***Brassica rapa* expansin-like B1 gene (*BrEXLB1*) regulate growth and development in transgenic Arabidopsis and elicits response to abiotic stresses**

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Abstract

The functional role of expansin-like A and B family members remains unclear in plants. In this study, we investigate the functional role of *Brassica rapa* expansin-like B1 (*BrEXLB1*) by overexpressing *BrEXLB1* sense, antisense and *BrEXLB1* specific promoter: *GUS* in Arabidopsis. The overexpressors of sense *BrEXLB1*, antisense *BrEXLB1* showed variable and unstable pleiotropic effects on leaf growth. Interestingly, overexpressors of sense *BrEXLB1* enhances plant height than antisense overexpressors and wild types. *GUS* reporter-aided analysis of *BrEXLB1* promoter showed their activity prominently in seeds, roots and carpels. This is further confirmed by relative quantification of *BrEXLB1* among various tissue samples of *B. rapa* by semi-quantitative Reverse transcription-polymerase chain reaction assay (RT-PCR). We found that *BrEXLB1* promoter has several cis-acting elements including light-responsive, phytohormone-responsive, environmental/stress-responsive and tissue-specific elements in promoter sequences. Hence, we analysed the promoter activity of *BrEXLB1* by *GUS* reporter-aided approach, in which stress, phytohormones and other factors regulated changes in *GUS* transcripts were measured using quantitative RT-PCR. This study suggests that promoter activity is inducible to exogenous application of phytohormones such as indole-3-acetic acid, jasmonic acid, and other factors such as white light and drought stress. These results suggest that *BrEXLB1* under its specific promoters may participate in regulation of leaf, plant growth and responds to hormone availability, light quality, dark periods, developmental stages, and drought conditions.

Keywords *Brassica rapa* · Expansin-like B1 gene · Plant growth and development · *GUS* · Promoter analysis · Transgenic Arabidopsis

Abbreviations

<i>BrEXLB1</i>	<i>Brassica rapa</i> expansin-like B1 gene
GUS	β-Glucuronidase
IAA	Indole acetic acid
GA	Gibberellic acid
JA	Jasmonic acid
SA	Salicylic acid
ABA	Abscissic acid
qRT-PCR	Quantitative reverse-transcription PCR

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Introduction

Expansins are cell wall proteins, present in all plants, play crucial role in cell wall remodeling (Marowa et al. 2016). Plant expansins are necessary for growth and development as it helps the plants to uptake water, cell stress relaxation

and or cell enlargement through its cell wall loosening activity (Cosgrove 2001; Sasidharan et al. 2011). It is a non-enzymatic proteins that extend cell walls by weakening non-covalent linkages between cellulose microfibrils and xyloglucans (Santiago et al. 2018; Wang et al. 2013). Based on the phylogenetic relationships, plant expansins are classified into four families: α -expansin (EXPA), β -expansin (EXPB), expansin-like A (EXLA), and expansin-like B (EXLB) proteins (Kende et al. 2004). Members of EXPA and EXPB families have been clearly shown to be involved in most plant growth and developmental processes, whereas EXLA and EXLB proteins are only known from their homologous conserved gene sequences and their functions yet to be discovered (Cosgrove 2015).

Following initial investigations of expansins in cucumber hypocotyls (McQueen-Mason and Cosgrove 1994; McQueen-Mason et al. 1992), numerous studies have shown that expansins are involved in all cell wall modification processes, from abscission (Cho and Cosgrove 2000), seed number increase (Bae et al. 2014), root formation (Greenwood et al. 2006), fruit ripening (Brummell et al. 1999), plant architecture definition (Dal Santo et al. 2011) to plant-microbe interactions (Wieczorek et al. 2006). Although the exact mechanism underlying expansin activity is not fully understood (Cosgrove 2015), several studies have successfully correlated the involvement of expansins with leaf growth and development using sense and antisense transgenic plants. Examples in *Arabidopsis* include overexpression of *NtEXPA5* increasing leaf and stem sizes (Kuluev et al. 2013), sense and antisense overexpression of *AtEXPA10* effectively altering leaf and petiole sizes (Cho and Cosgrove 2000), and overexpression of *AtEXPA10* and *PnEXPA1* producing larger leaves and longer stems (Kuluev et al. 2012). Overexpression of *NtEXPA1* increases tobacco leaf size (Kuluev et al. 2014), and *PttEXPA1* overexpression increases stem internode elongation and leaf expansion in aspen (Gray-Mitsumune et al. 2008). Furthermore, *CsEXPI* overexpression has been found to reduce leaf sizes and internodes in tomato plants (Wieczorek et al. 2006). Overexpression of *OsEXP4* sense and antisense constructs significantly increases and decreases, respectively, the number of leaves in rice plants (Choi et al. 2003). Because *EXLA* and *EXLB* gene sequences are conserved across many plant species and are highly homologous to those of *EXPA* and *EXPB* members (Sampedro and Cosgrove 2005), *EXLA* and *EXLB* genes may be directly and/or indirectly involved in plant growth and developmental processes. Concurrence with this hypothesis, *Arabidopsis EXLA2* gene has been recently reported to involve in plant development and defense (Abuqamar et al. 2013).

In our previous comparative genomic analysis, we identified three *EXLB* genes in the Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) genome (Krishnamurthy et al. 2015). In

this study, we cloned and characterized the physiological effect of the *BrEXLB1* gene in transgenic *Arabidopsis* expressing *BrEXLB1* sense and antisense constructs driven by the CaMV35S promoter. We also isolated the *BrEXLB1* promoter sequence and characterized its activity under various environmental stimuli, phytohormones, and stress conditions. The promoter activity was effectively measured using reporter gene expression levels in transgenic *Arabidopsis* via quantitative reverse-transcription PCR (qRT-PCR). This study will help to understand the likely function of *BrEXLB1* in plant growth and development.

Materials and methods

Sequence analysis

Full-length coding, amino acid, and upstream (promoter region: – 1600 bp to + 1 bp) sequences of *EXLB* genes of *B. rapa* (*BrEXLB1*) and *Arabidopsis thaliana* (*AtEXLB1*) were retrieved from Brassica (<http://brassicadb.org/brad/index.php>; *B. rapa* genome version 1.5) and Phytozome v10.3 (<http://phytozome.jgi.doe.gov/pz/portal.html#>) databases, respectively. To identify putative cis-acting elements of *BrEXLB*, the promoter region (– 1500 bp to + 1 bp) was searched against known motifs of plantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) database. All tools and programs mentioned in this study were used with default settings unless otherwise specified.

Construction of expression cassette for *BrEXLB1*-sense, antisense and *BrEXLB1* promoter::GUS

The *BrEXLB1* coding sequence was amplified by polymerase chain reaction (PCR) with gene-specific forward (5'-AATATGAAGACATTTAACGTCTTG-3') and reverse (5'-GGAATCAAGTAAGTAGAATGTTGG-3') primers. The EcoR I digested PCR amplicons (0.773 kb) were inserted in the transgene orientation between the cauliflower mosaic virus 35S promoter (CaMV35Sp) and the nopaline synthase terminator of a pCAMBIA1390 vector to generate binary vectors pCAMBIA1390::35S-Pro + *BrEXLB1*sense (pLSI09) and pCAMBIA1390::35S-Pro + *BrEXLB1*antisense (pLSI12). The sense and the antisense orientation of *BrEXLB1* was confirmed by Sanger sequencing. Similarly, the *BrEXLB1* promoter region was PCR amplified using forward and reverse primers 5'-GAAACGAACACGGCTATTATACG-3' and 5'-CATATTGTTATATGTAAGTATTGTA-3', respectively. The amplified promoter sequence (1.5 kb) was ligated into a pGEM-T Easy vector followed by transformation into *Escherichia coli* DH-5 α cells for amplification, and then introduced into a pCAMBIA1391Z

vector at EcoR I site to generate the binary vector pCAMBIA1391Z + BrEXLB1promoter::GUS (pLSI13). All primer pairs were obtained from GenoTech (Daejeon, Korea).

Plant materials, *Agrobacterium* mediated transformation and morphology of transgenic lines

Arabidopsis thaliana Columbia (Col-0) seeds were surface-sterilized by 70% ethanol for 15 min followed by 100% ethanol for 2 min and plated on Murashige and Skoog (MS) medium supplemented with vitamins, 1.5% sucrose, and 0.25% phytagel. Plates were stratified at 4 °C under dark for 2 days to induce synchronous germination and transferred to a growth chamber (16-h light/8-h dark photoperiod at 23 °C) for transformation and morphological character analyses. Transformation (*A. tumefaciens* strain GV3101) and transformant selection with hygromycin B were performed according to Hong et al. (2012). Leaf length, leaf width, and petiole length were measured on 15, 20, and 30 days after transferring the pots into the growth chamber. Measurements were taken from three independent biological replicates. Mean significant differences were compared by Duncan Multiple Range Test at $P \leq 0.05$ using SAS package 9.1.3 service pack 4.

Histochemical localization of β -glucuronidase (GUS) activity

To evaluate the regulation of BrEXLB1 promoter under different developmental stages, seeds of transgenic *Arabidopsis* (pLSI13) lines were germinated as mentioned above. Samples were collected from the first day of germination, 7-, 14-, and 20-days old seedlings and reproductive organs of mature plants. Samples were directly used for the GUS assay. An assay was performed using a β -Glucuronidase Reporter Gene staining kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Phytohormone, stress treatments, and quantitative reverse-transcription real-time PCR (qRT-PCR)

Transgenic T_1 *Arabidopsis* seedlings harboring pLSI13 cassette were surface sterilized as mentioned above and plated on MS medium supplemented with vitamins, 1.5% sucrose, 0.25% phytagel and 30 μ g/ml hygromycin B. After 2 days stratification under dark at 4 °C and 5 days under growth chamber, transformants were selected based on the hypocotyl length (Harrison et al. 2006). Selected seedlings were divided for triplicate and independently

grown hydroponically in MS solution for 3 days in *Arabidopsis* growth chamber. Then, three plates each (each having three seedlings) were moved to different light qualities namely white (photosynthetic photo flux density: 59 μ mol m⁻² s⁻¹), blue (59 μ mol m⁻² s⁻¹), red (10 μ mol m⁻² s⁻¹), far-red (2 μ mol m⁻² s⁻¹) and dark in our customized LED chamber. Concurrently, phytohormones, namely IAA (50 μ M), GA (50 μ M), JA (100 μ M), SA (100 μ M), and ABA (100 μ M), as well as heavy metal (CdCl₂; 200 μ M) and polyethylene glycol (PEG6000; 4%) were added to the respective triplicate hydroponic cultures and kept in the growth chamber with gentle shaking (60 rpm). After incubation for 6 h, entire seedlings from all the treatments were harvested, frozen in liquid nitrogen, and stored at -80 °C. Seedlings grown in growth chamber (80 μ mol m⁻² s⁻¹) in MS solution were used as control. Temperature (25 $\pm$ 0.5 °C) and humidity (33 $\pm$ 2%) were maintained same in both the growth chamber and LED chamber.

Total RNA was extracted using Ambion Purelink[®] RNA Mini Kit (Thermo Fisher Scientific, MA, USA) from ground tissues samples (Qiagen TissueLyser II (Hilden, Germany). RNA (1 μ g) was reverse transcribed into cDNA using Qiagen's QuantiTect[®] Reverse Transcription Kit (Hilden, Germany) which included genomic DNA elimination step. cDNA products were diluted in a 1:9 ratio with nuclease-free water (Promega) and used as template for qRT-PCR. QRT-PCR was performed using the SYBR Premix Ex Taq kit[®] (TaKaRa, Japan) with GUS-specific primers (forward: 5'-GAATACGGCGTGGATACGTTAG-3' and reverse 5'-GATCAAAGACGCGGTGATACA-3') on StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) under the following cycling profile: 95 °C for 30 s, 95 °C for 5 s (40 cycles), 60 °C for 30 s, and melting curve analysis at 65 °C for 10 s with 61 cycles. The reaction mixture volume for qRT-PCR was 20 μ L. Experimental repeat runs for three biological and three technical replicates were included in the analysis. The *Arabidopsis* Actin2 (*AT3G18780*) gene (forward primer: 5'-TCGGTGTTCCATTCTTGCT-3'; reverse primer: 5'-GCTTTTAAAGCCTTT GATCTTGAGAG-3') was used as an internal control to normalize the expression level of the target GUS gene. An analysis of the relative quantitation (RQ) values were directly inferred from the StepOnePlus Real-Time PCR System that mean significant differences were compared by Duncan Multiple Range Test at $P \leq 0.05$ using SAS package 9.1.3 service pack 4.

Relative quantification of BrEXLB1 transcripts in different tissues of *Brassica rapa*

Brassica rapa 'Chiifu-401-42' seeds were germinated and grown according to Hong et al. (2010). Samples of apical

meristems, cotyledons and hypocotyls were collected from 2-week-old *B. rapa* seedlings whereas leaves, roots, pollen, carpels, and siliques were harvested from mature *B. rapa* plants. All samples were frozen in liquid nitrogen and stored at -80°C throughout the sampling period and were ground using Qiagen TissueLyser II (Hilden, Germany). Total RNAs and cDNA were prepared as mentioned above and used as template for semi-quantitative (reaction volume 25 μL) PCR with *BrEXLB1* primers under following cycling parameters: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension step of 72°C for 7 min. *B. rapa* β -actin (forward: 5'-TGGCATCACACTTTTCTACAA-3'; reverse: 5'-CAACGGAATCTCTCAGCTCC-3') was used as an internal control to check the RNA quality (Hong et al. 2010).

Results and discussion

Implications on the structural characteristics of *B. rapa* EXLB genes and their promoter regions

Of the four expansin families, EXPA is the largest, followed in order by EXPB, EXLB, and EXLA. The highest numbers of EXLA ($n = 49$) and EXLB ($n = 15$) genes have been found in the soybean genome whereas the highest number of EXPB ($n = 48$) and EXLA ($n = 4$) genes have been identified in the maize genome (Cosgrove 2015). The Arabidopsis genome contains 36 EXPA, 6 EXPB, 3 EXLA and 1 EXLB genes. The whole genome triplication event generated at least 53 expansin genes in the *B. rapa* genome that have occurred after the speciation of Brassica from Arabidopsis (Krishnamurthy et al. 2015). The *B. rapa* genome consists of three EXLB genes namely *BrEXLB1* (gene id: *Bra040107*), *BrEXLB2* (*Bra012687*), and *BrEXLB3* (*Bra012684*) that were orthologous to *AtEXLB1* (*At4G17030*) ((Krishnamurthy et al. 2015); Table S1). Coding sequences of all *BrEXLBs* were of similar length (753 bp). *BrEXLB2* and *BrEXLB3* were tandem duplicates that shared 95.9% nucleotide and 97.5% amino-acid sequence similarity, while nucleotide and amino acid sequences of *BrEXLB1* shared $\sim 90\%$ similarity with those of *BrEXLB2* and *BrEXLB3*. All three *BrEXLB* protein sequences comprised 250 amino acids with nearly identical molecular weights of 27.8 kDa. The isoelectric points of *BrEXLB2* and *BrEXLB3* were the same (6.51) and slightly different from that of *BrEXLB1* (6.30). Multiple sequence alignment revealed that *AtEXLB1* shared 88–90% sequence similarity at nucleotide and amino acid levels with *BrEXLBs* suggesting that *AtEXLB1* and *BrEXLBs* may share common function. Further to understand evolutionary relationships between *BrEXLB1* and

homologs found in other closely related species of Brassicaceae families such as *Brassica oleracea* var. *oleracea* (*BoEXLB1*), *Arabidopsis thaliana* (*AtEXLB1*), *Capsella rubella* (*CrEXLB1*), *Camelina sativa* (*CsEXLB1*), *Brassica napus* (*BnEXLB1*) and *Raphanus sativus* (*RsEXLB1*) were compared through multiple sequence alignment followed by phylogenetic tree analysis (Fig. 1). *BrEXLB1* was more similar to *BoEXLB1* than *BnEXLB1*. The close relationship among *EXLB1* of the species can be ranked as *BoEXLB1* followed by *BnEXLB1*, *RsEXLB1*, *AtEXLB1*, *CsEXLB1* and *CrEXLB1*. Interestingly, the similarity between *BrEXLB1* and *RsEXLB1* is higher than *AtEXLB1*.

Comparative sequence alignment among the promoter regions (-1600 bp to $+1$ bp of the upstream sequence) of *BrEXLBs* revealed that the *BrEXLB1* promoter share only 48–50% similarity to *BrEXLB2* and *BrEXLB3* promoters, whereas 72.31% similarity was found between the latter two. The *AtEXLB1* promoter showed 43–49% similarity to the *BrEXLB* promoters. Due to the limitation of PlantCARE database, the -1500 bp to $+1$ bp of the obtained promoter sequences were submitted to identify and compare *cis*-acting elements. In each sequence, 83–96 conserved *cis*-acting elements were identified corresponding to 55 unique elements that could be broadly classified into five types: (1) promoter-related, (2) light-responsive, (3) plant hormone-responsive, (4) environmental- and stress-responsive, and (5) tissue-specific (Table S2). Of the 55 *cis*-acting elements, 35–47% is contributed by promoter-related elements followed by light-responsive (20–33%) and phytohormone-responsive elements. *BrEXLB* promoters has several elements like phytohormone responsive (ABA and SA), fungal elicitor-responsive (Box-W1 (TTGACC), wound and pathogen responsive (W box: (TTGACC)), meristem expression-specific (CAT-box: GCCACT), endosperm-specific expression (Skn-1_motif: GTCAT), circadian control and element involved in negative regulation of phloem expression and responsible for restricting vascular expression (AC-II; [(C/T)T(T/C)(C/T)(A/C)(A/C)C(A/C)A(A/C) C(C/A) (C/A)C]. The comparative analysis with *BrEXLB2*, *BrEXLB3* and its orthologous promoter *AtEXLB1* shows some elements are specific to respective promoter sequences. For instance, *BrEXLB1* has unique elements like Box-W1 and the W box (TTGACC), similarly, *BrEXLB2* has MBS motif (CAACTG; involved in drought inducibility), *BrEXLB3* has 5'-untranslated region Py-rich stretch element (TTTCTTCTCT) and *AtEXLB1* has auxin, JA-, GA-responsive, and AT-rich sequence (TAAAAATACT; element for maximal elicitor-mediated activation).

Elements such as anaerobic induction-responsive (ARE motif: TGGTTT), stress-responsive (TC-rich repeats: GTTTTCTTAC) were commonly found in all *BrEXLB* promoters while CAT-box was found in *BrEXLB1* and

Fig. 1 Phylogenetic relationship between *Brassica rapa* EXLB1 and homolog EXLB1 gene sequences of closely related species of Brassicaceae families. The proteins compared are *Brassica rapa* (BrEXLB1), *Brassica oleracea* var. *oleracea* (BoEXLB1), *Arabidopsis*

thaliana (AtEXLB1), *Capsella rubella* (CrEXLB1), *Camelina sativa* (CsEXLB1), *Brassica napus* (BnEXLB1) and *Raphanus sativus* (RsEXLB1)

BrEXLB3 promoters. Similarly heat stress-responsive elements (HSE motif: AAAAAATTTC and AGAAAATTCG) were present in *BrEXLB2* and *BrEXLB3* promoters. Further comparison with *AtEXLB1* revealed that AC-II as well as the Skn-1 motif: GTCAT are common among all the promoters, whereas circadian control elements were unique to *BrEXLB* promoters. As indicated in the present study and by the reports of Zhu et al. (2014), we expect that the presence of diverse *cis* elements may facilitate the differential expression of EXLB genes depending upon the environmental stimuli, presence of phytohormones or its exogenous application, developmental stages and stress conditions.

GUS reporter-aided analysis of *BrEXLB1*-specific promoter activity during plant development, application of phytohormones and stress in *Arabidopsis*

To investigate how BrEXLB1 regulated in different tissues, a GUS reporter-aided approach was adopted to elucidate

the promoter activities of BrEXLB1 in transgenic *Arabidopsis* in the present study. The identification of putative *cis*-acting elements using PlantCARE revealed that *BrEXLBs* has several elements including specific and common elements found in other BrEXP and *Arabidopsis*. Moreover, studies shown that abiotic stresses (Chen et al. 2018) including drought, light, phytohormones and hard metals regulates the expression of expansin (Marowa et al. 2016; Santiago et al. 2018). We thus generated transgenic *Arabidopsis* homozygous lines harboring the cassette pCambia1391Z + BrEXLB1promoter::GUS (pLSI13) to assess the functional regulation of the *BrEXLB1* promoter during developmental stages and in response to various light qualities, phytohormones, and drought stress. GUS activity was clearly detectable immediately after radicle elongation, on the first day of germination (Fig. 2a). Staining was observed in the hypocotyls, cotyledonary leaves, and radicles, with the strongest staining found in the apical points of 3- and 7-day-old seedlings (Fig. 2b, c). GUS staining was uniformly present in rosettes and hypocotyls but was unevenly detected in the root systems

Fig. 2 Histochemical localization of GUS expression driven by the *BrEXLB1* promoter in transgenic Arabidopsis. Expression at 1 (a), 3 (b), 7 (c), and 20 (d) days after germination and in flowers (e), and stigmas (f) of mature transgenic Arabidopsis plants

of 20-day-old seedlings (Fig. 2d). In addition, GUS activity was detected in pollen (Fig. 2e); activity was also detected in stigmas, with intense staining restricted to the abscission zone and stigmatic papillae with no expression in the middle part (Fig. 2f). These results indicate *BrEXLB1* likely to play a role in development of apical points in early development stages, hypocotyls, cotyledonary leaves, radicles, abscission zone and stigmatic papillae and pollen and to some extent in root.

Further to test the importance of cis-acting elements of *BrEXLB1* promoter under different light qualities, phytohormone and PEG induced drought stress was evaluated by quantitate the abundance of GUS transcript levels in each treatment using transgenic pLSI13 lines (Fig. 3). The results show that the transcript levels of GUS was 0.3-fold increased by white light and 0.3–0.4-fold decreased by red, far-red and dark conditions while no significant change was observed under blue light. It is therefore conceivable that light qualities and dark periods mediate plant growth through regulation of *EXPLB1*. The previous reports on *EXPA* and *EXPB* from various studies showed that phytohormones (auxin, gibberellin, cytokinin, and ethylene) regulate *EXPA* and *EXPB* gene expression at the transcript level (Cho 1997; Hutchison et al. 1999; Kim et al. 2000; Wrobel and Yoder 2001).

Similarly, the exogenous application of IAA, JA, and exposure to PEG6000 mediated drought stress and CdCl_2 influence the activities of *BrEXLB1* promoter. The

Fig. 3 GUS expression in *pBrEXLB1::GUS* transgenic Arabidopsis by qRT-PCR. Ten-day-old transgenic seedlings were treated with different light qualities (white, blue, red, far-red and dark), phytohormones [indole-3-acetic acid (IAA), gibberellic acid (GA), jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA)], heavy metal (CdCl_2), and drought (PEG) stress for 6 h. Arabidopsis *Actin2* gene expression was used to normalize the expression of GUS. Values shown are the mean and SD of three independent biological and technical experiments. Asterisks indicate statistical significant differences from the control (Duncan Multiple Range Test at $P \leq 0.05$) (color figure online)

quantitative RT-PCR analysis showed that application of IAA increased GUS transcripts by 2.8-fold and, JA and PEG exhibited 2.5-fold increase in pLSI13 lines. Intriguingly, cis-elements responsive to IAA, JA- and drought stress were not observed from the sequence analysis (Table S2). However,

both IAA (auxin), JA-responsive elements were found in *AtEXLB1* which is a homolog of *BrEXLB1*. It is reasonable to think that the technical limitation for sequence length with PlantCARE might reduce our chance in identification of auxin-, JA-responsive elements. Moreover, standardization of time periods for each treatment may provide most conceivable data. Further, the synergistic relationship between JA- and wound-responsive elements (W-box) may facilitate the induced activity of *BrEXLB1* promoter against JA (Bari and Jones 2009). Up-regulation by PEG6000 and slightly down-regulation by CdCl_2 suggests *BrEXLB1* is sensitive to plant water balance, as both CdCl_2 and PEG6000 decrease plant water potential and thereby inhibit plant growth and development (Lagerwerff et al. 1961; Perfus-Barbeoch et al. 2002). As evident from the results (Fig. 3), *BrEXLB1* promoter mediated expression of GUS transcript is also regulated by

exogenous application of GA, SA, ABA and CdCl_2 , although their exposure did not significantly affect the promoter activity. PEG induced activity of *BrEXLB1* are encouraging as it may result in improved drought stress response as noted in tobacco (Chen et al. 2016). Nevertheless, our results show that *BrEXLB1* promoter activity is up-regulated by white light, IAA, JA and PEG treatments and reduced under red, far-red and dark treatments indicating the possible involvement of *BrEXLB1* gene in plant growth and development.

Impact of *BrEXLB1* sense and antisense overexpressing transgenic Arabidopsis on plant growth and development

Transcript levels of endogenous *BrEXLB1* in *B. rapa* were examined using semi-quantitative RT-PCR. As inferred

Fig. 4 Relative quantification of *BrEXLB1* across different tissues of *B. rapa*. Impact assessment in overexpressors of sense and antisense constructs on leaf growth in Arabidopsis. **a** Expression pattern of *BrEXLB1* in different tissues (apical meristem, cotyledon, hypocotyl) of two-week old mature *B. rapa* 'Chiifu-401-42' plants (leaf, root, pollen, carpel, silique) analyzed by semi-quantitative RT-PCR. Relative quantification of *BrEXLB1* in sense (**b**) and antisense (**c**) lines (whole-seedlings of two-week old transgenic Arabidopsis) by

semi-quantitative RT-PCR. Blade length (**d**), blade width (**e**), and petiole length (**f**) in transgenic Arabidopsis lines were measured at 15, 20 and 30 days after transferring the pots into the growth chamber. Measurements shown are the mean and SD of three independent biological with three seedlings per line/wild type. Statistical significance between WT (wild type) and transgenic plants in each test at $P \leq 0.05$ (*)

from the amplicon intensity, the expression of *BrEXLB1* was high in seeds, roots, and carpels, less in apical meristems and hypocotyls, and undetectable in cotyledons, leaves, pollen, and siliques (Fig. 4a).

One effective approach to elucidate the physiological functions of expansin is comparison of morphological characteristics of control plants with their transgenic counterparts expressing sense and antisense constructs of target expansin genes (Cho and Cosgrove 2000). A number of previous studies showed a positive/negative effect of EXPA genes on leaf, petiole sizes and number of leaves (Cho and Cosgrove 2000; Choi et al. 2003; Gray-Mitsumune et al. 2008; Kuluev et al. 2012, 2013, 2014; Rochange et al. 2001). Hence, in this study, we validated the effect of overexpression of *BrEXLB1* sense and antisense constructs driven by the CaMV35S promoter in transgenic Arabidopsis. Following hygromycin selection, eight independent T₁ transgenic lines were randomly selected for leaf-morphology statistical analysis: four lines harboring the sense construct and four carrying the antisense construct, each differed in *BrEXLB1* expression (Fig. 4b, c). Measurements were taken at three time points (15, 20, and 30 days after transferring the pots into the growth chamber). The blade width of the sense line pLSI9-7 was significantly higher than the wild type at day 30; whereas, the antisense line pLSI12-2 showed increased and decreased blade width at days 15 and 30, respectively, than the wild type and these values were statistically significant (Fig. 4e). Concurrently, the blade length of both sense and antisense lines at all three time points were not significantly different from that of wild type except antisense line pLSI12-4 which blade length was significantly lower than the wild type (Fig. 4d). Moreover, we observed inconsistent length in petioles of sense and antisense lines of *BrEXLB1* transgenic Arabidopsis in comparison with wild types (Fig. 4f). The petiole length was significantly high in the sense lines of pLSI9-8 at day 20, pLSI9-7 at day 30 and in the antisense line of pLSI12-4 at day 15. Surprisingly, the sense line pLSI9-2 showed significantly decreased petiole length at day 30 than the wild type. mRNA accumulation not correlated with phenotypic changes observed in transgenic Arabidopsis. This is clear with sense line pLSI9-9 where relatively high number of *BrEXLB1* transcripts found, but do not show any significant phenotypic change (Fig. 4). Moreover, the phenotypic changes induced by sense and antisense constructs are not uniform. However, it is evident that transgenic lines did affect the leaf morphology in comparison with wild types indicating other unknown factors might also be involved in leaf growth or morphology. The results also suggest that the ectopic expression of *BrEXLB1* gene results differential and unstable pleiotropic effects on the growth of leaves. Interestingly, plant height were visually increased in

Fig. 5 Comparative analysis of plant height upon overexpression of sense *BrEXLB1* (pLSI9-9) and antisense *BrEXLB1* (pLSI12-4) constructs with wild type plants

BrEXLB1 (sense) overexpressing transgenic lines than wild type and *BrEXLB1* antisense overexpressing lines. It is clear that *BrEXLB1* either directly or indirectly regulate plant growth and development including leaf, and plant height/plant growth by its known cell wall loosening activity in Arabidopsis (Fig. 5). To understand the mechanisms behind *BrEXLB1* mediated growth further in-depth analysis is necessary.

Moreover, the expression of GUS driven by promoter of *BrEXLB1* in leaves and cotyledons of transgenic Arabidopsis was abundant while the expression of endogenous *BrEXLB1* in *B. rapa* was undetectable. This difference in *BrEXLB1* promoter activity suggests the likely interference of other factors and the different genetic constitution. Unlike in *A. thaliana*, three *EXLB* (*EXLB1*, 2 and 3) genes were present in *B. rapa* (Krishnamurthy et al.). Possibly, *BrEXLB2* and *BrEXLB3* would have direct or indirect effect in expression of *BrEXLB1* in host plant, *B. rapa*. However, the effect of *BrEXLB2* and *BrEXLB3* is unlikely in transgenic Arabidopsis due to the absence of *EXLB2* and *EXLB3*. In addition, other endogenous and exogenous factors including white light, red light, dark, IAA, JA and drought stress in *B. rapa* and *A. thaliana* would modulate the expression of *BrEXLB1* differently as shown by transgenic promoter lines in the present study.

In conclusion, our findings suggest that *BrEXLB1* may participate in germination, leaf development, and plant growth depending upon the phytohormone availability (esp. IAA, JA), interaction with other *EXLB* genes, transcriptional regulators, light types and intensity, dark periods and drought stress in a given physiological conditions.

Moreover, BrEXLB1 may exhibit tissue preference as evidenced by GUS reporter-aided analysis of *BrEXLB1*-specific promoters in this study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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