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ORIGINAL PAPER

Identification of wheat non-specific lipid transfer proteins involved in chilling tolerance

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Abstract

Key message **Three TaLTPs were found to enhance chilling tolerance of transgenic *Arabidopsis,* which were characterized by analyzes of promoter-GUS activity, subcellular localization, chromosomal location and transcriptional profile.**

Abstract Non-specific lipid transfer proteins (nsLTP) are abundantly expressed in plants, however, their functions are still unclear. In this study, we primarily characterized the functions of 3 type I *TaLTP* genes that were localized on chromosomes 3A, 3B, and 5D, respectively. The transcripts of *TaLTPIb.1* and *TaLTPIb.5* were induced under chilling, wound, and drought conditions, while *TaLTPId.1* was only up-regulated by dark treatment. All the 3 *TaLTP* genes could be stimulated by the in vitro treatment of salicylic acid, while *TaLTPId.1* was also positively regulated by methyljasmonic acid. Furthermore, the promoter-reporter assay of *TaLTPIb.1* in the transgenic brachypodium showed a typical epidermis-specific expression pattern of this gene cluster. When fused with *EGFP*, all the 3 proteins were shown to localize on the plasma membrane in transgenic tobacco, although a signal in chloroplasts was also observed for *TaLTPId.1*. Heterogeneous overexpression of each of the *TaLTP* genes in *Arabidopsis* resulted in longer root length compared with wild type plants under chilling condition. These results suggest that type I TaLTPs may have a conserved functionality in chilling tolerance by lipid permeation in the plasma membrane of epidermal cells. On the other hand, the type I TaLTPs may exert functional divergence mainly through regulatory subfunctionalization.

Keywords Non-specific lipid transfer protein (nsLTP) · Epidermis · Chilling · Subfunctionalization

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Introduction

Plant non-specific lipid transfer proteins (nsLTPs) are encoded by a multigene family, and are known for their ability to reversibly bind and transport hydrophobic molecules in vitro (Vergnolle et al. 1992). These cationic peptides contain a conserved eight-cysteine motif backbone (C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C), in which the cysteine residues are engaged in four disulfide bonds to stabilize a tertiary structure of hydrophobic cavity (Lee et al. 1998; Samuel et al. 2002; Shin et al. 1995). Computational and biochemical analyzes have demonstrated that this hydrophobic cavity of nsLTPs is plastic or flexible to allow loading of a great variety of lipid compounds such as phospholipids (Vergnolle et al. 1992; Zachowski et al. 1998), palmitic acid (C16:0), and acyl chains of 1,2-dimyristoylphosphatidylglycerol (Han et al. 2001; Shin et al. 1995). According to protein molecular masses, the nsLTP genes had been divided into two families of 7 and

9 kDa which was thought to transport different kinds of lipids in plant cells. Recently, plant *nsLTP*s were extended over nine types using genomic information from *Arabidopsis*, *Oryza sativa* and *Triticum aestivum*, in which type I *nsLTP*s comprise the maximum number of members belonging to the classical 9 kDa nsLTPs (Boutrot et al. 2008). In cereal, a certain number of type I *nsLTP*s are preserved in genome via the concerted evolution between homeologous chromosomes, however, their functional redundancy or diversification is an open question (Paterson et al. 2009; Wang et al. 2012).

NsLTPs were reported to be present in abundant quantities in plants, up to as much as 4 % of the total soluble proteins of wild cabbage leaves (Pyee et al. 1994). The expression pattern of *nsLTP*s from different plants has shown a large divergence covering various tissues and against environmental stimulates (George and Parida 2010; Kader 1996; Pitzschke et al. 2013; Pyee et al. 1994). Consistent with their complex expression profiles, the pleiotropic functions of nsLTPs have been previously suggested to be involved in cuticle synthesis (Lee et al. 2009; Pyee et al. 1994), catabolism in lipid storage (Tsuboi et al. 1992), somatic embryogenesis (Kader 1996), stigma-pollen interaction (Huang et al. 2013; Mollet et al. 2000; Tian et al. 2013), defense signaling (Blein et al. 2002; Maldonado et al. 2002), nodule organogenesis (Lei et al. 2014) development, and antimicrobial activities (Caaveiro et al. 1997; Kristensen et al. 2000). However, the exact function of nsLTPs remains to be clearly established. Several lines of evidence support the function of nsLTPs in cuticle synthesis such as their extracellular localization, the cutin monomer binding capability (Pyee et al. 1994; Thoma et al. 1994), and the epidermis-specific expression in young aerial growing tissues, where the cuticlar wax usually localizes (Thoma et al. 1994; Wang et al. 2012). Recent research data demonstrated that the disruption of a nsLTP-like protein (LTPG1) simultaneously altered the cuticular lipid composition and enhanced the susceptibility to the fungal pathogen, *Alternaria brasssicola*, supporting the overlapping involvement of the nsLTP and cuticle in defense mechanism (Lee et al. 2009). However, it is unclear whether the susceptibility to the pathogens of *LTP*-knockout plants is attributed to cuticular lipid changes or its antimicrobial activities (Kristensen et al. 2000). An alternative explanation of nsLTPs involved in the defense against fungal pathogens is that the cutin monomer-nsLTP complex is released by the fungal cutinase at the time of infection, which can act as signaling molecules and bind to the plasmalemma receptor triggering plant defense responses (Blein et al. 2002). The role of nsLTP in defense signaling was subsequently confirmed by the study of two important nsLTP genes. *DIR1*, a 7 kDa nsLTP, can release a systemic signal responsible for systemic acquired resistance (SAR) in the vascular system, while an *AZI1* mutation resulted in the specific loss of systemic immunity in priming defenses (Maldonado et al. 2002; Pitzschke et al. 2013). It was presumed that nsLTPs may bind to signal molecules and translocate SAR signals across long distances. Furthermore, nsLTPs were reported to be involved in pollen development. One *LTP* (SCA) from the Lily, sharing 56.4 % identity with *AtLTP1*, was proved to function in pollen tube adhesion both in vivo and in vitro (Mollet et al. 2000). Some *nsLTP* genes, especially in type III, were found specifically expressed in the pollen and anther tapetum, and seemed to play important roles in pollen development by the transportation of lipids between the pollen and the tapetum (Carvalho Ade and Gomes 2007; Huang et al. 2013). Although a bulk of data demonstrate that the expression of some *nsLTP* genes show broad responses against abiotic stresses, such as drought, chilling, and high salt, the evidence that indicates their direct association with the tolerance to these stresses is rare (Carvalho Ade and Gomes 2007; George and Parida 2010; Guo et al. 2013; Jung et al. 2005; Pitzschke et al. 2013). In a previous study, the transgenic *A. thaliana*, overexpressing a *CALTP1* from *Capsicum annuum*, resulted in enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000, *Botrytis cinerea*, and exhibited tolerance to NaCl and drought stresses (Jung et al. 2005). Similarly, the overexpression of *AtLPT3* increased the drought tolerance of the transgenic *Arabidopsis* that was regulated by *MYB96* (Guo et al. 2013).

In wheat, a total of 156 putative wheat *nsLtp* genes had been identified using the EST sequences of *T. aestivum*. None of these members had been functionally identified before. Previous studies of wheat *nsLTP*s mainly focused on the transcript level, in which a complex expression pattern was shown during the entire life cycle of the plant (Boutrot et al. 2008; Wang et al. 2010). In this study, we attempted to examine the possible functions and functional redundancies of 3 type I *TaLTP*s involved in abiotic stresses. The phylogeny, transcriptional response, and subcellular localization were characterized. The functionality of these genes was then identified by observing the phenotypic changes of the transgenic *Arabidopsis* under stress conditions.

Materials and methods

Phylogenetic analysis and chromosomal location identification

The putative sequences of *nsLTP*s were downloaded from National Center for Biotechnology Information. The deduced amino acid sequences of all the *nsLTPs* were

aligned by using the ClustalW program with default parameters. The phylogenetic tree was created with MEGA using the maximum like algorithm, with 1,000 boot strap replicates (Kumar et al. 2008). The chromosomal distribution of *TaLTPIb.1*, *TaLTPIb.5* and *TaLTPId.1* was verified by a set of Chinese Spring (CS) nulli-tetrasomic lines. The specific primer pair for each gene was employed for PCR amplification using the genomic DNA of CS nulli-terasomic lines as templates.

Quantitative RT-PCR for abiotic stress and hormone treatments

Seeds of *T. aestivum* were allowed to germinate on filter papers and then moved to containers as previously described by Wang et al. (2012). After 36 h, the water was replaced with the Murashige and Skoog (MS) medium (Duchefa Biochemie B.V., Haarlem, Netherlands) in order to grow the plants for 10 days with the photoperiod of 14/10 h and temperature of 28/25 °C (day/night) before stress treatments. For drought stress, the healthy young seedlings were transferred to containers with 25 % (w/v) of polyethylene glycol (PEG, MW 10,000). The chilling stress was applied by subjecting plants to 4 °C, and the wounding stress was applied by piercing the leaves with 0.3 mm-diameter needles. For hormone treatments, the seedlings were transferred to containers with 100 μM of methyljasmonic acid (MeJA), 2 mM of salicylic acid (SA), 100 μM of abscisic acid (ABA), and 100 μM of indole-3-acetic acid (IAA). Leaves of 10 or more seedlings from the above treatments were harvested at different time points after treatments, frozen immediately in liquid nitrogen, and stored at −80 °C. RNA isolation and quantitative RT-PCR (qRT-PCR) were performed as described previously (Wang et al. 2010, 2012). The sequence specificity of each amplified band was cloned and confirmed by sequencing.

Construction of *TaLTPIb.1* promoter::uidA fusion and transformation in brachypodium

The sequences of the *TaLTP* promoters were previously identified elsewhere (Jang et al. 2008). The promoter of *TaLTPIb.1* (−723 to +3) was amplified from the genomic DNA of the wheat cultivar, Sumai 3, and cloned into the pMD-19 vector (Takara, Japan) with the *Hin*dIII restriction enzyme site at the 5′ end and the *Bam*HI restriction enzyme site at the 3′ end. The plasmid was digested with the above restriction enzymes and then sub-cloned into a binary vector of pCambia1391Z. The *Agrobacterium* strain, AGL1, was transformed with the *TaLTPIb.1* promoter::uidA construct following a freeze–thaw method (Lai et al. 2011). The brachypodium of Bd21-3 was then used for *Agrobacterium*-mediated transformations using the procedure described by Bragg et al. (2012). The pCambia1391Z vector was also transformed into brachypodium plants as negative controls. Histochemical analyzes were performed with the T_2 plants according to the procedures previously described (Wang et al. 2012). For fluorimetrical assays of plant tissues, the concentration of protein extracted in the β-glucuronidase (GUS) assay buffer was determined by the Bradford (1976) method using a Multiskan (Labsystems, Thermo Fisher Scientific Inc., MA, USA) spectrophotometer. GUS specific activity (hydrolysis of 4-methylumbelliferone glucuronide per mg protein per hour) was determined at 460 nm with a Fluoroscan (Labsystems, Thermo Fisher Scientific Inc., MA, USA) apparatus (Hull and Devic 1995). For the sections, the GUS-stained tissue samples were fixed in the FAA buffer containing EtOH (50 %, v/v), formaldehyde (3.7 %, v/w), and acetic acid (5 %, v/v). Dehydration was performed with the graded ethanol series (80–95 %, v/v), followed by additional *tert*-butanol batches (25, 50, and 100 %, v/v). The samples were initially incubated in 50 % paraplast (dissolved in *tert*-butanol) and finally embedded in 100 % paraplast. Subsequently, the samples were sectioned by a microtome, each with a thickness of 10 μm. Deparaffinization was performed by the immersion of the slides in xylene for 5 min. The fluorescence inverted microscope system (Carl Zeiss, Oberkochen, Germany) was used to generate the images.

Subcellular localization

The coding regions of *TaLTPIb.1*, *TaLTPIb.5* and *TaLTPId.1* were amplified and cloned into the pBIN35S:EGFP vector that was then transformed into the *Agrobacterium*, EAH105. For transient expression of the fusion proteins in *Nicotiana benthamiana*, the resultant *Agrobacterium* culture was resuspended in an infiltration medium [10 mM 4-morpholineethanesulfonic acid hydrate (MES), pH 5.6, 10 mM $MgCl_2$, and 200 mM acetosyringone], then injected into 3-week-old *N. benthamiana* leaves with an optical density of 0.6 OD at 600 nm. The transformed 35S:EGFP was used as a negative control. For colocalization, the *Agrobacterium* harboring TaLTP:EGFP and the *Agrobacterium* harboring pm-rb CD3-1008 were mixed in a 1:1 ratio and co-injected in tobacco leaves as described above (Nelson et al. 2007). Transient expression of TaLTPs for the subcellular localization in onion (*Allium cepa*) epidermal cells was conducted according to Das et al. with a Bio-Rad PDS-1000/He biolistic particle delivery system (Das et al. 2009). Confocal microscopy was used to assess the results, 3 days following the infection. Fluorescent images were obtained using an LSM 510 META NLO system (Carl Zeiss, Oberkochen, Germany).

Analysis of phenotypic changes in transgenic *Arabidopsis*

Agrobacteria GV3101 containing either pBIN35S:TaLTPIb.1, pBIN35S:TaLTPIb.5, or pBIN35S:TaLTPId.1 were used to transform *Arabidopsis* plants according to the floral-dip method (Clough and Bent 1998). Transgenic lines were selected on MS agar plates containing 30 mg/ml kanamycin and the T_3 lines were used for further phenotypic analysis. Two independent overexpressing *Arabidopsis* lines were tested to observe the effects of chilling, freezing, and drought. For the chilling treatment, the transgenic seeds were germinated on 1/2 MS medium for 3 days and moved to 4 °C for 10 days. For freezing treatment, the 10 days old seedlings were subjected to −10 °C for 1–5 h, followed by 2 days of recovery under normal growth conditions (Chinnusamy et al. 2003). For drought treatment, the wild type and transgenic plants were grown in the soil for 3 weeks, and then watering was stopped until the observation of a phenotype.

Results

Phylogeny and genomic localization

The nsLTP genes in wheat, rice, and *Arabidopsis* were previously classified (Boutrot et al. 2008). To clarify the phylogeny of *TaLTPIb.1*, *TaLTPIb.5,* and *TaLTPId.1*, putative 9 KDa nsLTP genes from wheat and rice were downloaded from NCBI (Boutrot et al. 2008). All the 3 *TaLTP* genes were found to belong to the type I category. *TaLTPIb.1* and *TaLTPIb.5* fell in a wheat-specific gene cluster with extremely high homology, which contains 23 LTP members due to independent duplications in wheat (Figure S1). *TaLTPId.1*, *TaLTPId.1* and *TaLTPId.3* localized on a sub-branch with two pairs of rice homeologs (ex. Os11g02400.1 and Os12g02330.1), which had undergone concerted evolution (Wang et al. 2012).

To determine the genomic distribution, wheat nulli-tetrasomic stocks and gene-specific primers were employed to examine the chromosomal location of *TaLTPIb.1*, *TaLTPIb.5,* and *TaLTPId.1*, and all the amplified bands were confirmed by sequencing. The results showed that *TaLTPIb.1* and *TaLTPIb.5* were located on chromosomes, 3B and 3D (Fig. 1), while *TaLTPId.1* was observed to be located on chromosome 5D (Fig. 1).

Expression pattern of TaLTPIb.1, TaLTPIb.5, and TaLTPId.1

Previous studies indicated that the transcripts of *TaLTP*s were extensively induced by abiotic stresses, implying their possible functional roles. To study the biological function of *TaLTPIb.1*, *TaLTPIb.5*, and *TaLTPId.1*, we first examined their expression pattern in chilling, drought, dark, and wounding-treated wheat seedlings by qRT-PCR. Under control conditions, no apparent changes of these transcripts were observed from 0 to 72 h (Figure S2). It was observed that *TaLTPIb.1* and *TaLTPIb.5* showed very similar expression patterns that were distinct from that of *TaLTPId.1*. Under dehydration conditions, the transcripts of

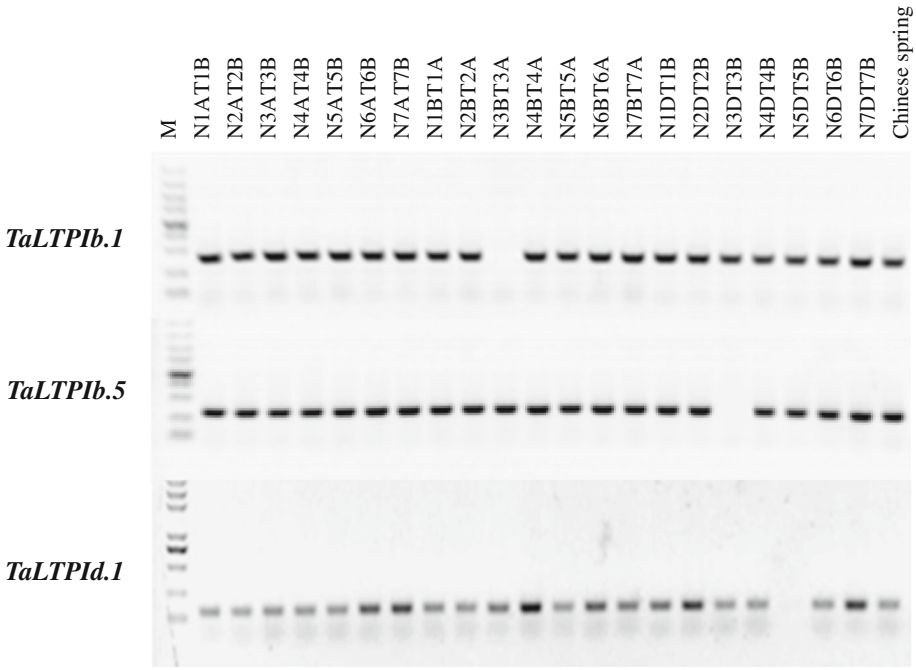

Fig. 1 Chromosome distribution of *TaLTPIb.1*, *TaLTPIb.5* and *TaLTPId.1*. Gene specific primers were used to amplify a Chinese Spring (CS) nulli-tetrasomic set. N3BT3A (nullisomic 3B–tetrasomic 3A), N3DT3B (nullisomic 3D–tetrasomic 3B), and N5DT5B (nullisomic 5D–tetrasomic 5B); M, 1 kbp ladder

TaLTPIb.1 and *TaLTPIb.5* were both up-regulated from 9 h, while *TaLTPId.1* showed a down-regulated response to this stress (Fig. 2a). *TaLTPIb.1* was strongly up-regulated by chilling stress, 9 h after treatment, reaching the peak of 12.5-fold at 24 h, while *TaLTPIb.5* was induced at a lower expression level of 3.6-fold. The expression of *TaLTPId.1* was only slightly induced in the first 24 h (2.8-fold) and recovered subsequently (Fig. 2$a_{1–3}$). Similarly, *TaLTPIb.1* and *TaLTPIb.5* were slightly down-regulated by dark treatment for 24 h, while, *TaLTPId.1* was strongly up-regulated (Fig. 2$a_{1–3}$). For wounding, the expression of *TaLTPIb.1* and *TaLTPIb.5* were strongly induced in the first hour, while no response was observed for *TaLTPId.1* (Fig. 2$a_{1–3}$).

In order to investigate the possible involvement of a regulatory pathway, the transcriptional response of *TaLTPIb.1*, *TaLTPIb.5*, and *TaLTPId.1* against various hormones was carried out by qRT-PCR. The expression of *TaLTPIb.1* and *TaLTPIb.5* were both sharply induced by SA in the first 3 h. The expression of *TaLTPIb.1* was depressed by MeJA and IAA, while the expression of *TaLTPIb.5* was depressed by ABA (Fig. 2b_1, b_2). *TaLTPId.1* seemed to be gradually stimulated by SA and MeJA, but depressed by IAA (Fig. 2b_3).

Localization of *TaLTPIb.1*, *TaLTPIb.5,* and *TaLTPId.1*

Type I *TaLTP* genes were found to be expressed in various organs but the expression feature was found to be highly conserved in epidermal cells (Wang et al. 2012). Here, we tested the promoter activity of *TaLTPIb.1* in a transgenic brachypodium. The vector, pCambia1391Z, was transformed in plant as a negative control that showed no GUS activity (Fig. 3a, b). The promoter activity of *TaLTPIb.1* was observed mainly in young leaves (Fig. 3c), shoots, and spikes (Fig. 3d), but was not observed in roots. A section of

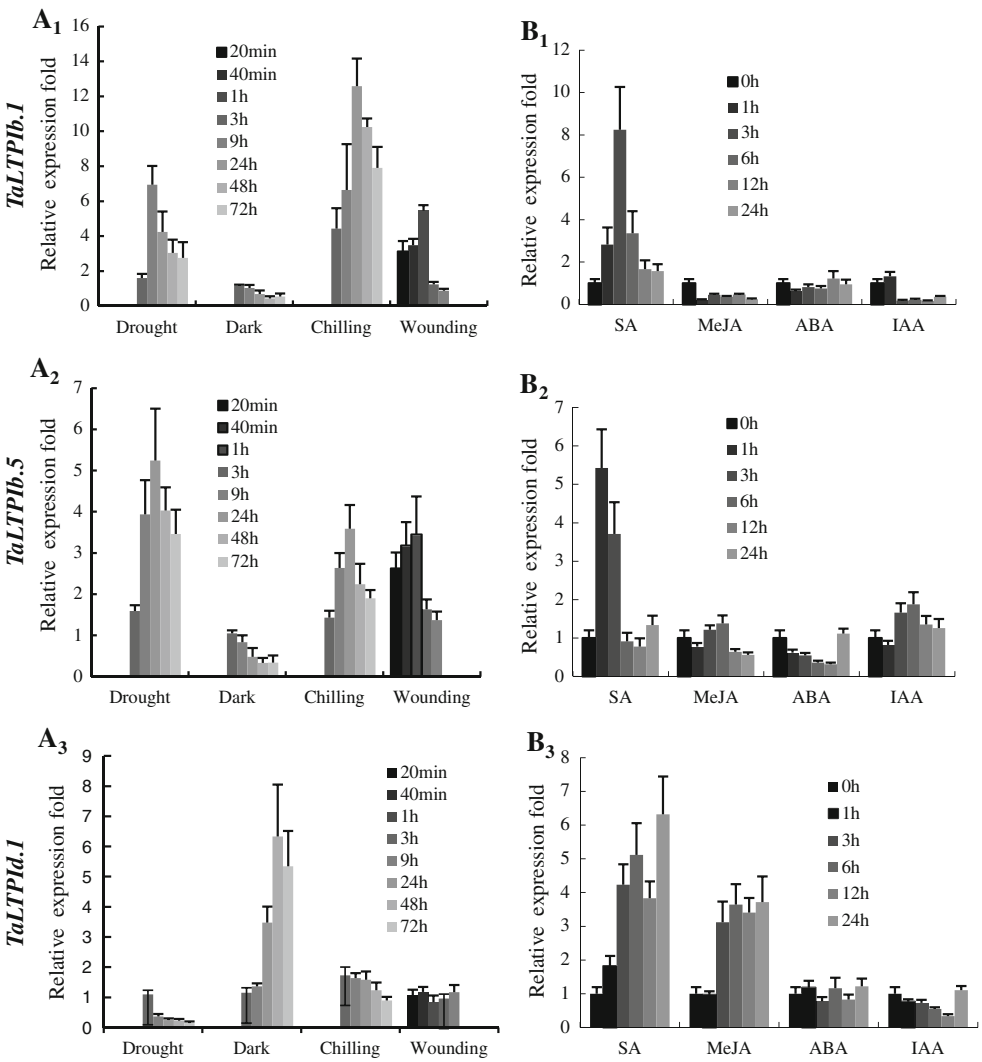

Fig. 2 Transcription profiles of *TaLTPIb.1*, *TaLTPIb.5* and *TaLTPId.1* against abiotic stresses and hormone stimuli. **a_1–a_3** The stress treatments were applied by subjecting 10-day-old seedlings to *dark* (3, 9, 24, 48 and 72 h), *wounding* (20, 40 min, 1, 3 and 9 h), *drought* (PEG, 25 %; 3, 9, 24, 48 and 72 h), *chilling* (4 °C; 3, 9, 24, 48 and 72 h) conditions; **b_1–b_3** For hormone treatments, the material samples were harvested from the plants treated with *MeJA* (100 μM), *SA* (2 mM), *ABA* (100 μM), and *IAA* (100 μM) at 1, 3, 6, 12 and 24 h after the treatments. All of the expression values were presented as fold changes compared with control. The wheat 18 s rRNA was used internal control (Supplementary Table 1). The gene expression was shown as mean ± SEM for three to four independent experiments

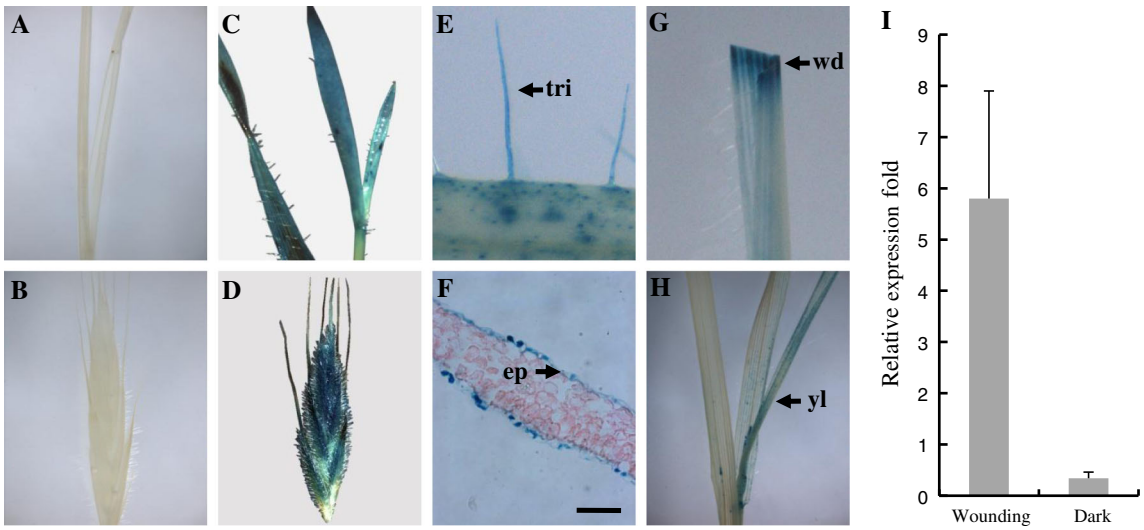

Fig. 3 Tissue specific expression of *TaLTPIb.1* promoter::*uidA* fuisions in brachypodium. The promoter region of 723 bp upstream start codon was employed for promoter-reporter construction and transformed in brachypodium. T_2 seedlings were used for GUS activity observation during the plant whole life cycle. **a**, **b** The GUS staining pattern of transgenic brachypodium harboring vector of pCambia1391Z; **c** the expression of *TaLTPIb.1*p:*uidA* at seedling stage; **d** the expression of *TaLTPIb.1*p::*uidA* in spike; **e** the expression of *TaLTPIb.1*p::*uidA* on trichome; **f** the leaf section; **g** the wound shoot; **h** the expression of *TaLTPIb.1*p:*uidA* at seedling stage under dark condition; **i** the GUS quantities under stress conditions was evaluated using leaf tissues. *wd* wouding, *ep* epidermis, *bar* = 50 μm

leaf revealed that the expression of *TaLTPIb.1* was located in epidermal cells (Fig. 3f), including trichomes (Fig. 3e). Consistent with the results of qRT-PCR, the promoter activity could be induced by wound stress, where over fivefold of GUS activity on the leaf was stimulated 1 hour after application of the wounding treatment (Fig. 3g, i). Moreover, the major GUS activity was restricted to only young emerging leaves when applying dark treatment for 3 days. The GUS activity was depressed as much as 88 % compared with the control (Fig. 3h, i).

To examine the subcellular localization of these TaLTPs, we constructed the EGFP fusion proteins under the control of the 35S promoter and transitionally expressed the proteins in *N. benthamiana* leaves. For the negative control, the transient expression of *35S:EGFP* was observed in the cytosol and nuclei with a weak signal (data not shown). When fused with the EGFP, all of the 3 TaLTPs were exclusively expressed in the plasma membrane (Fig. 4a, b), which was confirmed by the well-merged signal with the marker line of pm-rb CD3-1008 (Nelson et al. 2007). Furthermore, small vehicle like structures were observed for *TaLTPId.1* in the cytosol, which overlapped with chloroplast autofluorescence (Fig. 4c). To further confirm the localization of these TaLTPs, we transformed the three *TaLTP:EGFP* constructs into the onion epidermis and all of them directed the same signal pattern. For example, the TaLTP1:EGFP fusion was originally found around the cell wall, while it was subsequently confirmed in plasma-membrane via plasmolysis of onion epidermis (Figure S3).

Overexpression of *TaLTPIb.1*, *TaLTPIb.5,* and *TaLTPId.1* in *Arabidopsis*

To further examine their functions, we generated transgenic *Arabidopsis*, overexpressing each gene, for stress tolerance test. Two independent transgenic lines (T_3) were selected depending on the expression levels under normal conditions (Fig. 5a_2, b_2, c_2). When grown in normal conditions in soil or plates, no significant difference in phenotypes was observed between the wild type and transgenic plants (data not shown). For drought stress, irrigation of 2-week-old transgenic plants was stopped for 10 days and no significant phenotypes were observed compared with the control plants (data not shown). For the chilling tolerance test, the root length test showed that the transgenic seedlings of all the 3 genes grew faster and their roots were significantly longer (*t* test, $P < 0.01$) than the wild type plants (Fig. 5a_1, a_3, b_1, b_3, c_1, c_3). This result implied that the heterogeneous overexpression of *TaLTPIb.1*, *TaLTPIb.5*, and *TaLTPId.1* in *Arabidopsis* can promote seedling growth under conditions of chilling. Interestingly, compared with wild type plants, we observed no significant changes of these transgenic lines in their phenotype when subjecting plants to freezing condition (data not shown).

Discussion

Many type I *nsLTP*s were found to be abundantly expressed on the surface of plant aerial tissues and showed a

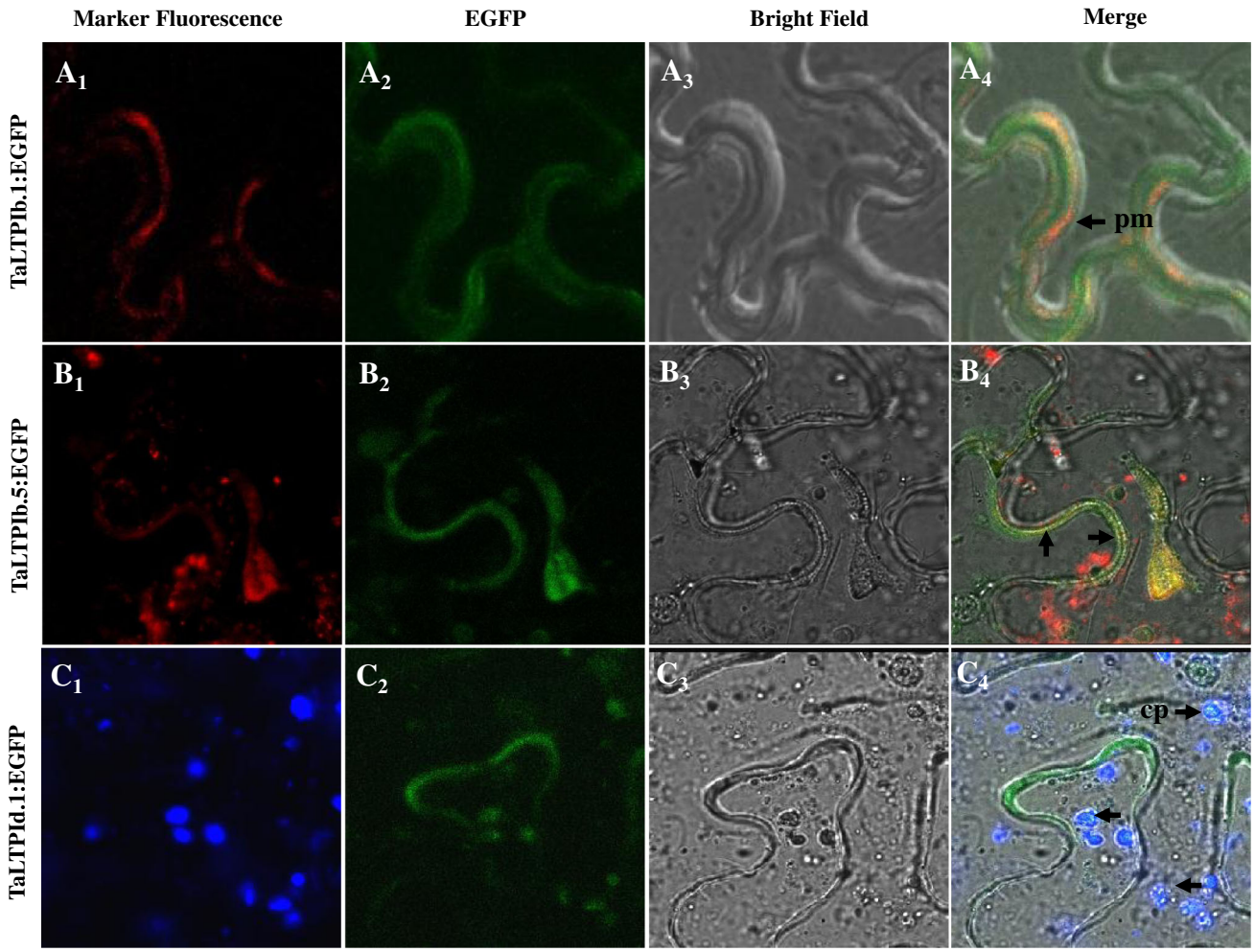

Fig. 4 Subcellular localization of *TaLTP:EGFP* fusion proteins in tobacco leaves. *Agrobacterium* strains GV3101 harboring each construct of TaLTP:EGFP were transiently expressed in *Nicotiana* leaves. $\mathbf{a_1}$–$\mathbf{a_4}$ Subcellular localization of *TaLTIb.1:EGFP* in tobacco leaf cells; $\mathbf{b_1}$–$\mathbf{b_4}$ subcellular localization of *TaLTIb.5:EGFP* in tobacco leaf cells; $\mathbf{c_1}$–$\mathbf{c_4}$ subcellular localization of *TaLTId.1:EGFP* in tobacco leaf cells. Images were captured and merged by *z* series optical sections after 3 days of agro-infiltration. *pm* plasma membrane, *cp* chloroplast, *bar* = 20 μm

broad transcriptional response to abiotic stresses. However, direct evidence of these small peptides in abiotic stress tolerance was rarely known. In this study, overexpression of three *TaLTP*s in *Arabidopsis* resulted in enhanced chilling tolerance of the transgenic plants, supporting their functional involvement.

The mechanism by which plants perceive changes in temperature, transfer signals and switch on the chilling tolerance pathway is relatively unknown. Numerous physiological and biochemical changes in plants were changed under chilling conditions, including the ABA level, the membrane lipid composition, and the accumulation of compatible osmolytes (Sanghera et al. 2011). The cell membranes are thought to be the primary damage sites under chilling stresses and may play important roles in signal transduction and in the chilling resistance machinery, in which the increase in unsaturated fatty acids is thought to be an effective strategy for living cells to adapt to chilling temperatures (Sanghera et al. 2011). Computational and biochemical analysis has demonstrated that some type I nsLTPs were able to accommodate various kinds of lipids such as phospholipids (Shin et al. 1995; Vergnolle et al. 1992), palmitic acid (C16:0), and acyl chains of the 1,2-dimyristoylphosphatidylglycerol (Shin et al. 1995). In this study, we found that all the 3 *TaLTP* genes were mainly located on the plasma membrane although small differences were found for *TaLTPId.1* that was also localized in the chloroplast (Fig. 4). Thus, it is possible that these TaLTPs may function by transferring corresponding lipids and modifying the permeation of plasma membrane in order to reduce the damage caused by chilling stress. Previously, an epidermis-specific expression

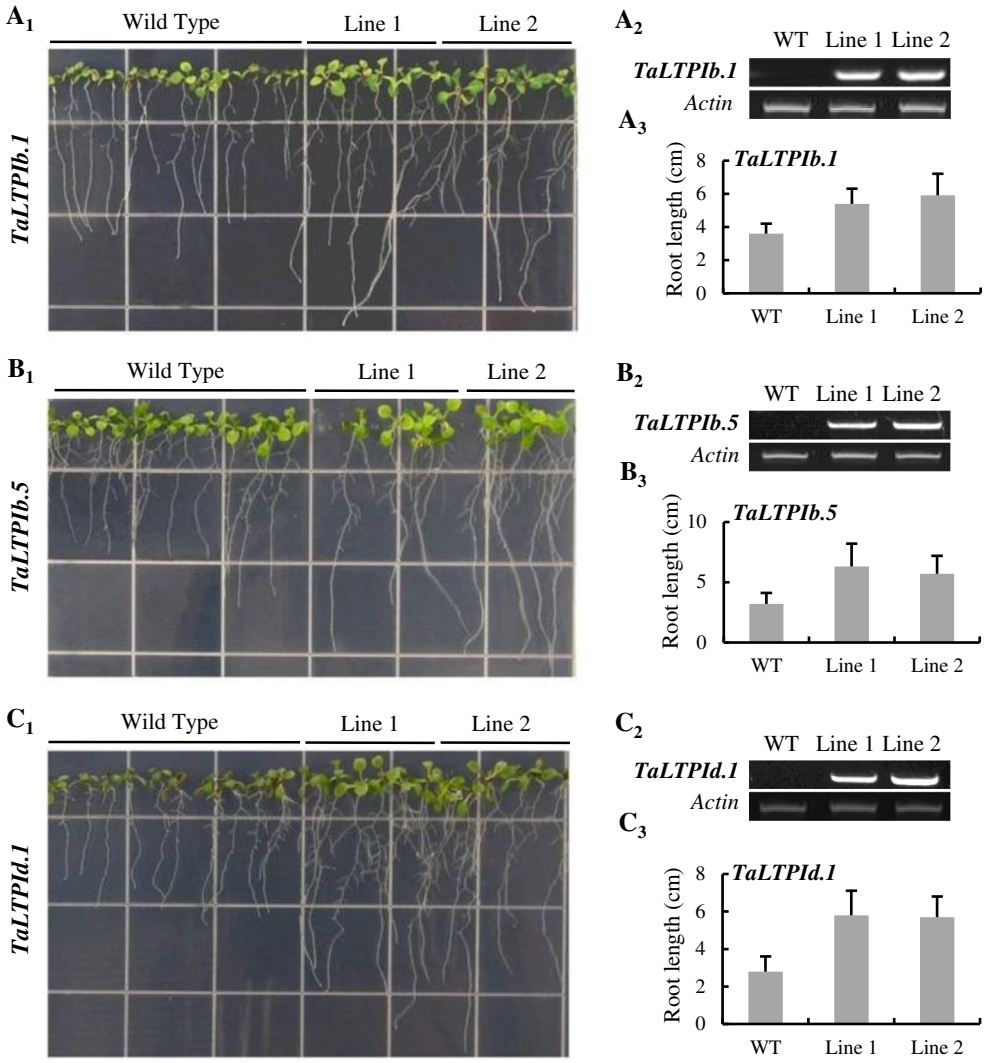

Fig. 5 *TaLTPIb.1*, *TaLTPIb.5* and *TaLTPId.1* contribute to the chilling tolerance in *Arabidopsis*. **a$_1$–c$_1$** Phenotype of the transgenic plants under chilling stress (4 °C); **a$_2$–c$_2$** the expression level of transgenic lines by semi-quantitative RT-PCR; **a$_3$–c$_3$** root length of the transgenic plants under chilling stress (4 °C). Each data point is average of three experiments and *bars* indicate SD

of type I *nsLTP* genes had been revealed, supporting their functional involvement in cuticle synthesis. This expression pattern was further verified in this study when transforming the construction of *TaLTPIb.1::uid*A in brachypodium (Fig. 3). It is not known whether these TaLTPs contribute to the chilling tolerance as well as cuticle synthesis. However, the cuticle composition in plants was also found to associate with cold and drought tolerance, indicating the common underlying mechanisms (Amid et al. 2012).

Indeed, a bulk of previous data and the current study demonstrate a strong transcriptional response of *nsLTP* genes against drought stress, while we observed no phenotypic changes when testing the *TaLTP* overexpressing *Arabidopsis*. One possible explanation may be the functional redundancy in *Arabidopsis* itself that contains over 30 members of *nsLTP* genes. Moreover, none of the transgenic lines in this study exhibited significant improved tolerance for freezing, which should be similar to chilling tolerance. Recently, overexpression of *AtLTP3*, in a native plant, was found to enhance the drought and freezing tolerance, which was observed to be located in the whole cell cytoplasm (Guo et al. 2013). Considering the different subcellular localization of these nsLTP genes, it may be presumed that the lipids transferring to different membranes may result in a functional difference in these gene copies.

The type I *nsLTP*s were extensively duplicated in cereal, many of which were preserved in genome via concerted evolution resulting in functional redundancy. For instance, six pairs of type I *OsLTP*s located on a duplication block of chromosomes 11 and 12 (Paterson et al. 2009; Wang et al.

2012), which fell together with the three *TaLTP*s in this study. *TaLTPId.1* located in the same branch with two pairs of *OsLTP*s undergoing concerted evolution (Os11g02400 and Os12g02330, Os11g02424 and Os1202340). However, only three wheat members were found in the sub-branch where *TaLTPId.1* located and no concerted evolution signature was observed for these *TaLTP*s. Considering the hexploid nature of wheat, it was supposed that some wheat members in this sub-clade were lost. In contrast, *TaLTPIb.1* and *TaLTPIb.5* were in a wheat-specific gene cluster containing 23 copies (Figure S1), which should be extensively duplicated recently but not only due to the polyploidy process during evolution of wheat (Brenchley et al. 2012). It is rather difficult to clarify the duplication story of these gene copies. At the transcript level, clear redundancy was found for *TaLTPIb.1* and *TaLTPIb.5*. Both the genes were up-regulated by drought, chilling and wounding, but depressed by darkness, indicating high conservation of these 2 genes in both the coding and non-coding regions during evolution (Fig. 2a). On the other hand, *TaLTPId.1* showed a relatively distinct expression profile compared with *TaLTPIb.1* and *TaLTPIb.5*, which was up-regulated by MeJA, darkness, and showed a signal in the chloroplast, implying different biological roles. However, the transgenic *Arabidopsis* lines overexpressing these 3 gene copies all constitutively enhanced the tolerance to chilling stress, demonstrating the functional redundancy of *TaLTPIb.1*, *TaLTPIb.5,* and *TaLTPId.1* at the protein level. Perhaps, the functional divergence between *TaLTPId.1* and the other two genes may be partial through regulatory changes in the non-coding and coding regions.

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Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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