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PECTATE LYASE-LIKE 9 from *Brassica campestris* is associated with intine formation

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ABSTRACT

Brassica campestris pectate lyase-like 9 (*BcPLL9*) was previously identified as a differentially expressed gene both in buds during late pollen developmental stage and in pistils during fertilization in Chinese cabbage. To characterize the gene's function, antisense-RNA lines of *BcPLL9* (*bcpll9*) were constructed in Chinese cabbage. Self- and cross-fertilization experiments harvested half seed yields when *bcpll9* lines were used as pollen donors. *In vivo* and *in vitro* pollen germination assays showed that nearly half of the pollen tubes in *bcpll9* were irregular with shorter length and uneven surface. Aniline blue staining identified abnormal accumulation of a specific bright blue unknown material in the *bcpll9* pollen portion. Scanning electron microscopy observation verified the abnormal outthrust material to be near the pollen germinal furrows. Transmission electron microscopy observation revealed the internal endintine layer was overdeveloped and predominantly occupied the intine. This abnormally formed intine likely induced the wavy structure and growth arrest of the pollen tube in half of the *bcpll9* pollen grains, which resulted in less seed yields. Collectively, this study presented a novel *PLL* gene that has an important function in *B. campestris* intine formation.

1. Introduction

In flowering plants, microspores undergo meiosis to produce a tetrad of four haploid microspores, each of which will develop into a pollen grain. During pollen development, several layers of pollen walls are synthesized and degraded in a specific order and in turn produce a mature bi-layered structure pollen wall that includes the intine and exine. This pattern of pollen development has been clearly identified along with the identification of various important genes involving in male gametogenesis [1–3] and pollen wall development processes [4].

Pectin is a class of polysaccharide polymer typically characterized by the linear backbone of the α-1,4-linked galacturonic acid (GalA) residues, which include homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) [5]. Pectinases target methyl-esterified HG to yield substrates for polygalacturonases (PGs) and pectate lyases (PLs, or pectate transeliminases) to cleave GalA backbones. PLs cleave α-1,4-glycosidic linkages in HG to produce unsaturated oligosaccharides [6–8]. PLs have been extensively studied in several plant pathogenic microorganisms. *Erwinia chrysanthemi*, for example, is an extracellular causal agent in soft root disease that affects a wide range of plant species [9]. In plants, pectate lyase-like (*PLL*) genes Late Anther Tomato 56 (*LAT56*) and *LAT59* that present strong sequence homology with the PelC isoform of bacterial PLs were first isolated to be expressed at maximal levels in mature anthers in tomatoes (*Solanum lycopersicum*) [10]. Since then, several other *PLL* genes were identified as expressed in pistils, tracheary elements, ripening fruits and latex [11–19]. *PLL* genes were also expressed in response to plant hormones, environmental stress, cell separation and pathogen infections [20–22].

PLL genes are abundant in plants, including 26 *PLL* homologous genes in *Arabidopsis* and 12 in rice (*Oryza sativa*) [20]. These genes are also abundant in tomato, tobacco, alfalfa and Chinese cabbage (*Brassica campestris* ssp. *chinensis*) [10,12,14,23]. Out of the 26 *Arabidopsis PLL* genes, 14 are expressed in the pollen [20]. Our previous study also revealed that two Chinese cabbage *PLL* genes

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were exclusively expressed in the pollen and pistil [4]. Researchers have proposed that the tomato pollen expressed genes, *LAT56* and *LAT59*, are related to a requirement for pectin degradation during pollen tube growth [12,14]. In addition, numerous promoter activities of the *PLL* genes are similar to those exhibited by multiple PGs, which suggests a close functional association among PLLs and PGs, particularly in the digestion of the pollen grain cell wall prior to germination and during pollen tube growth [24–26]. However, to our knowledge, no direct experimental evidence has been presented regarding the involvement of *PLL* genes in pollen development or in remodelling the pollen tube wall. Little is known about the spatial and temporal regulation of *PLL* genes and their functions during reproductive growth in plants.

In this study, we provide evidences that a novel *PLL* gene, *BcPLL9*, has an important function in pollen wall development. Ectopic expression of *BcPLL9* antisense gene in *B. campestris* ssp. *chinensis* resulted in abnormal intine formation during pollen wall development as well as pollen tube growth retardation, partial male sterility and reduced seed set.

2. Materials and methods

2.1. Plant material and sampling

A Chinese cabbage pak-choi 'Aijiaohuang' (*B. campestris* ssp. *chinensis* syn. *B. rapa* ssp. *chinensis* cv. Aijiaohuang) GMS AB line named '*Bajh97-01A/B*' was used in this study. '*Bcajh97-01A*' completely failed in pollen formation but several of its tissues were the same as those of '*Bcajh97-01B*' [27–29]. '*Bajh97-01A/B*' was developed through continuous backcrossing within the population for more than ten generations and was the sibling (sister) line segregated with homozygous male sterile plants (*Bcajh 97-01A*, genotype: *msms*) and heterozygous male fertile plants (*Bcajh 97-01B*, genotype: *Msms*) in a 1:1 ratio. The plants were grown at natural light conditions (12 h light/12 h dark) at 14–25 °C during early April in Hangzhou, Zhejiang, China.

2.2. Cloning and analysis of Chinese cabbage BcPLL9 gene

DNA was isolated from the leaves of *Bajh97-01B* plants via the CTAB method. Total RNA was extracted from inflorescence of *Bajh97-01B* by using Trizol reagent (Invitrogene, USA) treated with DNase (TaKaRa, Japan) and reverse-transcribed into first strand complementary DNA by using the PrimeScript 1st strand cDNA synthesis kit (TaKaRa, Japan) from 1 μg of total RNA. The DNA and cDNA sequences of At2g02720 (*AtPLL9*) were downloaded from the *Arabidopsis* website (http://www.arabidopsis.org/) and used for basic local alignment search in the *Brassica* database (http://brassicadb.org/brad/) with the basic local alignment search tool (BLAST) with default settings. The BAC clone AC189452 showed the highest identity. Second, the full length of the cDNA sequence, including 5′-untranslated region (UTR) and 3′UTR, was amplified with the forward primer, P9-fwd, and the reverse primer, P9-rev. The DNA sequence of the gene was cloned with the forward primer, PD9-fwd, and the reverse primer, PD9-rev. Gene-specific primers were designed using the Primer Premier 5.0 software and are listed in Table S1. The PCR products were sub-cloned into a pGEM-T Easy vector (Promega, USA) and sequenced.

The DNAstar software was used to analyse the gene structure, including the largest open reading frame (ORF) and the character of the deduced amino acids. The secondary structure prediction of the protein was performed in the NPSA-MLRC website (http://npsa-pbil.ibcp.fr). Motif prediction was carried out in the SignalP-4.0 (http://www.cbs.dtu.dk/services/SignalP/) and Pfam (http://pfam.sanger.ac.uk/) websites. Default parameters were used.

2.3. Quantitative RT-PCR (qRT-PCR) analysis

First-strand cDNA was reverse-transcribed using the PrimeScript 1st strand cDNA synthesis kit (TaKaRa, Japan) as described above. A sample of cDNA (100 ng) was subjected to qRT-PCR in a final volume of 20 μL by using a SYBR Premix Ex Taq Kit (TaKaRa, Japan) with CFX96 Real Time System (Bio-Rad, France). Data was normalized to the expression levels of the internal control gene, *BcUBC10* [30]. In addition, the end-time PCR products were separated on agarose gel and sequenced to verify the amplification product. Three biological and three technical replicates for each sample were performed. Relative expression levels were calibrated via the $2^{-\triangle\triangle Ct}$ method [31]. The primers (P9q-fwd and P9q-rev) are listed in Table S1. Error bars represent ±SE, $^{**}P < 0.01$, by χ^2 test.

2.4. In situ hybridization

Flower buds at uninucleate and mature pollen stages, as well as pollinated pistils at 24 h after pollination (hap), were fixed in 4% formaldehyde–PBS solution, dehydrated with 30% sucrose solution, transited with 30% sucrose:optimal cutting temperature (OCT) compound (1:1), and then embedded in pure OCT compound. The embedded samples were sectioned at 14–16 μm in thickness with Thermo Shandon Finesse 32 freezing microtome (Thermo Scientific, USA) and were hybridized to digoxigenin (DIG)-labelled RNA probes. A 190-bp fragment was amplified with a primer pair (P9i-fwd and P9i-rev, listed in Table S1) specific to *BcPLL9*. Sense and antisense probes were synthesized and labelled using a DIG RNA Labelling Kit (Roche Diagnostics, New Jersey, USA). Flower buds at uninucleate and mature pollen stages were used in the present experiment [28]. The male sterile line was used as the female parent in conducting hand-pollination experiments to prevent extraneous pollen contamination. Unpollinated pistils were collected from inflorescences next to pollinated inflorescences and used as negative control samples. The flower buds at mature stages were collected from the bagged shoots.

2.5. Antisense-RNA construction and plant transformation

A 190-bp fragment within *BcPLL9* gene (from 287 bp to 476 bp) was amplified with primers (P9i-fwd and P9i-rev) containing the restricted enzyme sites of *Xba* I and *Bam*H I, respectively. The PCR product was cloned into pGEM-T Easy vector and then sub-cloned into the binary vector, pBI121, which contained a CaMV35S promoter. The construct was confirmed via PCR assay and restriction enzyme digestion, sequenced, and named as pBI121-*35S::aBcPLL9*. This antisense-RNA construct was then transformed into flowering Chinese cabbage (*B. campestris* ssp. *chinensis* var. *parachinensis*) by using the *Agrobacterium tumefaciens* EHA105-mediated plant transformation system [32]. Empty vector pBI121 was transformed using the same method as negative control sample.

2.6. Molecular confirmation of the transgenic plants

To confirm that the T-DNA fragment in pBI121-*35S::aBcPLL9* construct was migrated into the plant genome, PCR and Southern blot were performed. Genomic DNA was extracted from young leaves of the putative antisense-RNA transformed plants and the control plants. PCR and Southern blot were performed to detect a 371-bp fragment within the *GUS* gene in the pBI121 vector (primer: GUS-fwd and GUS-rev, listed in Table S1). For PCR assay, 35 cycles of amplification were performed using 20 ng of each gDNA sample. For