i>	Bol016897	-
<i>AtLSH6</i>	AT1G07090	<i>BrLSH6-1</i>	Bra015551	<i>BoLSH6-1</i>	Bol041101	Locus_13192
		<i>BrLSH6-2</i>	Bra031552	<i>BoLSH6-2</i>	-	Locus_21750
<i>AtLSH7</i>	AT1G78815	<i>BrLSH7-1</i>	Bra035066	<i>BoLSH7-1</i>	Bol038483	Locus_51379
		<i>BrLSH7-2</i>	Bra008382	<i>BoLSH7-2</i>	Bol021549	Locus_15825
		<i>BrLSH7-3</i>	Bra003637	<i>BoLSH7-3</i>	-	-
<i>AtLSH8</i>	AT1G16910	-	-	-	-	-
<i>AtLSH9</i>	AT4G18610	<i>BrLSH9-1</i>	Bra021000	<i>BoLSH8-1</i>	Bol009398	Locus_39945
		<i>BrLSH9-2</i>	Bra013330	<i>BoLSH8-2</i>	Bol029905	Locus_9841
		<i>BrLSH9-3</i>	Bra012584	<i>BoLSH8-3</i>	Bol024421	Locus_14109
<i>AtLSH10</i>	AT2G42610	<i>BrLSH10-1</i>	Bra016865	<i>BoLSH10-1</i>	-	Locus_52096/Locus_40164/ Locus_55288
		<i>BrLSH10-2</i>	Bra000269	<i>BoLSH10-2</i>	-	Locus_25548/Locus_27130
		<i>BrLSH10-3</i>	Bra004711	-	-	-

manufacturer's instructions (Invitrogen, U.S.A.). Complementary DNA was diluted 10-fold, and 1 µl of the diluted cDNA was used in each 20 µl PCR mixture. RT-PCR primers were designed using sequence information from *Arabidopsis thaliana*, *B. rapa* and *B. oleracea* (Table 1). Primer sequences used in this experiment are listed in Table 2, and the primer set used for the control was designed based on *B.*

rapa actin1 (*BrACT1*) (Forward sequence = 5'-ACACC-ATGATGTCTTGGCCTACCA and reverse sequence = 5'-AATGGTACCGGAATGGTCAAGGCT). Standard PCR was performed, with 5 min denaturation at 94°C followed by 25 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 60 s. The PCR products were analyzed following electrophoresis on a 1.5% agarose gel.

Table 2. Primer sequences used in RT-PCR experiments. Three to four reverse primer sequences for *LIGHT-SENSITIVE HYPOCOTYLS (LSH)* genes (except *LSH8*) were used to distinguish alleles, but one common forward primer was used. Among reverse primers, LSH-R primers were used to amplify all *LSH* genes from any *Brassica* species. LSH-R primers were designed with identical sequences among *Arabidopsis*, *B. rapa* and *B. oleracea*. However, BrLSH primers were specific for each allele of each *LSH* gene (to distinguish each allele). LSH8 primers were designed based on *Arabidopsis* and *Eutrema salsugineum* genes.

Gene name	Name	Forward primer Sequence (5'→3')	Name	Reverse primer Sequence (5'→3')
<i>LSH1</i>	LSH1-F	CCGCCTACGAGGAGAACGGT	LSH1-R	TTGCACCCGAGTAATTAGCCATC
			BrLSH1-1	CGGCTGCGTCTGCGATAG
			BrLSH1-2	GCTGCTGTTCTTGTGTGTGCTGC
			BrLSH1-3	GTGGCTGCGTCTGCGGTAA
<i>LSH2</i>	LSH2-F	GAYTGGAACACTTTCTGCCAATACCT	LSH2-R	TCCACCGTCTCCTCGTAGGC
			BrLSH2-1	GGCCTTGCTGTTGCTGCTCTA
			BrLSH2-2	CTTCTCTCTCATGCTGATGCTGTTGT
			BrLSH2-3	TGTAGAGGCGGCTGCAAACAC
<i>LSH3</i>	LSH3-F	TCGGTAAGACAAAAGTCCACACGA	LSH3-R	CTAGCTACGGCGGAGGAAGAAGA
			BrLSH3-1	TCACTTCTCAAGGTTTGATATTTGGG
			BrLSH3-2	GAGGCCGACAACCTGAGGAACC
<i>LSH4</i>	LSH4-F	RTTYCTCAGGTACCTCGACCAATT	LSH4-R	CACGYGCTTTAGCCTGCGA
			BrLSH4-1	TTAGGGCTACTGGAATCACCGAG
			BrLSH4-2	CTATTCGAAATCCCCGGCTGA
<i>LSH5</i>	LSH5-F	AGTACCTCGACCAGTTTGGTAAGACC	LSH5-R	AAGGGTTTGACTCNGGAAGACCAC
			BrLSH5-1	TCCATCATCGACGTTGGGAGTT
			BrLSH5-2	CTCCATCATCGGCGTTTAGAGTC
			BrLSH5-3	ATCCATCATGGGGATTTTGAGCT
<i>LSH6</i>	LSH6-F	CGTTCTTGCAGTACCTCAAGAACC	LSH6-R	GACTYTCTCTGACTTCYCTCAAGTAAATCC
			BrLSH6-1	TCACGGTGGTAACAGTTGTCGGT
			BrLSH6-2	CGGTGGTGACAGTTGGTGCC
<i>LSH7</i>	LSH7-F	ATGGCTAGTCMTAGCAACAAAGGC	LSH7-R	CTTSGCCTGTGAATCTCTCACTTC
			BrLSH7-1	CAAGTTGGAGCTACTAGTCCCTGTAGTAC
			BrLSH7-2	GTCCCTGCAGTACCATCTTCAACAT
			BrLSH7-3	GCTACTAGTCCCCGTAGTATCATCTTCAA
<i>LSH8</i>	LSH8-F	GCCGMGACTGGAACACGTTT	LSH8-R	AGCCGTCCGATCAAAGCATC
<i>LSH9</i>	LSH9-F	CAAGCTTGTGTCTTCTTCGGACAA	LSH9-R	CACGAGCCTTGCTTGAGATT
			BrLSH9-1	CCTGTCTTTTTCTGTACGGAATC
			BrLSH9-2	TCTCCTCTTCTTCTTCTGTACGAAATT
			BrLSH9-3	CCTCCTCTTCTTCTTCTGTAAAGGTATC
<i>LSH10</i>	LSH10-F	ATCATCAGGATCAGAGCCACCGGT	LSH10-R	GCCTGACACTCCCTAACCTCCC
			BrLSH10-1	CCACCACTCATCTCCGTCTTTG
			BrLSH10-2	CAACCATCTCCGTAGTTGGCTG
			BrLSH10-3	ACCCATCTCCGTGCTCGTTG

Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignments were conducted using ClustalX2.0 (Larkin *et al.* 2007). The phylogenetic tree, based on the *LSH* coding sequences (CDs), was constructed using the neighbor-joining (NJ) method. NJ trees were constructed using MEGA6 (<http://www.megasoftware.net/>) with the 'pairwise deletion' option and 'Kimura 2-Parameter' model, with a bootstrap test of 1,000 replicates (Tamura *et al.* 2013).

Promoter analysis

To analyze *cis*-acting elements of *BrLSH2*, a ca. 3,200 bp sequence upstream of the *LSH2* ATG start codon, which

was obtained from BRAD (<http://brassicadb.org/>), was analyzed with PlantPAN (Chang *et al.* 2008) and PLACE (Higo *et al.* 1999). Several important motifs are indicated in the figure.

RESULTS

LSH genes from *B. rapa* and *B. oleracea*

A total of 24 *B. rapa* *LSH* (*BrLSH*) genes corresponding to ten *Arabidopsis* genes were found in the BRAD database (<http://brassicadb.org/brad/>), with no ortholog of *AtLSH8* detected (Table 1). A similar number of *LSH* genes may be present in another *Brassica* species, *B. oleracea*. To

Fig. 2. Phylogenetic relationships of *LIGHT-SENSITIVE HYPOCOTYLS* (*LSH*) gene families from several species. Trees were constructed using the NJ (neighbor-joining) method in MEGA (version 6.0). Red squares indicate genes from *Arabidopsis*. Br and Bo stand for *B. rapa* and *B. oleracea*, respectively. Numbers 1 to 10 indicate that the *LSH* gene families can be classified into ten groups.

examine the relationship among the members of the *LSH* multigene family, we constructed an NJ tree using ClustalX2.0 and MEGA6 software (Larkin *et al.* 2007; Tamura *et al.* 2013) (Fig. 1). As shown in Figure 1, all *LSH* family genes could be divided into two large groups; one group includes *LSH1*, 2, 3 and 4, while the other includes *LSH5*, 6, 7, 8, 9 and 10. The genes were further divided into four groups: *LSH1* and 2; *LSH3* and 4; *LSH5* and 6; and *LSH7*, 8, 9 and 10. This classification reflects both the functional redundancy and divergence of the *LSH* gene family.

Expression of *LSH* genes from various *Brassica* species

To examine the expression characteristics of *LSH* genes

from diverse *Brassica* species, we performed RT-PCR analysis with a common primer set and an allele-specific primer set for each *LSH* gene of *B. rapa* (Table 2 and Fig. 3). The common primer set could be applied to all *Brassica* species because it was designed from a highly conserved region among *Brassica* genes, including *Arabidopsis* sequences. As shown in Figure 3, *LSH* genes were specifically induced in the midribs of all *Brassica* species examined. However, *LSH5-2*, *LSH5-3*, *LSH7s* and *LSH9s* were expressed at very low levels. Unexpectedly, although *LSH8* is not present in the *B. rapa* genome, we detected *LSH8* transcripts in this species, implying possible contamination of other *LSH* gene products, such as *LSH7*, which is most closely related to *LSH8* (Fig. 2).

Fig. 3. Expression levels of *LIGHT-SENSITIVE HYPOCOTYLS (LSH)* genes in midrib (M) and leaf blade (L) tissues of various *Brassica* species. Abbreviations are as follows: Chiifu (*Brassica rapa* ssp. *pekinensis*, DH line Chiifu), Kenshin (*Brassica rapa* ssp. *pekinensis*, DH line Kenshin), Pakchoi (*Brassica rapa* ssp. *chinensis*), Brapha (*Brassicoraphanus*), Kc (Korean cabbage), Bo-10 (*B. oleracea* 10 days old), M (midrib) and L (leaf blade). Gene names without *Br* indicate transcripts amplified by common primer sets.

To investigate whether the expression of *LSH* genes occurs in photosynthetic or sink tissues, various tissues of *B. rapa* were subjected to RT-PCR analysis (Fig. 4). Outer leaves, middle leaves and inner non-photosynthetic leaves from 'Heissen' (F_1 Korean cultivar) and green cabbage (F_1 Chinese cultivar) were sampled when they formed heads. In addition, the midribs of 'Heissen' leaves were further dissected into vascular bundles and mesophyll cells. *LSH* genes were predominantly expressed in midribs from all parts of the plant. There was no difference in *LSH* gene expression between mesophyll cells and vascular bundles, while *LSH6* was more highly expressed in vascular bundles than in mesophyll cells. Detection of *LSH* transcripts in blades may have been due to the presence of veins in this tissue or PCR cycles. These results indicate that the expression of *LSH* is not related to the photosynthetic status of the tissue but is, instead, midrib-specific.

To examine *LSH* gene expression in different parts of the petiole, we performed RT-PCR analysis of Korean cabbage leaves, which have relatively long petiole-like midribs (Fig. 5). As shown in Figure 5, *LSH* genes were

evenly expressed throughout all areas of the petiole region but not in the blade. The levels of *LSH3* and *LSH9* expression were relatively low, indicating that different *LSH* genes are expressed at different levels in various *Brassica* species.

Expression of *LSH* genes in *B. oleracea*

Changes in leaf morphology in *B. oleracea* are quite dramatic throughout development, ranging from long petioles in young seedlings to blade-like petioles to no petiole-like structures during head formation (left panel of Fig. 6). The expression levels of *LSHs* were high in the midrib and petiole and relatively low in the leaf blade. The most interesting finding was that we could detect expression of *LSHs* in both the midrib and leaf blade in samples harvested from the bottom halves of leaves. In particular, *LSH1* and *LSH2* were highly expressed in the 6th to 13th leaf blade portions (*Bo-2L*, *Bo-3L* and *Bo-4L*) in the bottom halves of leaves. These results indicate that the *LSH* genes are highly expressed in the leaf base region near the stem or border area in *B. oleracea*.

Fig. 6. Expression of *LIGHT-SENSITIVE HYPOCOTYLS (LSH)* genes in various *B. oleracea* tissues, beginning at the heading stage. Midrib (M) and leaf blade (L) tissues were sampled from 20-day-old *B. oleracea* plants (Bo-M, L, no photograph). *Bo1*, *Bo2*, *Bo3* and *Bo4* indicate 3rd, 6th, 10th and 13th leaves of 80-day-old plants (top middle), respectively. The sampled parts are indicated in the photographs.

To further dissect the gene expression patterns in *B. oleracea*, we separated a cabbage leaf into 14 sections and subjected the sections to RT-PCR analysis (Fig. 7). All *LSH* genes were highly expressed in the midrib, and the expression of most of the genes was relatively high in the basal parts of the leaf blade. Only low or very low transcript levels were detected in parts 9 and 10. Compared to *B. rapa*, cabbage leaves contain relatively large veins derived from the midrib, which may affect the expression of *LSH*. These results, along with those shown in Figure 6, indicate that *LSH* genes are highly expressed in the basal parts of cabbage leaves.

Analysis of the *BrLSH2* gene promoter

To understand why *LSH* genes were specifically expressed in the midrib, we analyzed *cis*-acting elements in the *B. rapa LSH2* gene (*BrLSH2*) promoter, which shows the most prominent expression in the midrib compared to the leaf blade. The region 3,159 nucleotides upstream of *BrLSH2* contains 527 motifs corresponding to 67 transcription factors (data not shown). Among these, we identified the binding sites of eight transcription factors (auxin response: *ARF* and *ARFAT*, cytokinin response: *ARR10* and *ARR1AT*, leaf development: *ATHB1*, *ATHB1AT* consensus, *ATHB2* and *ATHB9*), whose positions are shown in Figure 8. These

Fig. 7. Expression of *LIGHT-SENSITIVE HYPOCOTYLS (LSH)* genes in different parts of a cabbage leaf. Samples were selected from 14 parts of a leaf, as indicated in the photograph.

Fig. 8. Organization of *cis*-elements in the promoter region of *BrLSH2* (Bra040188). *Cis*-elements were identified using PlantPAN (Chang *et al.* 2008) and PLACE (Higo *et al.* 1999).

elements appear to be associated with leaf development or differentiation. Except for *ATGB-2* and *ATHBIAT* consensus, most elements are present at multiple sites. All *cis*-elements are present within 1,700 nt of the ATG start codon, indicating that this region may confer midrib-specific expression of the *BrLSH2* gene.

DISCUSSION

Functions of DUF640 domain proteins

Although the precise functions of DUF640 domain (or ALOG) proteins have not yet been defined, there are four possible roles for these proteins based on published information, as follows: regulation of inflorescence architecture, determination of organ identity and differentiation, transport of RNA, and sensing of invading DNA. *ALOG* genes control inflorescence architecture in rice and tomato (Yoshida *et al.* 2009, 2013; Li *et al.* 2012; MacAlister *et al.* 2012; Yan *et al.* 2013; Teo *et al.* 2014). *Arabidopsis* genes are expressed at the boundary region of the SAM and lateral organs, indicating their function in organ identity (Cho *et al.* 2011; Takeda *et al.* 2011). Moreover, sequence analysis of ALOG domains suggests that they help establish organ identity and differentiation by binding to specific DNA sequences and acting as transcription factors or recruiters of repressive chromatin (Iyer and Aravind 2012). The current expression data also support the notion that all *LSH* genes play a role in leaf formation and in determining the boundary between the leaf and the stem.

Solanum tuberosum LSH10 (*StLSH10*) encodes an RNA-binding protein (B5RBP3) that binds to the 3'UTR of *StBEL5*, a mobile RNA that controls tuber formation (Cho *et al.* 2012; Lin *et al.* 2013). *StBEL5* RNA is highly abundant in petioles, where it moves long distances through the phloem (Cho *et al.* 2012). RBPs are commonly detected in companion cells and sieve elements of leaf veins where they serve as chaperones of mobile RNAs. The RNA levels of *B5RBP3* (*StLSH10*) are remarkably low in leaves but extremely high in petioles, stolons and young tubers, suggesting that this protein plays a role in RNA transport as well as tuber development (Cho *et al.* 2012). The expression patterns of *LSH* genes detected in the

current study, i.e., high levels of *LSH* transcripts in petioles and low levels in leaf blades, suggest that *Brassica* LSHs function in long-distance RNA transport. However, the levels of *BrLSHs* transcripts were high in both bundle sheath cells and mesophyll cells of the midrib (Fig. 4), suggesting that *BrLSHs* play different roles from those of potato LSH10.

The ALOG domain is present in certain plant defense proteins, and domain analysis can help predict whether proteins might function as DNA sensors to detect invading DNA (Iyer and Aranid 2012). Based on the expression data, we cannot yet determine whether *Brassica* LSHs also participate in this defense response.

Midrib development

Midrib, the largest vein in a leaf, runs through the middle of the leaf and sometimes becomes the petiole. The midrib helps keep the leaf in an upright position and conducts foods and water *via* its vascular bundles. Several recent reviews have focused on the development of leaf morphology (Byrne 2012; Fambrini and Pugliesi 2013), but no study on midrib development in dicots has been reported. Several such studies have been performed in monocotyledons, such as rice and sorghum. In rice, the well-studied *DROOPING LEAF* (*DL*) gene regulates midrib development (Yamaguchi *et al.* 2004; Ohmori *et al.* 2011). Moreover, the morphologies of the leaves of several *Arabidopsis* mutants are similar to that of mature Chinese cabbage leaves, including plants harboring mutations in *LEAFY PETIOLE* (*LEP*) (van der Graaff *et al.* 2000), *JAGGED* (*JAG*) (Ohno *et al.* 2004), *BLADE-ON-PETION1* (*BOP1*) and *BOP2* (Ha *et al.* 2003, 2007; Hepworth *et al.* 2005). In the current study, the expression pattern of *BOP1* resembled that of *LSH* (data not shown), suggesting a possible interaction between these genes. Perhaps, LSHs interact with known gene products that regulate leaf shape and development.

Recent work has revealed that *CUC1* activates the expression of *LSH3* and *LSH4*, which are specifically expressed in the boundary cells of various shoot organs, such as cotyledons, leaves and floral organs (Takeda *et al.* 2011), indicating that *CUC1* is an upstream transcription factor of *LSH3* and *LSH4*. Therefore, further studies should focus on identifying other upstream genes, as well as

downstream genes, of *LSHs*.

Promoter analysis and possible applications

As shown in Figure 8, major transcription factor-binding sites are related to the auxin response, the cytokinin response and leaf development. The plant hormone auxin affects plant growth and differentiation. The meristem-active regulatory genes *WUSCHEL* (*WUS*) and *SHOOTERMISTEMLESS* (*STM*), which contain cytokinin-response regulator binding motifs (ARR1AT) and auxin-responsive elements, are highly expressed in leaf veins (Bao *et al.* 2009), indicating that these elements may be related to midrib specificity or midrib differentiation. *ATHB1* and *ATHB2* are involved in the regulation of leaf development (Aoyama *et al.* 1995) and the adaxial identity of subsequently formed organs (Turchi *et al.* 2013), respectively. These observations suggest that the region 1,700 nt upstream of the ATG start codon of *BrLSH2* may contain a *cis*-element required for leaf boundary formation or identity that can be used as a midrib-specific promoter.

Bacterial soft rot is the most severe and destructive disease of various crops including *Brassica* family members. Chinese cabbage is highly susceptible to soft rot disease caused by the Gram-negative bacterium *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (Ren *et al.* 2001; Zhang *et al.* 2007). Infection tests have largely been carried out by midrib inoculation of *Brassica* (Vanjildorj *et al.* 2009; Park *et al.* 2012), because infection starts at the stem or petiole. The analysis of the *BrLSH2* promoter performed in the current study suggests that this promoter can be used for the generation of transgenic plants exhibiting soft rot resistance. We are currently analyzing the *BrLSH2* promoter and the up- and down-stream of genes of *LSHs*.

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