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The involvement of an expansin gene*TaEXPB23* from wheat in regulating plant cell growth

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Abstracts

Expansins, found in the cell wall, have the unique ability to induce immediate cell wall extension. In this study, a β-expansin gene (*TaEXPB23*) isolated from wheat (*Triticum aestivum* L.) coleoptiles was transformed to tobacco (*Nicotiana tabacum*) to investigate its role in plant growth and development. *TaEXPB23* was preferentially expressed in wheat coleoptile and a close correlation between *TaEXPB23* expression and coleoptile growth was observed. The over-expression of *TaEXPB23* in tobacco also resulted in accelerating growth of leaves and internodes at earlier developmental stages, and it was involved in regulating plant development.

Additional key words: coleoptile, gene expression, *Nicotiana tabacum*, RT-PCR, transgenic tobacco, *Triticum aestivum*.

Introduction

Plant growth is the result of cell division and cell enlargement, accompanied by an increase in the plasma membrane and cell wall surface area. The regulation of cell extension is critical for plant growth and morphology (Matin *et al.* 2001, Smith 2003). Previous research has suggested that the direction and magnitude of primary cell wall extension are determinants of the expansion pattern and the final cell shape and size (Matin *et al.* 2001, Smith 2003). The plant cell wall is a rigid network of interwoven polymers, with substantial mechanical strength, which must be overcome or reduced to permit wall extension and cell growth (Carpita and Gibeaut 1993, Cosgrove *et al*. 1997). Proteins in the cell wall are believed to play important roles in the regulation of cell wall extensibility, a key parameter in determining cell expansion (Zenoni *et al.* 2004).

Plant cell walls extend or relax by a process of molecular 'creep', in which the cellulose microfibrils and associated matrix polysaccharides separate one from another (Marga *et al*. 2005). Such molecular creep occurs only when the cell wall is loosened by expansins or by other factors. Expansins were first identified in 1992 by Cosgrove and his co-workers (McQueen-Mason 1992) and are thought to control plant cell growth by a variety of mechanisms, such as loosening the cell wall (Wu *et al.* 1996) and stimulating cell enlargement (Cho and Kende 1997a,b). Expansin's effects on cell walls are distinct from those expected of hydrolytic enzymes, they do not have hydrolytic activity or any of the other enzymatic activities yet assayed (Li and Cosgrove 2001, Sampedro and Cosgrove 2005). It is proposed that expansin disrupts noncovalent bonds that tether matrix polysaccharides to the surface of cellulose microfibrils or to each other (Cosgrove 2000). In this model, the expansin is thought to act like a zipper that enables microfibrils to move apart from each other by ungluing the chains that stick them together, but without causing major covalent alterations of cell walls (for review see, Sampedro and Cosgrove 2005).

The expansin expression patterns are consistent with their involvement in plant growth (Cho and Cosgrove 2000, Pien *et al.* 2001). The correlation of expansin activity with growth rates was observed, *e.g*., in oat coleoptile (Cosgrove and Li 1993). Additionally, Cho and Kende (1997a,b) also implicated expansin gene expression in internode elongation of deepwater rice when submerged.

Expansins are encoded by a large superfamily, which is made up of four families, designated α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). EXPA and EXPB proteins are known to have cell-wall loosening activity and to be involved in cell expansion and other developmental events during which cell-wall modification occurs (Sampedro

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Abbreviations: RT-PCR - reverse transcription - polymerase chain reaction; WT - wild type.
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and Cosgrove 2005), sharing only about 20 to 30 % of amino acid identity, however, their predicted secondary structures are up to 75 % identical (Cosgrove *et al.* 1997, Cosgrove 2000). They are typically 250 - 275 amino acids long and are made up of two domains (domain 1 and domain 2) preceded by a signal peptide. EXPA proteins and some, but not all, EXPB proteins are implicated as catalysts of the enlargement of plant cells stimulated by low extracellular pH. While the role of α-expansins in cell extension has been reported in many plants (Cosgrove 2000, McQueen-Mason and Rochange 1999), β-expansins were originally represented by group I allergens of grass pollen (Cosgrove *et al.* 1997), with additional research suggesting β-expansins are more abundant in grasses than in dicots (Cosgrove *et al.* 1997, McQueen-Mason and Rochange 1999). Research has also demonstrated that the maize (*Zea mays*) pollen allergen can loosen the cell wall of maize silk and wheat (*Triticum aestivum*) coleoptile, as well as cucumber hypocotyls (*Cucumis sativus*), albeit to a lesser extent (Li *et al.* 2003). These data suggest that β-expansins may be the primary wall-loosening proteins in grasses (Lee and Kende 2001) which have a significantly different cell wall composition compared to dicots (Carpita 1996). Others have reported that α-expansins had lower activity on grass cell walls than on dicot cell walls, with grasses containing far greater numbers of putative β-expansin genes than dicots (Cho and Kende 1997a, McQueen-Mason and Rochange 1999, Lee and Kende 2001).

The plant cell wall not only plays a central role in protection, but also in shape development of the individual cells, thereby establishing the morphology of the whole plant, and ultimately determining the production of the economic important plants. Many biotic and abiotic stresses interfere with plant growth by impacting cell wall (Liu *et al*. 2007, Šimonovičová *et al*. 2004, Procházková and Wilhelmová 2007). Although expansins are currently believed to play important roles in regulating plant cell wall extensibility, the exact mechanism by which these proteins mediate plant growth and development is still unclear. Most of the published research on expansin proteins has focused on α-expansins, with few studies investigating β-expansins. Wheat coleoptile is a good experimental unit to study cell elongation regulation as the elongation of wheat coleoptile is primarily attributed to cell expansion after seed germination (Wang *et al.* 1999, Huang *et al.* 2000). In this study, a β-expansin gene (named *TaEXPB23*, GenBank accession No. AY260547) was isolated from wheat coleoptiles (a grasses species), and transformed into tobacco (dicotyledonous species) to investigate the roles of β-expansin in plant growth and development. Our results suggested that *TaEXPB23* is not only involved in plant cell growth but also in plant development.

Materials and methods

Plants and growth conditions: Wheat (*Triticum aestivum* L. cv. HF0621) seeds were surface-sterilized in 0.1 % $HgCl_2$ (m/v) for 5 min, rinsed with tap water and distilled water and germinated in glass plates containing two layers of filter paper saturated by distilled water. The seedlings were cultivated in a growth chamber at dark and temperature of 25 °C. Coleoptile length was measured at 0, 4, 8, 12, 20, 24, 36, 48 and 72 h after the first day of germination. Experiments were independently replicated at least three times with 10 plants per experiment. The growing profile curve was portrayed with *SigmaPlot 2000*. Statistical analysis was conducted using the procedures of DPS (Zhejiang University, China).

Sterilized tobacco (*Nicotiana tabacum* L. cv. NC89) seeds were germinated on Murashige and Skoog (MS) medium in a growth chamber (25 °C, 16-h photoperiod with irradiance of 300 μmol m^{-2} s^{-1}) and their leaves were used for transformation.

Positive T_0 transgenic tobacco seedlings were grown on MS medium in the growth chamber for 3 weeks and then transferred to earthen pots (30 cm in height and 25 cm in diameter) filled with 7.5 kg soil.

Tobacco T_1 seeds, including wild-type, transgenic plants (pB121XB1S or Ln) and controls (or L_0) were initially grown on MS medium. Phenotypic parameters such as plant height, number of leaves and length of internodes were observed at the 14th week after planting.

RT-PCR analysis of gene expression: Plant tissue samples were stored in liquid N_2 at -70 °C until used for RNA isolation. Total RNA from wheat coleoptiles, which were grown in dark for 0, 1, 2, 3, 4, 8, 12, 20, 24, 36, 48 and 72 h, was isolated according to the manufacturer's protocol (ADM1701, *Promega*, USA), and subsequently used for reverse transcription polymerase chain reaction (RT-PCR). Total RNA from coleoptiles was treated with DNase I (RNase-free, *Promega*) and reverse transcription was performed using the primer (5′-TTTTTTTTT-TTTTTTTTT-3′) and the M-MLV reverse transcriptase (*Promega*). Independent PCR reactions with equal aliquots (0.2 mm^3) of cDNA samples were performed using special primes *EXP5* (5′-GGTCAGTGC-GCTGGTACA-3′) and *EXP6* (5′-ATACCAAATCAA-GCCAAT-3′). Primer sets resulted in a product containing at least an intron, allowing a direct confirmation that RT-PCR products were of mRNA origin. Annealing conditions were empirically determined. Preliminary experiments demonstrated that the amount of PCR product increased with increasing numbers of cycles, indicating that the reaction components were not a major limiting factor (datum not shown). Amplification of the *a-tubulin* gene (using primers *Tubulin1*, 5′-ATCTGTGCC-TTGACCGTATCAGG-3′ and *Tubulin2,* 5′-GACATC-AACATTCAGAGCACCATC-3′) exhibiting constitutive expression, was used as a positive control, from which a linear relationship between the amount of RNA used for

amplification and the amount of cDNA fragment amplified, as well as the quality of both extracted RNA and RT-PCR reactions were determined. The RT-PCR products were separated on a 1.0 % (m/v) agarose gel.

The amplification conditions were as follows: 28 cycles; 1 min at 94 °C, 45 s at 55 °C, and 1 min at 72 °C. Experiments were independently replicated at least three times and the fragment was sequenced by *Invitrogen Life Technologies*, which proved that the fragment amplification was specific. The relative signal density was analyzed by a bio-light *Tanon4100* (Shanghai, China).

Construction of pBI121XB1S vectors: A fragment containing the coding region of *TaEXPB23* was cloned into a vector pBI121 downstream of the cauliflower mosaic virus (CaMV) 35S promoter and plasmid DNA constructs were isolated from cells cultured overnight, digested with restriction endonucleases (XbaI and SalI), and used to confirm desired sequences. Constructs were further verified by PCR, sequenced by *Invitrogen Life Technologies*, and then introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw (An *et al.* 1988). Transformed *Agrobacterium* cells were screened on solidified Luria-Bertani (LB) medium containing 100 mg cm^{-3} kanamycin after incubation (3 d, 28 °C, 200 rpm, in the dark). The colonies were picked and cultured in 2 cm^3 liquid LB medium overnight at 28 °C in the dark, with vigorous shaking (200 rpm).

Transformation of pBI121XB1S to tobacco plants: Recombinant plasmids and the pBI121 containing the CaMV 35S promoter and β-glucuronidase (GUS) without TaEXPB23 as control were introduced into *Nicotiana tabacum*. Transformants were verified using PCR with the primer sets (EXPB5 and EXPB6), antibiotics (100 mg dm^{-3} kanamycin) and RT-PCR. The transformants harboring recombinant plasmids were named pBI121XB1S and Ln.

Results

Characterization of *TaEXPB23* full-length cDNA: An expansin gene, named *TaEXPB23* (GenBank accession No. AY260547), was isolated from wheat coleoptiles, based on the obtained cDNA sequences. The similarities of its amino acid sequence with other 25 deduced expansin amino acid sequences from GenBank were analyzed and phylogenetic tree was constructed by neighbor joining. The data (Fig. 1) suggested that TaEXPB23 belonged to the β-expansin subfamily. The amino acid sequences of TaEXPB23 also displayed higher similar characteristics to other wheat β-expansins (Lin *et al.* 2005), slight lower similarity to other β-expansins from dicots, and the lowest

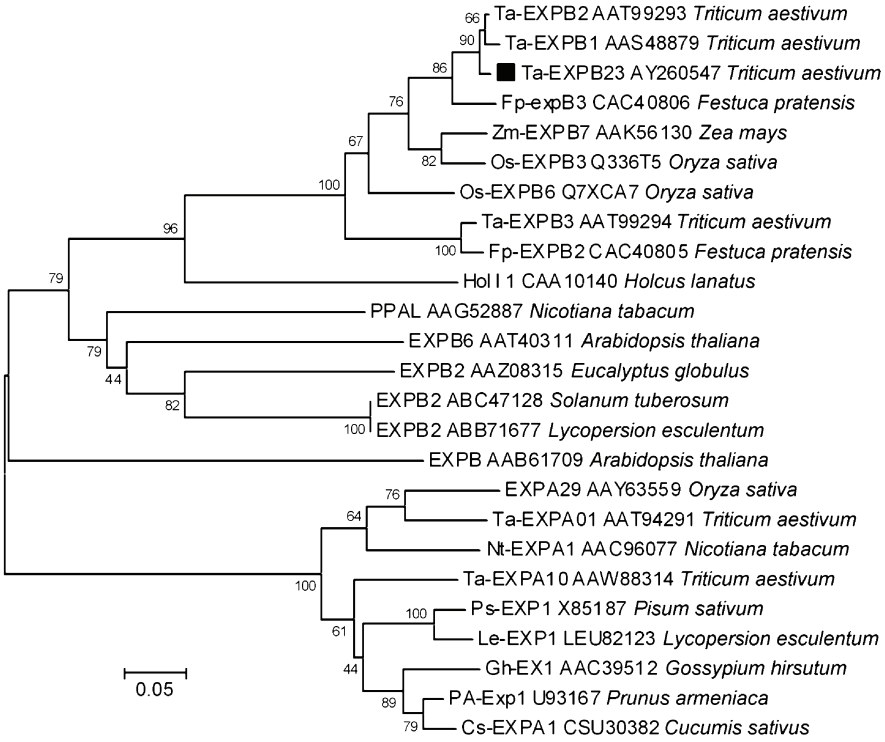

Fig. 1. Phylogenetic tree of wheat coleoptile TaEXPB23 protein sequence and other expansins. Constructions are performed based on the predicated amino acid sequence of protein regions from GenBank. The tree was constructed using a neighbour-joining method. Gaps are completely deleted. The scale bar is 0.05, which refers to percentage of divergence.

similarity to α-expansins. Structure identity of TaEXPB23 to other expansins was also displayed according to amino acid sequences, such as two domains characteristic of expansin proteins, a cysteine-rich region and a tryptophan-rich C terminal portion, five conserved cysteines, a "GACG" motif in the cysteine-rich region, and three tryptophans near the C terminus in the deduced amino acid of TaEXPPB23 (data not shown).

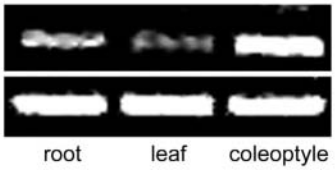

Fig. 2. Expression patterns of *TaEXPB23* in young seedlings by RT-PCR in coleoptiles, leaves and roots: mRNA abundance of *TaEXPB23* gene transcription (*above*), RT-PCR reactions with *α-tubulin* gene as control (*below*).

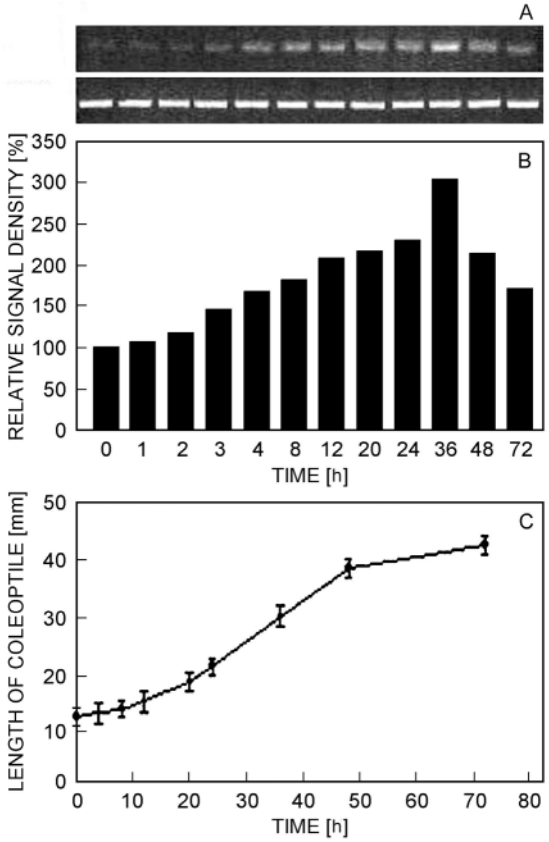

Fig. 3. The temporal expression of the *TaEXPB23* gene and cell extension speed during coleoptile growth. *A* - mRNA abundance of *TaEXPB23* gene expression detecting by RT-PCR in wheat coleoptile (*above*), RT-PCR with *α-tubulin* gene as control (*below*). *B* - The relative signal density of *TaEXPB23* gene expression at different time points of coleoptile growth. The expression abundance at 0 h was used as control. *C* - coleoptile growth curve.

Expression of *TaEXPB23* in wheat: The organ-specific expression pattern of *TaEXPB23* transcripts was characterized in wheat coleoptiles, leaves and roots at seedling stage utilizing RT-PCR (Fig. 2). High transcript levels of *TaEXPB23* were detected in wheat coleoptiles, intermediate levels in roots, with little noted in leaves under the same conditions at this stage. This data suggested *TaEXPB23* gene was preferentially expressed in wheat coleoptiles, with almost a 2.5-fold increase compared to that in roots.

The temporal expression profile of *TaEXPB23* during the development of wheat coleoptile was analyzed (Fig. 3*A*), and the relative signal density was shown in Fig. 3*B*. The results demonstrated that the expression levels began to increase after germination, and mRNA accumulation increased notably at about 36 h and then decreased, corresponding to the growth speed of coleoptiles (Fig. 3*C*). The slope of growth profile curve was indicative of growth speed (Fig. 3*C*), indicating that the fastest speed of coleoptile growth presented at about 36 h after germination, consistent with the highest level of *TaEXPB23* mRNA accumulation (Fig. 3*A*).

Plant transformation, identification and phenotypic alteration of *TaEXPB23* transgenic tobacco plants: *TaEXPB23* gene was transformed to tobacco. In T_0 lines, 30 transgenic plants harboring the sense construct of *TaEXPB23* gene (shown as pB121XB1S or Ln) and 18 plants harboring the vector alone without *TaEXPB23* gene sequence (shown as control or L_0) were obtained. The data suggested that almost all the sense transgenic plants grew faster at early developmental stages (data not shown). However, no significant differences in morphology were observed between wild-type plant (WT) and control at seedling stage. Total RNA was extracted from leaves of L1 to L6 transgenic lines, control and WT (Fig. 4). The presence of the *TaEXPB23* gene was confirmed by RT-PCR with *TaEXPB23* specific primer sets. Different levels of *TaEXPB23* gene mRNA were shown in L1 to L6. Among them the higher levels were in L1 and L2, demonstrating the effective transcription in T1 lines. There was not *TaEXPB23* gene expression in WT and control.

L1 and L2 transgenic tobacco plants were used to investigate phenotypic alterations at different developmental stages. The number of leaves and roots, plant height and length of internodes and roots were detected at the 3rd (Fig. 5) and the 14th (Fig. 6) week after planting. It was shown that TaEXPB23 transgenic plants grew faster than control tobacco plants and that the transgenic plants

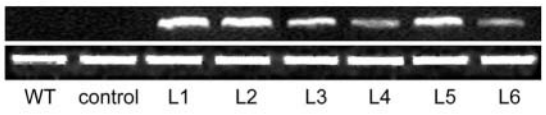

Fig. 4. *TaEXPB23* gene expression in different T1 lines transgenic tobacco plants by RT-PCR. WT, wild-type tobacco; control, transformed tobacco plants harboring 35S::GUS vector alone; L1 - L6, different lines of *TaEXPB23* gene transformed tobacco plants. mRNA abundance of *TaEXPB23* gene transcription (*above*); positive control of RT-PCR reactions with *α-tubulin* gene (*below*).

had more leaves and roots than control plants at seedling stage (an average of seven leaves in L1 plants and five in control plants). Additionally, four roots were observed in L1, but only one was developed and almost no branch roots emerged in controls.

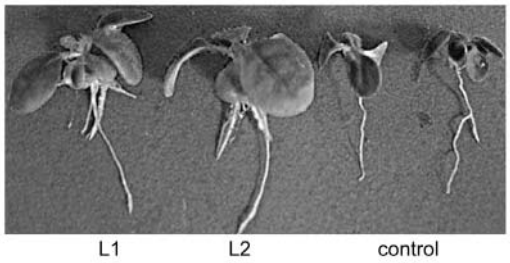

Fig. 5. Phenotypic alterations of T1 transgenic plants (L1, L2) in comparison with control at the 3rd week.

Significant phenotypic alterations of tobacco plants (L1 of T1 lines) especially in plant height and leaf size were induced by the transformation of *TaEXPB23* at the 14th week age (Fig. 6), and the similar changes were also found in T_0 and T_2 lines (data not shown). At the 14th week after planting, L1 plant height was averagely 44.5 cm, approximately 20 cm higher than controls; longer stalk internode length was averagely 3.95 cm compared to 1.58 cm in controls, and the average number of leaves was 20 compared to only 14 in controls.

Fig. 6. Phenotype of T1 transgenic plants carrying the *TaEXPB23* gene, controls and wild-type plants (WT) at the 14th week.

Discussion

Expansin genes have been identified in a range of plants including wheat (Lin *et al.* 2005). Expansin proteins participate in developmental processes (Lee and Kende 2001, Cosgrove *et al.* 2002). There is also strong evidence in transgenic plants implicating endogenous expansins in the regulation of vegetative growth (Pien *et al.* 2001, Choi *et al.* 2003). In this paper, the expression patterns and effects of overexpression a β-expansin (*TaEXPB23*) isolated from wheat on transgenic tobacco were studied. The phylogenetic analysis data (Fig. 1) suggested that TaEXPB23 was highly conserved in β-expansin and the higher mRNA level was observed in coleoptiles compared to roots and leaves at the seedling stage (Fig. 2). The temporal expression profile of TaEXPB23 corresponded to the growth speed profile in wheat coleoptiles (Fig. 3). This implicated that TaEXPB23 played a key role in the modulation of the growth in wheat coleoptiles, consistent with previous research (Cho and Kende 1997b, Choi *et al*. 2003, Lin *et al*. 2005, Belfield *et al*. 2005).

Transgenic plants overexpressing *TaEXPB23* gene had higher plant height, longer stem internode, larger leaf blades, and more leaves and roots than controls at seedling stage (Figs. 5, 6). This suggested that TaEXPB23 was involved in cell expansion and organ growth, consistent with previous research (Wu *et al.* 1996). The results obtained (Fig. 6) also suggested a role of expansins in modulating developmental progress, because the transgenic plants developed flowers earlier than WT and control plants. Overexpression of the *TaEXPB23* gene resulted in enhancing plant growth speed and increasing height of transgenic seedlings, primarily due to increased internode length. Additionally, there was an increase in the number of leaves. These data supported the hypothesis that expansin was an important control point for regulation of plant cell growth (Lin *et al.* 2005, Belfield *et al.* 2005) and plant development.

There are two types of cell walls, type I (dicots and of non-commelinoid monocot species) and type II (Carpita and Gibeaut 1993, Carpita 1996). Previous research has demonstrated that α-expansins from both dicots and grasses were more effective in inducing extension *in vitro* (Cho and Kende 1997a). Li *et al.* (2003) suggested that α- and β-expansins acted on different matrix polysaccharides; β-expansins had a more dominant role in grasses than in dicots and other type I wall species. Lee and Choi (2005) reported differences in subcellular localization and solubility between α- and β-expansins, as α-expansins were bound to the cell wall and were soluble in extraction buffer containing 1 M NaCl, while extraction of OsEXPB3, a β-expansin of rice, required SDS. Immunogold labeling and electron microscopy showed that OsEXPB3 protein was located in the primary cell walls. Sampedro and Cosgrove (2005) showed that β-expansin proteins have some characteristics similar to that of α-expansins, *e.g*. pH dependence. In this study, the β-expansin TaEXPB23 from coleoptile also had some characteristics similar to α-expansins. There were some significant effects of TaEXPB23 on the growth and development of tobacco, a dicot species with a type I cell wall, for the constitutive expression of *TaEXPB23*, a β-expansin

gene from monocot species with a type Ⅱ cell wall.

In conclusion, our data suggested that β-expansin TaEXPB23 functions not only in cell growth but also in plant development. Further research is needed to fully elucidate the mechanism of it.

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