The AtXTH28 Gene, a Xyloglucan Endotransglucosylase/Hydrolase, is Involved in Automatic Self-Pollination in Arabidopsis thaliana

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Successful automatic self-pollination in flowering plants is dependent on the correct development of reproductive organs. In the stamen, the appropriate growth of the filament, which largely depends on the mechanical properties of the cell wall, is required to position the anther correctly close to the stigma at the pollination stage. Xyloglucan endotransglucosylase/hydrolases (XTHs) are a family of enzymes that mediate the construction and restructuring of xyloglucan cross-links, thereby controlling the extensibility or mechanical properties of the cell wall in a wide variety of plant tissues. Our reverse genetic analysis has revealed that a loss-of-function mutation of an Arabidopsis XTH family gene, AtXTH28, led to a decrease in capability for self-pollination, probably due to inhibition of stamen filament growth. Our results also suggest that the role of AtXTH28 in the development of the stamen is not functionally redundant with its closest paralog, AtXTH27. Thus, our finding indicates that AtXTH28 is specifically involved in the growth of stamen filaments, and is required for successful automatic self-pollination in certain flowers in Arabidopsis thaliana.

Keywords: Arabidopsis • Cell wall • Self-pollination • Stamen filament • XTH.

Introduction

For successful fertilization in flowering plants, the stamen and pistil act as key components of male and female gametophytes, respectively. In the stamen, the development of the anther and stamen filament is key to ensuring successful fertilization from the point of view of morphogenesis of the male gametophyte (Goldberg et al. 1993). After differentiation of the male germline, pollen mother cells undergo meiosis to form tetrad cells, the haploid microspores, which then mature into pollen grains during cell division and formation of the complex pollen wall. During development of pollen grains, the stamen filament elongates in the flower bud, by harmonizing an extension of the stamen. In self-pollinating plants, such as Arabidopsis thaliana, these developmental events occur at the correct time when the stigma is receptive, so that the anthers are positioned above the stigma to release the pollen grains for effective fertilization (Goldberg et al. 1993).

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Synthesis, assembly and modification of the plant cell wall constitute fundamental processes in regulation of both cell expansion and differentiation during morphogenesis, thereby representing primary determinants of the anther and stamen structures for reproductive competence. During the course of meiosis, the microsporocytes secrete a callose wall between the plasmalemma and the original cellulosic wall. At the end of meiosis, the external and interporal walls of the tetrads are dissolved by enzymes secreted by the tapetum, such as endoglucanase and exoglucanases, to release individual microspores (Ariizumi et al. 2004, Scott et al. 2004, Dong et al. 2005, Yang et al. 2007). Anther dehiscence requires secondary wall thickening, which generates the tensile force necessary for the rupture of the stomium (Keijzer 1987, Steiner-Lange et al. 2003, Mitsuhashi et al. 2005). These studies identified a relatively small number of cell wall genes that probably play a role in pollen grain maturation and anther dehiscence. However, recent comprehensive gene expression profile analysis identified a large number of cell wall genes working at different stages of anther development, most of which belong to gene families such as glycosyl hydrolases, glycosyltransferases and structural proteins (Amagai et al. 2003, Zik and Irish 2003, Mandaokar et al. 2006, Alves-Ferreira et al. 2007, Hobo et al. 2008). Despite rapid advances in the identification of cell wall genes expressed in the stamen, the role of most families of cell wall genes remains functionally unexplored.

Xyloglucan endotransglucosylase/hydrolases (XTHs) have been implicated in both the splitting and reconnection of xyloglucan cross-links (Fry et al. 1992, Nishitani and Tominaga 1992), and are considered to play a pivotal role in both the construction and the disassembly of cell wall architecture in a wide variety of plant tissues (Rose et al. 2002). XTHs are typically encoded by large multigene families in land plants. In Arabidopsis, 33 genes that encode XTH proteins have been identified (Yougoyama and Nishitani 2001). Comprehensive gene expression analyses of the XTH family in Arabidopsis revealed that most family members exhibit spatially and temporally distinct expression profiles, and some of the AtXTH genes are predicted to be expressed in flower development (Yougoyama and Nishitani 2001, Hyodo et al. 2003, Imoto et al. 2005, Becnel et al. 2006). Despite extensive studies on this gene family, little is known about the role of AtXTH genes in flower development.

To identify the XTH genes responsible for reproductive competence in flower development, we analyzed the reproductive phenotypes of Arabidopsis mutants in each AtXTH gene by a reverse genetic strategy. We found that a loss-of-function mutation of the AtXTH28 gene causes a defect in self-pollination in certain flowers. In this report, we propose that AtXTH28 has a specific role in the formation of cell wall architecture that is essential during stamen development in Arabidopsis plants.

## Results

### Isolation of the atxth28 mutant

Using a reverse genetic strategy, we analyzed reproductive phenotypes of Arabidopsis mutants in which each AtXTH gene has been disrupted, and found that a loss-of-function mutant line for the AtXTH28 gene (atxth28) showed a phenotypic defect in silique development. A T-DNA insertion mutant of the AtXTH28 gene was obtained from the Salk Institute, which contains the insertion between the second and third exons of the coding region (Fig. 1A). Semi-quantitative reverse transcription–PCR (RT–PCR) analysis showed that no transcript of AtXTH28 was detected in the atxth28 mutant, whereas AtXTH28 mRNA accumulated substantially in wild-type plants (Fig. 1B). Thus, we concluded that atxth28 is a null mutant line for the AtXTH28 gene.

A characteristic phenotype of the atxth28 mutant is shorter siliques with very few seeds and no readily discernible phenotype at any developmental and reproductive phase. To determine the effect of the mutation more precisely, we investigated the relationship between the silique position and the length of siliques in terms of inflorescence development. Most of the first to fifth siliques on the primary stem of the mutant line were shorter than those of the wild type, and fewer numbers of seeds were in these siliques, although longer siliques containing normal seed number were found occasionally near the fifth silique. For sixth and later siliques, the number of longer siliques gradually became larger, and all upper siliques after the eighth on the primary stem were equivalent to those of the wild type (Fig. 1C). Although the first and second siliques were sometimes shorter in wild-type plants, the pattern of shorter silique formation apparently differed for the atxth28 mutant (Fig. 1C).

For complementation tests, a 5.7 kb genomic DNA fragment containing the entire AtXTH28 sequence, including 3.0 kb of the 5’- and 3’-flanking regions, respectively, was introduced into an atxth28 mutant line by Agrobacterium-mediated transformation. The AtXTH28 transcript was restored in the transgenic plants (Fig. 1B), and the silique length phenotype of these transgenic plants coincided with that of the wild type (Fig. 1C). These observations indicate that the shorter silique phenotype in the atxth28 mutant is caused by a defect in AtXTH28.

We generated various combinations of double mutant lines between other closely related AtXTH genes using T-DNA insertion mutants. The severity of the phenotype found in the atxth28 mutant was not enhanced in these double mutants compared with the single atxth28 mutant. It is worth noting that the atxth27/atxth28 double mutant did not increase the severity of the atxth28 phenotype (Fig. 1C).
Self-pollination ability

In order to determine the viability of male and female organs in the atxth28 mutant, hand self-pollination and reciprocal crossing between atxth28 mutant and wild-type plants were performed. The atxth28 pollen had normal viability by hand pollination, and normal siliques were produced on reciprocal crossing (Fig. 2). To investigate automatic self-pollination in atxth28 and wild-type flowers, pollen grains on stigma papilla were observed using aniline blue staining. On the second and/or third flowers of the wild-type plants, a large number of pollen grains and penetration of pollen tubes were observed on the stigma papillae (Fig. 3A, B). In contrast, at the same position of the inflorescence in the atxth28 mutant, most flowers did not have pollen grains on the stigma (Fig. 3B). In the upper position of the inflorescence of the atxth28 mutant, which could form longer siliques, pollen grain number and pollen tube penetration resembled those of the wild type (Fig. 3B). To check the release of pollen grains from the anther, we compared anther development and the timing of anther dehiscence for wild-type and atxth28 flowers. No differences were observed in the maturation of anther tissues (e.g. tapetum and pollen development) or in the timing of anther dehiscence, and, furthermore, no morphological differences were observed in the outer structure of anthers (data not shown). These results indicate that the atxth28 mutation affects self-pollination and consequently induces a shorter silique phenotype.
Growth of stamen filaments

Rapid growth of the stamen filament is closely linked to flower development, and correct timing of anther dehiscence is essential for successful self-pollination (Fel and Sawhney 1999). To examine whether the atxth28 mutation affects the physical position of anthers, we measured the length of stamen filaments. For an evaluation of the relative position between anther and stigma, the length ratio between stamen and pistil is a good indicator. Fig. 4A shows that, at the pollination stage (stage 14, Smyth et al. 1990), the atxth28 mutant exhibited a significantly lower stamen/pistil ratio compared with that of wild-type plants, indicating that the atxth28 stamen filaments did not elongate sufficiently to the correct position close to the stigma at the pollination stage. At the flowering stage (stage 15), the stamen/pistil ratio showed no significant difference between wild-type and atxth28 flowers (Fig. 4B). The lengths of the pistil were not significantly different between wild-type and atxth28 flowers at both the pollination and flowering stages [Fig. 4A(c), B(c)]. These results indicate that atxth28 is a mutant defective in timing of the growth of the stamen filament that is required for automatic self-pollination.

To observe the spatial positions of the anther and stigma at the pollination stage, we analyzed the inside three-dimensional (3D) architecture of the flowers of the atxth28 mutant and the wild type non-destructively, by a microfocus X-ray CT system. By use of this technology, we could measure the...
angle between the stamen and pistil based on the 3D images obtained. Quantitative measurements of the angle showed that the atxth28 mutant often showed an increase in the angle of the stamen compared with the wild type (Fig. 5A, C). Consequently, the anthers were positioned relatively more distant from the stigma in the atxth28 mutant.

The side of the anther that dehisces generally faces the stigma in wild-type flowers of A. thaliana; however, in the atxth28 mutant flower, this side did not always face the stigma (Fig. 5B).

These results suggest that aberrant development of the stamen, its angle and the orientation of the dehiscing side are associated with the decreased capability for self-pollination in the atxth28 mutant.

Expression of the AtXTH28 gene in stamen filaments

We examined the expression profile of the AtXTH28 gene during flower development in transgenic plants expressing the AtXTH28 promoter::β-glucuronidase (GUS) fusion gene (pAtXTH28::GUS). A high level of GUS activity was observed in stamen filaments, although occasional GUS activity was detected in the entire area of the flower (Fig. 6A). It should be noted that a high level of GUS activity continued during flower development only in the stamen filaments.

To examine more precisely the expression pattern of AtXTH28 during the development of the stamen, the cellular localization of AtXTH28 mRNA was investigated by in situ hybridization (Fig. 6B, C). At early stages of stamen development, '1–2 mm
Discussion

Using a T-DNA insertion mutant of the AtXTH28 gene, we showed that a defect in this gene led to the formation of a small number of seeds and short siliques on the proximal region of the inflorescence stem.

The atxth28 mutant has no immediately discernible phenotype with regard to the morphology of the stamen at the flowering stage compared with that of the wild type. However, detailed analysis of developing flowers of atxth28 revealed three characteristic phenotypes: a significantly lower elongation ratio of the stamen at the pollination stage (stage 14); an increased angle between the stamen and pistil; and an aberrant orientation of anther dehiscence (Fig. 4, 5).

In the developing flower bud, male and female reproductive organs develop independently and mature at around stage 13, just before anthesis. Before this stage, the gynoecium and stamen lengthen in concert, and, after that, stamen filaments extend even faster and the length of the long stamens outstrips that of the gynoecium at the beginning of stage 14. During the elongation of the stamen, anthers dehisce and release pollen, and then pollination is accomplished when mature pollen and stigma brush against each other. For successful pollination by delivery of pollen grains to the surface of the stigma, the morphological structure is one of the key features for successful reproduction, in addition to the viability of the reproductive components, the pollen and pistil. The atxth28 mutant lacked accurate development of a male component, i.e. timing of the elongation of the stamen filament, the distance between the anther and stigma, and orientation of anther dehiscence. That is, the atxth28 mutant failed to keep the correct position of the anthers relative to the stigma at the pollination stage, due to a defect in the stamen filaments. Due to this defect, the capability for automatic self-pollination was decreased.

Expression of the AtXTH28 gene was observed in stamen filaments, and its mRNA accumulated intensively in the vascular tissue of the filaments at the pollination stage (Fig. 6), indicating that the AtXTH28 gene product takes part in cell wall construction of the vascular tissue in stamen filaments during the early stages of flower development. Given that xyloglucan modification is mediated exclusively by the XTH family of proteins, AtXTH28 is responsible for cutting and rejoining xyloglucan chains within the vascular cell walls, thereby controlling the extensibility or mechanical properties of the cell wall in vascular cells during stamen filament elongation. A defect in the properties of the vascular cell walls would cause a change in the timing of elongation of the stamen filament. In addition, the AtXTH28 gene was expressed extensively at the junction between anther and filament, and in the receptacles at a later stage (Fig. 6B). Proper construction of cell walls by AtXTH28 would also be
required for development of the mechanical properties of the tissues that stabilize the growth direction of the stamen. Alternatively, the mechanical properties of the junction regions, controlled by AtXTH28, might be associated with a twist in the stamen filament. In the atxth28 mutant, the anther dehiscence side does not always face the stigma: anthers faced the stigma with various angles, such as 180°, 45°, 30° and 0° (as in the wild type). Subtle changes in atxth28 expression spatially and temporally in stamen filaments would lead to significant differences in the capability for automatic self-pollination.

The relatively subtle phenotype of the atxth28 mutant, shorter siliques for the first couple of flowers in the main inflorescence, suggests that there is a functional redundancy among members of the AtXTH family of genes. In fact, expression of AtXTH28 was null in all flowers of the atxth28

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**Fig. 6** AtXTH28 expression analyses. (A) pAtXTH28::GUS expression in the stamen filament at several developmental stages. (B) In situ hybridization analysis of AtXTH28 mRNA in stamen filaments of wild-type flowers at several developmental stages. A DIG-labeled antisense probe of AtXTH28 was used in each experiment. Arrowheads indicate relatively intensive signals. Bars indicate 50 µm (100 µm in receptacles). (C) Negative control with a DIG-labeled sense AtXTH28 RNA probe.
mutant (Fig. 1B), although later developing siliques lengthened normally. We generated various combinations of double mutant lines between other closely related AtXTH genes using T-DNA insertion mutants. The severity of the phenotype found in the atxth28 mutant was not enhanced in these double mutants compared with the single atxth28 mutant. It is worth noting that the atxth27/atxth28 double mutant did not increase the severity of the atxth28 phenotype (Fig. 1C).

AtXTH27 and AtXTH28 originated from a gene duplication event and maintain a high degree of nucleotide sequence identity (Yokoyama and Nishitani 2001). The mutation in AtXTH27 causes a cell type-specific defect in the development of trachaeary elements in expanding rosette leaves, but no observable defects in the developing inflorescence (Matsui et al. 2005). This phenotype was also not enhanced in atxth27/atxth28 double mutants, indicating that AtXTH28 is not functionally redundant with AtXTH27 and instead has evolved a non-overlapping, member-specific role in the development of stamen filaments.

The self-compatibility (SC) of A. thaliana is mainly caused by a disruption of the S locus genes SP11/SCR and SRK, components of a pollen–stigma interaction (Shimizu et al. 2008). This indicates that the ancestor of Arabidopsis should have self-incompatibility (SI), and, in fact, A. lyrata, a wild relative of A. thaliana, is an SI species. In addition, S-locus gene mutants showing SC were recently identified in Brassica (S. Isokawa, K. Suwabe and M. Watanabe, unpublished data), a species classified in the same family with Arabidopsis, the Brassicaceae. These results indicate that disruption of S-locus genes leads to SC in several species of the Brassicaceae. SI is one of the genome barriers in plant reproduction and is controlled by the physiological mechanism mentioned above. In the case of the atxth28 mutant, male and female components for reproduction are normal and functional, and a disruption of the gene for an enzyme involved in restructuring of the vascular cell wall in stamen filaments leads to repression of automatic self-pollination. Thus, physiological and morphological properties are critical for successful plant reproduction, and the atxth28 mutant will offer an opportunity to explore the morphological aspects of pollination biology.

In conclusion, a gene potentially regulating cell wall construction and modification, AtXTH28, is involved in a self-pollination mechanism through a change in cell wall formation in stamen filaments, and the formation of the cell wall is important for correct stamen development. Thus, the AtXTH28 gene can act as a model for linking a reproductive phenomenon to regulation of the cell wall structure.

Materials and Methods

Plant materials and growth conditions

The A. thaliana (L) Heynh Columbia (Col-0) background was used throughout the experiments. The seeds were sown and grown on rockwool moistened with MGRL medium (Tsukaya et al. 1991) or on a solid Murashige and Skoog (MS) salt and vitamin medium (Murashige and Skoog 1962) containing 3% sucrose. Plants were grown in a chamber at 22°C under continuous light conditions (70–80 μmol m⁻² s⁻¹) before bolting. When bolting was observed, plants were transferred to long-day conditions (16 h light/8 h dark, 70–80 μmol m⁻² s⁻¹).

The atxth28 mutant line (SALK_069319) was obtained from the Salk Institute Genomic Analysis Laboratory T-DNA insertion lines (http://signal.salk.edu). The atxth28 mutant was subjected to the following analyses after four backcrosses to the wild type. The genotype of the T-DNA insertion allele was determined by PCR of genomic DNA, using primer sets of an AtXTH28-specific forward primer (5′-GTGTTAGTGACT-CTACCTGTG-3′), a T-DNA left border-specific primer (5′-CGGTGGAGCCGTGTCACTGCAACT-3′) and an AtXTH28-specific reverse primer (5′-CGATTAAACTCCTGAGAAG-3′).

Plasmid construction and transformation of the AtXTH28 gene into A. thaliana

For complementation tests, a 5.7 kb genomic fragment of the AtXTH28 gene, which contains the S′ promoter region (3 kb) and 3′ untranslated region (1 kb), was amplified from genomic DNA of the Col-0 ecotype. After verifying the nucleotide sequence of the amplified fragment, it was subcloned into a SYN947-D binary vector containing a hygromycin resistance gene (TAKARA BIO INC., Shiga, Japan). This construct was transformed into Agrobacterium tumefaciens strain C58, and then introduced into an atxth28 homozygous mutant line using the floral dip method (Clough and Bent 1998). For evaluation of phenotypic complementation, five independent transformants having a single AtXTH28 genomic fragment were established by hygromycin selection.

The 1.5 kb promoter region of AtXTH28 was amplified from Col-0 genomic DNA with a primer set (5′-AGTTTGCGACCTTTGCGAGGATTAGGTTGC-3′ and 5′-GATTCTAGCGGTGGAGCCGTGTCACTGCAACT-3′). The amplified DNA fragment was digested with SalI and XbaI restriction enzymes to insert into a multicloning site of the binary vector pBI101; the resulting construct was named pXTH28::GUS. Agrobacterium-mediated Arabidopsis transformation was performed as described above. For evaluation of GUS expression, four independent transformants having a single pXTH28::GUS were established by kanamycin selection.

Measurement of silique length and seed numbers

For evaluation of pollination and fertilization, measurements of silique length and seed number were performed. The siliques of 5- to 6-week-old rockwool-grown plants (approximately 25–30 cm in height) were measured using a slide caliper (DIGIMATIC SOLAR CD-S15M; Mitsutoyo, Japan). Seed numbers were counted for each siliquid.
Pollination tests and measurement of stamen length

Pistils were stained with basic aniline blue solution, as described in Ishiguro et al. (2001), with a slight modification. Briefly, 30–35 pistils were fixed in a solution (ethanol: acetic acid 3: 1) for 1 h at room temperature. After washing with distilled water three times, pistils were softened in 1 M KOH for 10 min at 65°C and then stained in basic aniline blue solution (0.1% aniline blue in 0.1 M K₂HPO₄-KOH buffer, pH 11) for 5 h in complete darkness. The stained pistils were placed in a drop of 50% glycerol on a glass slide and were observed by fluorescence microscopy under UV light excitation (Leica DMRXP System, Heerbrugg, Switzerland: excitation filter, 360/340 nm; barrier filter, 420 nm). The number of pollen tubes that had penetrated a stigma’s papilla cells was counted for each pistil.

In order to measure stamen and pistil length at various developmental stages, flower buds and flowers at anthesis were stained with basic aniline blue as described above. The stamen length was measured by a microscope (Leica MZ16FA System).

Reverse transcription–PCR

For RT–PCR, flower buds from 4- to 5-week-old plants were collected into liquid nitrogen and subjected to total RNA extraction according to the conventional SDS–phenol protocol (Yokoyama and Nishitani 2001). An aliquot of cDNA synthesized from total RNA was used as a template for PCR amplification with a set of specific primers for the AtXTH28 gene (5’-CTCTGCAAGAGTGTATCG-3’ and 5’-AACGATAATTGTCTAAAATTGAGGAA-3’). PCR was performed with Taq DNA polymerase (Promega, Madison, WI, USA) for 27 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min.

In situ hybridization and GUS assay analysis

In situ hybridization was performed according to Nakamura et al. (2003). Briefly, plant tissue samples were fixed in an FAA solution [50% ethanol, 10% formaldehyde and 5% acetic acid (v/v)], and were dehydrated through an ethanol series before transfer to xylene. After permeation, tissues were embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). Microtome sections (8 µm thick) fixed on a glass slide were deparaffinized with xylene and rehydrated through an ethanol series. For synthesis of the AtXTH28-specific probe, an AtXTH28 fragment (242 bp) was amplified by PCR using the following primers, 5’-TGAGCTTGCTCTGACTGCTAT-3’ and 5’-TAATTCGACAATGGTCTTATAAACTGAAGGAA-3’. PCR was performed with Taq DNA polymerase (Promega, Madison, WI, USA) for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min.

Hybridization of DIG-labeled cRNA with anti-DIG alkaline phosphatase-conjugated antibody (Roche Diagnostics). For detection of hybridized antibody, a nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl-phosphatase (BCIP) solution was used. Photographs were taken with a differential interference contrast (DIC) microscope system (DMRXP; Leica).

Histological assays for GUS activity in transgenic plants were performed as described in Jefferson et al. (1987) with slight modifications. Plant tissues were gently fixed with 90% acetone on ice for 10 min and were rinsed in 100 mM phosphate buffer (pH 7.0). Tissues were placed in a staining solution [100 mM phosphate buffer (pH 7.0), 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronyl), 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆ and 0.01% Triton X-100]. Plant tissues were vacuum infiltrated briefly and then incubated at 37°C overnight. After staining, chlorophyll was cleared by 70% ethanol treatment. Photographs were taken using Leica MZ16FA and DMRXP microscope systems.

X-ray CT analysis

Images of flowers were collected using a microfocus X-ray CT system (SMX-100CT; Shimadzu, Kyoto, Japan) with the following parameters: X-ray tube voltage, 21 kV; tube current intensity, 84 mA; SID, 520 mm; SOD, 28 mm; and CT scan mode, normal; reconstruction matrix, 512×512×512; slice thickness, 0.005912 mm; number of views, 600; number of average, 10. For quantitative analysis of CT images, angles between the stamen and pistil were measured using VG Studio MAX 1.2.1. Software (Nihon Visual Science, Japan). Specimens for analysis were pre-anthesis flowers and flowers at anthesis at the pollination stage.

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