**BraLTP1**, a Lipid Transfer Protein Gene Involved in Epicuticular Wax Deposition, Cell Proliferation and Flower Development in *Brassica napus*

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Abstract

Plant non-specific lipid transfer proteins (nsLTPs) constitute large multigene families that possess complex physiological functions, many of which remain unclear. This study isolated and characterized the function of a lipid transfer protein gene, *BraLTP1* from *Brassica rapa*, in the important oilseed crops *Brassica napus*. *BraLTP1* encodes a predicted secretory protein, in the little known VI Class of nsLTP families. Overexpression of *BnaLTP1* in *B. napus* caused abnormal green coloration and reduced wax deposition on leaves and detailed wax analysis revealed 17–80% reduction in various major wax components, which resulted in significant water-loss relative to wild type. *BnaLTP1* overexpressing leaves exhibited morphological disfiguration and abaxially curled leaf edges, and leaf cross-sections revealed cell overproliferation that was correlated to increased cytokinin levels (tZ, tZR, iP, and iPR) in leaves and high expression of the cytokinin biosynthesis gene *IPT3*. *BnaLTP1*-overexpressing plants also displayed morphological disfiguration of flowers, with early-onset and elongated carpel development and outwardly curled stamen. This was consistent with altered expression of a number of ABC model genes related to flower development. Together, these results suggest that *BraLTP1* is a new nsLTP gene involved in wax production or deposition, with additional direct or indirect effects on cell division and flower development.

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Introduction

Plant non-specific lipid-transfer proteins (nsLTPs) are small, abundant, basic, secreted proteins in higher plants [1,2]. nsLTPs contain an 8 cysteine motif (8 CM) structure comprising eight cysteine residues interleaved in the 8 CM structure. Recently, fifty two rice *nsLTP* genes, 49 Arabidopsis *nsLTP* genes and 156 putative wheat *nsLTP* genes were identified through genome-wide analyses [3,4]. Members of this 8 CM structure are encoded by multigene families that were originally subdivided into type I (9 kDa) and type II (7 kDa) on the basis of molecular mass. More recently, several anther specific proteins in *Brassica* (Asteraceae) have been classified by Edstam et al [9], functionally overlaps with *LTPG1* during cuticular wax export or accumulation, and the total cuticular wax load was reduced in both *ltpg2* and *ltpg1* *ltpg2* siliques [11]. These *LTPG* genes belong to type G nsLTPs classified by Edstam et al [28], and are not included in Boutrot’s classification system [7].

nsLTPs are encoded by multigene families that were originally classified by Edstam et al [9], functionally overlaps with *LTPG1* during cuticular wax export or accumulation, and the total cuticular wax load was reduced in both *ltpg2* and *ltpg1* *ltpg2* siliques [11]. These *LTPG* genes belong to type G nsLTPs classified by Edstam et al [28], and are not included in Boutrot’s classification system [7].
Plant epidermal wax forms a hydrophobic layer covering aerial plant organs. This constitutes a barrier against nonstomatal water loss, as well as biotic stresses, and provides protection against pathogens [29]. Cuticular wax contains very-long-chain fatty acids (VLCFA) and their derivatives, such as alkanes and alkohols, with chain lengths of 20–34 carbons, and wax composition varies with species, organ, and developmental stage [30]. Synthesis of VLCFA in the epidermis via acyl-CoA dehydratase PAS2 is essential for species, organ, and developmental state [30]. Synthesis of VLCFA, and their derivatives, such as alkanes and alcohols, with chain lengths of 20–34 carbons, and wax composition varies with species, organ, and developmental stage [30]. Synthesis of VLCFA in the epidermis via acyl-CoA dehydratase PAS2 is essential for species, organ, and developmental state [30].

Moreover, it was suggested that VLCFA, or its downstream derivatives or metabolites might function as signaling molecules to suppress cytokinin biosynthesis in the vasculature, thus fine-tuning cell division in internal tissue [31].

Despite progress in the functional analyses of these nsLTPs involved in cuticular wax deposition, the exact functions of most nsLTPs remain unclear, and complex expression profiles suggest disparate and unpredictable gene functions of unknown nsLTPs to the CaMV 35S sequence (35S-F: 5'-GGAGCTCAACATCTCCGTCAAAGGCCACA-3' and 35S-R: 5'-GGATCCCAACCCCTATGGGACAAATGTA-3'), containing 5' restriction enzyme sites for SauI and BamHI respectively. PCR was carried out in 50 μL, with 50 ng DNA, 0.4 mM dNTPs, 0.2 μM each primer, 0.5 U LA Taq (TaKaRa, Japan) and 1× LA Taq buffer II (TaKaRa, Japan). Conditions were: 94°C for 3 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min each. PCR product was checked by gel electrophoresis and target fragment was recovery and purified. The purified PCR product was cloned into pBluescript plasmid vector and was transformed to E.coli DH5a, and ligated between the CaMV 35S promoter and a terminal poly A sequence in the vector PBI121s (Fig. 1B), derived by modifying the multiple cloning site and deleting the GUS gene of PBI121 [33]. Positive clones by PCR using the above gene specific primers were chosen and sequenced (sangon company of Shanghai, China) to make sure they were correct. Standard molecular techniques [34,35] were used for DNA manipulation.

### Materials and Methods

**Plant material**

The plants used in this study were grown in pots containing mixture of moss peat (PINSTRUP, Danmark) and field soil with the proportion of 3:1 in a plant growth room set to 20°C±2°C under a 16/8 h photo-period at a light intensity of 44 μmol m⁻² s⁻¹ and 60–90% relative humidity.

**Vector construction**

The coding sequence of *BraslTP1* was amplified from *B. rapa* accession Chifu genomic DNA using primers designed to the published *B. rapa* sequence Bra011229 (http://brassicadb.org/brad/index.php) [32]. Primers were as follows: BraLTP1-F: 5'-GGAGCTCAACATCTCCGTCAAAGGCCACA-3' and BraLTP1-R: 5'-GGATCCCAACCCCTATGGGACAAATGTA-3', containing 5' restriction enzyme sites for SauI and BamHI respectively. PCR was carried out in 50 μL, with 50 ng DNA, 0.4 mM dNTPs, 0.2 μM each primer, 0.5 U LA Taq (TaKaRa, Japan) and 1× LA Taq buffer II (TaKaRa, Japan). Conditions were: 94°C for 3 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min each. PCR product was checked by gel electrophoresis and target fragment was recovery and purified. The purified PCR product was cloned into pBluescript plasmid vector and was transformed to E.coli DH5a, and ligated between the CaMV 35S promoter and a terminal poly A sequence in the vector PBI121s (Fig. 1B), derived by modifying the multiple cloning site and deleting the GUS gene of PBI121 [33]. Positive clones by PCR using the above gene specific primers were chosen and sequenced (sangon company of Shanghai, China) to make sure they were correct. Standard molecular techniques [34,35] were used for DNA manipulation.

### Genetic transformation

The 35S::*BraslTP1* fragment in PBI121s was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation, and positive clones were selected on on LB agar plates at 37°C, supplemented with appropriate concentration of antibiotics (gentamicin 50 mg L⁻¹, rifampicin 50 mg L⁻¹ and kanamycin 50 mg L⁻¹) and PCR verified. A single positive colony was used to transform *B. napus* cv. Zhongshuang 6, an elite Chinese cultivar in China, as follows: Seeds of Zhongshuang 6 were soaked in 75% ethanol for 1 min and for 10–15 min in a 1.5% mercuric chloride solution. Five to six days after germination under darkness, etiolated hypocotyls were cut in 7 mm segments and mixed with 50 mL *Agrobacterium* in liquid DM media (MS+30 g L⁻¹ sucrose+100 μM acetosyringone, pH 5.8) (OD ~0.3) for 0.5 h. Surface air dried hypocotyls were then transferred to co-cultured medium (MS+30 g L⁻¹ sucrose+18 g L⁻¹ manitol+1 mg L⁻¹ 2, 4-D+0.3 mg L⁻¹ kinetin+100 μM acetosyringone+8.5 g agrose, pH 5.0) for 2 days and then to a selection medium (MS+30 g L⁻¹ sucrose+18 g L⁻¹ manitol+1 mg L⁻¹ 2, 4-D+0.3 mg L⁻¹ kinetin+20 mg L⁻¹ AgNO₃+0.5 mg L⁻¹ agrose+25 mg L⁻¹ kanamycin+250 mg L⁻¹ carbenicillin pH 5.8) for proliferation. After 3 weeks, hypocotyl callus was transferred to regeneration medium (MS+10 g L⁻¹ glucose+0.25 g L⁻¹ xylose+0.6 g L⁻¹ MES hydrate+2 mg L⁻¹ zeatin+0.1 mg L⁻¹ indole-3-acetic acid,5.3 g L⁻¹ agrose+25 mg L⁻¹ kanamycin+250 mg L⁻¹ carbenicillin, pH 5.8) for 2 weeks. Hypocotyls were transferred to new regeneration media every 2 weeks for 3–4 regeneration cycles before transfer to radication medium (MS+10 g L⁻¹ sucrose+10 g L⁻¹ agar, pH 5.8) for rooting (about 3 weeks). Transformed plants with roots were transplanted into pots and grown as described. For the construct, more than 60 independent 35S::*BraslTP1* T₅ transgenic plants were generated, and more than 85% were positive transormants as detected using a forward primer designed to the CaMV 35S sequence (35S-F: 5'-AGGGACAGCTGAAAT-CACGA-3) and a reverse primer designed to *BraslTP1* (D-BralTP1-R: 5'-GGATCCCAACCCCTATGGGACAAATGTA-3'), T₅ seeds of PCR-positive transformants were harvested and grown to T₂ generation for phenotype identification.

### Protein sequence analysis

*BraslTP1* was aligned to homologous amino acid sequences from several cruciferae including *Arabidopsis*, *B. rapa*, *B. napus* and *B. oleracea*, using Align X multiple sequence alignment software (Vector NTI Advance 11.0, 2000 Invirotion corpora). Homology search were conducted using BLAST 2.0 program of the National Center of Biotechnology Information (NCBI). Conserved domains were identified using CDD (http://www.ncbi.nlm.nih.gov/cdd/) and the signal peptide was determined by SignalP (http://www.cbs.dtu.dk/services/SignalP/).

**Wax analysis**

Epicuticular waxes were extracted from ~100 mg *B. napus* leaf disks from the fourth fully-expanded leaf from the apex for each plant by mixing in chloroform for 1 min, with 150 μL 100 μg L⁻¹ triacontane added as an internal standard. The chloroform was then evaporated under gaseous N₂, and the following steps for wax analysis was as described previously [36].

**Water loss determination**

For water loss analysis of detached leaves, leaves were detached from 8-week-old plants, placed on a petri dish for 1, 2, 3, 4, 5, and 6 h, and weighed [37]. Three independent experiments were performed, with 5 plants for each line in each experiment.

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**Function Study of ** *BraLTP1* in *Brassica napus*

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Leaf phenotype analysis

*B. napus* leaves were fixed for 24 h in 4% paraformaldehyde. After dehydration using an ethanol series (75% for 4 h, 85% for 2 h, 90% for 2 h, 95% for 1 h and 100% for 30 min twice) the leaves were cleared twice with xylene for 2 h each. The leaves were infiltrated and subsequently embedded in paraffin wax according to the method of Hu et al [38]. Seven micromolar sections were obtained using a Leica RM 2016 microtome (Leica, Nanterre Cedex, France) and stained with 1% safranin for 6 h. After dehydration using an ethanol series (50%, 70% and 80% for Figure 1. Basic protein characteristics of *BraLTP1* and vector construction. (A) Analysis of the deduced amino acid sequences of *BraLTP1* with its homologous sequences in other cruciferae; variable sites (dark grey) the nsLTP-like conserved 8 CM domain (light gray) with conserved cysteine residues (asterisks) and putative extracellular secretory signals (underlined). Sequences are from *Arabidopsis thaliana* *AtLTP1* (AT4g30880), *B. rapa* *BraLTP1* (Bra011229) [32], *B. oleracea* *BolLTP1* (Bol018048) [60], *B. napus* *BnaLTP1* (AY208878), and *B. napus* *BnaLTP2* (KM062522). (B) T-DNA region of the *BnaLTP1* overexpression construct containing *BnaLTP1* driven by the CaMV 35S promoter. LB = Left border, RB = Right border, poly A = poly A terminator, nptII = kanamycin resistance, NOS Ter = nopaline synthase terminator, NOS Pro = nopaline synthase promoter. (C) Analysis of *BraLTP1* mRNA levels in 10-week-old wild type (WT) and T0 35S::*BraLTP1* transgenic plants by qRTPCR. Transgenic plants include *BraLTP1*-1, -6, -7, -19, -20, -21, -22, -28, -29, -32, -34 and -39. Standard errors were derived from three repeated experiment for the expression levels of each plants. doi:10.1371/journal.pone.0110272.g001
3 min each), sections were stained with 0.5% fast green for 30 min and then dehydrated with 100% ethanol for 5 min. Observations were made and images were acquired with a Leica DM 2500 (Leica microsystems, DFC420C). Six leaf disks with 1.5 cm diameter were hole-punched from the 4th leaf of 8-week-old plants and weighed to estimate weight/unit leaf area. For quantification of cytokinin, sampling of ~100 mg fresh leaves from 4-week-old seedlings was repeated three times, and extraction and determination of cytokinin content were conducted as per Nobusawa et al [31].

**Real-Time PCR**

RNA was extracted using a TIANGEN RNAprep Pure Plant Kit (DP 432) according to the manufacturer's instructions. First-strand cDNAs were synthesized from DNasel-treated total RNA using a TIANGEN FastQuant RT Kit (with gDNase) (KR106) according to the manufacturer's instructions. For transgene expression level and co-segregation experiments, real-time PCR was done using PCR SuperReal PreMix Plus (probe) (TIANGEN). The reaction system and process were followed by the manufacturer's instructions, and four replicates were performed for each cDNA sample. PCR primers and TaqMan probes were designed on the basis of the BraLTP1 cDNA sequences as follows: sense: RT-BraLTP1-F: 5'-ATCGGTCTAGAGAATGCATC-3', RT-BraLTP1-R: anti-sense: 5'-AGAGCACCCTGTCATGAG-3', RT-BraLTP1-probe: 5'-CTTGAGATCTGTCCCTTTGACAG-3'. Specific primers for the *B. napus* Actin gene (GenBank accession number: AF111812.1) were used as an internal control (Actin-F: 5'-CACAGGAATAACTTCTAG-3', Actin-R: 5'-GGATGGA-TATAGATGTCAG-3', Actin-probe: 5'-ACTGAGCACCAC-GAACCAGAA-3'). For transcriptional profiling of cell division and flower related genes, real-time PCR was done using PCR SuperReal PreMix Plus (SYBR Green) (TIANGEN). The reaction system and process followed the manufacturer's instructions, as above in four replicates for each cDNA sample. Table 1 provides information about the genes and primers used for the real-time PCR. Specially, IPT1, IPT4, IPT6 in *Arabidopsis* all correspond to the one *Brassica napus* IPT gene. Real-time PCR was performed in an optical 96-well plate with a Bio Rad CFX96 Real-Time System (C1000 Thermal Cycler) (Applied Biosystems, Hercules City, CA, USA).

**Results**

**Protein sequence characterization**

The *B. rapa* LTP1 coding region is predicted to encode a protein of 109 amino acid residues (Fig. 1A). Similarity searches revealed that *BraLTP1* has 84% overall amino acid identity with *AtLTP1* of *Arabidopsis*, 98% identity with both *BoLTP1* from *Brassica oleracea* and *BnaLTP1* from *B. napus* (which are 100% similar to each other), and 100% identity with *BnaLTP2* of *B. napus* (while gene sequences were 98% similar). *B. napus* (*Brassica* AC genome) is an allotetraploid species resulting from a cross between *B. rapa* (A genome) and *B. oleracea* (C genome) and thus it is not surprising that it contains LTP protein copies corresponding 100% to the A and C genomes derived from both of these diploid species (Nagaharu., 1935). All species possessed highest levels of sequence similarity in the *nsLTP*-like domain regions (light shaded amino acids 29 to 99) (Fig. 1A). A putative extracellular secretary signal (amino acids 1 to 22 in *Arabidopsis* and 1–19 in the other *Brassica* species) was 100% conserved in the *Brassica* species (Fig. 1A). Nine amino acid substitutions exist in the *nsLTP*-like domains between *AtLTP1* and the *Brassica* genes. The eight strictly conserved cysteine residues in all plant LTPs that form four intrachain disulfide bridges, were also 100% conserved among all aligned sequences.

In *Arabidopsis*, nine classes of *nsLTP* were identified that contain a variable number of inter-cysteine amino acid residues [7]. The number of amino acids between the eight cysteines in the 8 CM motif of *BrLTP1* and it’s aligned sequences are 10, 16, 0, 9, 1, 22 and 9, with a methionine and a valine residue present 10 and 4 aa before Cys2. These characteristics place the *BraLTP1*, *AtLTP1*, *BoLTP1*, and *BnaLTP1/2* proteins as secreted LTPs in class VI of plant *nsLTPs*.

**Overexpression of *BrLTP1* in *B. napus***

Given the 100% similarity between the *B. rapa* LTP1 and *B. napus* LTP2 proteins, and the relative agronomic importance of *B. napus*, we carried out functional analysis of *BraLTP1* in *B. napus*. Overexpression of *BraLTP1* driven by the CaMV 35S promoter in 12 independent transformants of the *B. napus* L. cultivar Zhonghuang 6 showed expression levels 415 times higher than in wild type using qRT-PCR (our primers could analyse *BraLTP1* from *Brassica rapa* and its homologous gene from *Brassica napus* without difference) (Fig. 1C). The most visually striking feature of the *BraLTP1* overexpression lines was a distinctly green leaf phenotype, with no waxy surface visible on either the abaxial or adaxial surfaces compared with the wild type (Fig. 2). Additional morphological differences in *BraLTP1* overexpressing plants included humpy and wrinkled leaf surfaces, abaxially curvature of leaf edges, and a significant reduction in total plant size relative to the wild type and negative segregants (Fig. 2 and Fig. 3A). These phenotypes were widespread in most lines, and we chose two 35S::*BraLTP1* transgenic lines (*BraLTP1-20* and *BraLTP1-22*) for further study due to their low copy numbers, morphologically clear phenotype and high transgene expression, enabling easy generation and comparison to negative segregates as controls.

To confirm that these phenotypes resulted from specific overexpression of *BraLTP1* rather than tissue culture or vector insertion effects, we performed co-segregation analysis. Leaf samples of positive and negative segregants of *BraLTP1-20* and *BraLTP1-22* lines, together with wild type, were selected based on genomic PCR for the insert (Fig. 3B) and real-time PCR of *BraLTP1* expression (Fig. 3C). Plants of *BraLTP1-20* and *BraLTP1-22* that were positive for the insert and had high *BraLTP1* transcript level also had the phenotypes described above, while negative segregates of these lines showed the same phenotype as the wild type controls (Fig. 3A, B and C). Therefore, 35S::*BraLTP1* transgenic phenotypes perfectly cosegregated with overexpression of the *BraLTP1* gene.

**Overexpressing *BraLTP1* reduces cuticular wax in leaves**

Mutant analyses have implicated a role for *nsLTPs* in the transport of waxes or cutin monomers [8,9,10,11], however overexpression of *nsLTPs* has seldom been reported. To determine whether the visible leaf phenotypes of the *B. napus* *BraLTP1* overexpressor lines resulted from decreased cuticle wax, the density of wax crystals on the leaf surface was assessed by scanning electron microscopy (SEM). A clear reduction in wax crystal density was observed on leaves of the *BraLTP1-22* plants, accompanied by altered crystal shape and form (Fig. 4). This suggested that altered epicuticular wax might lead to the visible phenotypes of *BraLTP1* overexpressing leaves.

To determine the chemical composition of *BraLTP1* overexpressing leaves in greater detail, gas chromatography mass spectrometry (GC-MS) analyses were performed. A 78% reduction in levels of the C31 alkane, hentriacontane, a major component of...
cuticular waxes, was seen in *BraLTP1*-22 leaves (49.6 μg g⁻¹) relative to wild type (221.3 μg g⁻¹). A second major wax constituent, C29 alkane (nonacosane), was decreased by 44% in the overexpressor line relative to wild type; from 1431.7 μg g⁻¹ to 82.8 μg g⁻¹. Other wax components were similarly reduced, ranging from 17% to 80% reductions (Fig. 5). Despite these defects in cuticular wax, *BraLTP1*-22 overexpressors did not display any organ fusions, unlike some other mutants with cuticle defects [39,40]. Together these results suggest a broad-range, non-specific reduction in wax deposition in *B. napus* plants overexpressing *BraLTP1*.

Distortion of the cuticular layer often results in an increased permeability of leaves [41,42,43]. To test this, water loss assays of detached leaves from *BraLTP1*-20, *BraLTP1*-22 and wild type were performed. A significantly higher rate of water loss occurred for the detached leaves of *BraLTP* overexpressing lines when compared with wild type (Fig. 6). This is consistent with the observed abnormal cuticular layer of overexpressor leaves.

| Gene name | Primer name | Sequence(5’→3’) |
|-----------|-------------|-----------------|
| IPT1,4,6  | Fna-75600-F | GAGGAGGCAAATGGAAATAG |
|           | Fna-75600-R | CGACGAACTCGAACATCATGA |
| IPT2      | Fna-41524-F | CAAACAGGAGCTGAATACC |
|           | Fna-41524-R | AGCGGAGCTATTTTGTGCTG |
| IPT3      | Fna-06414-F | TCAGGAAATGCGGCTTCTAA |
|           | Fna-06414-R | GTTTGCAAGCTACCAGGAAAG |
| IPT5      | Fna-36855-F | GAGGGGAGAAGCTGTATTAT |
|           | Fna-36855-R | CGGACATGCAGCAAGAACAG |
| IPT7      | Fna-44012-F | TTGGGTGCACTTCTTTATAC |
|           | Fna-44012-R | GCTTTGGATGTGACTTCT |
| IPT8      | Fna-63259-F | GCTTGCGCAAGAGAGAATAG |
|           | Fna-63259-R | CTCTCTGAGATGCTCCCTAA |
| IPT9      | Fna-63954-F | GCGGTAGCATAAAAGAGTGAAG |
|           | Fna-63954-R | CATTGGACGCTGTTACAGAG |
| CYP735A1  | Fna-68846-F | GAACTAGCCGACACTCTTCTC |
|           | Fna-68846-R | GCAGCCACATACCTCTTACT |
| CYP735A2  | Fna-08361-F | CCTCTAGCTCTTTGTCTATA |
|           | Fna-08361-R | TTGCTCAAGCGGAAGGATAC |
| URH1      | Fna-72547-F | GGGTGAGACTCAAGGAATAG |
|           | Fna-72547-R | CATGCCACGTAGCTGGAAAG |
| AP1       | Fna-16396-F | TTCTTAGGGCACAGCAGAG |
|           | Fna-16396-R | GCATGTATGATGCTGGATTTG |
| AP2       | Fna-27636-F | GCAGATGACGAAATTAAGCAAG |
|           | Fna-27636-R | CTCCCCAGGACACACCTTAT |
| AP3       | Fna-72759-F | ATCGAAGGATACGTTCTTAC |
|           | Fna-72759-R | AATGAGTCTAGAGCGAGATGG |
| PI        | Fna-60439-F | AATGTGGCAGGAGGAGAA |
|           | Fna-60439-R | GAATCGCGCTGACCTGTATC |
| AG        | Fna-37522-F | CTTATGCGCAAGAGGAAATAG |
|           | Fna-37522-R | ATGCCGGACAGTGGAAATA |
| CRC       | Fna-16399-F | AAGAGTGCCACATCGGAAAT |
|           | Fna-16399-R | GCTCAGGAAATGAAGGATAG |
| SPT       | Fna-54144-F | CTTTGAGACCTTTCCCTACTT |
|           | Fna-54144-R | CATCAAACGCGACATGTTCTC |
| LEUNIG    | Fna-27110-F | ACAGCAGTGAAAGGAAATG |
|           | Fna-27110-R | CACCAATACGAGTGGAAAG |
| ANTEGUMENTA | Fna-09982-F | AGACACAGATGCGTGGAG |
|           | Fna-09982-R | GAGCAGCTCTCTCTCCATAC |
| Actin     | BrActin 88-F | GCTGACCCTATGAGCAAGAAG |
|           | BrActin 88-R | AGATGGATCCATCCAATCAAAC |

Table 1. Primers for real-time PCR checking genes related to cytokinin synthesis and flower development.

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suggesting that wax defects can result in perturbed cuticle permeability. The result also hints that *BraLTP1* could be important for plant water relations and drought tolerance.

**Overexpression of *BraLTP1* promotes cell overproliferation in leaf**

In addition to the wax phenotype, the cellular morphology and weight of per unit leaf area was examined (Fig. 7A and B). *BraLTP1*-20 and *BraLTP1*-22 leaves were 0.033 g cm\(^{-2}\) and 0.031 g cm\(^{-2}\) respectively, significantly higher than negative segregants and wild type, which varied from 0.024 g cm\(^{-2}\) to 0.026 g cm\(^{-2}\) \((P<0.01)\) (Fig. 7A). To examine changes at the cellular, structural level, paraffin-wax-embedded leaf cross-sections stained with Safranin and fast green were examined. This demonstrated that in *BraLTP1* overexpressing lines, the cellular layer of both palisade tissue and parenchyma tissue was increased; with palisade cells, parenchyma cells and epidermic cells smaller and more compact than negative segregants and wild type sections (Fig. 7B). Thus, increased cellular layering and a compact cell arrangement likely led to the increase in weight for per unit leaf area. This underlying change in leaf cell layer number and density likely contributes to the visible morphological defects including leaf curling.

To correlate these changes to cytokinin composition, we quantified cytokinin content, including the levels of the cytokinins isopentenyladenine (iP) and trans-zeatin (tZ), and of their riboside...
As observed in BraLTP1-22 line, the BraLTP1 overexpressing leaves contained significantly higher amounts of tZ, tZR, iP, and iPR when compared with the wild type (P<0.01, Fig. 8), consistent with more cell accumulation in leaves (Fig. 7B). This indicates that active cytokinins are highly synthesized in leaves overexpressing BraLTP1.

**BraLTP1 affects flower development**

In the reproductive phase of the B. napus lifecycle, overexpressing BraLTP1 resulted in early development of longer carpels and outward bending stamen in the flower. Early in flower development, carpels grew out of the apical flower buds, with the outward bending stamens clearly visible from the slightly opened sepals, giving an observably distinct phenotype from wild type flowers (Fig. 9A, B and C). Microscopic examination of the petals in the bud showed a variable severity of this phenotype in different lines. In the moderate version of this phenotype, the four petals developed into nearly normal petals upon flower opening (Fig 9A, B and D), while in severe phenotype, poorly developed, shriveled petals were found resulting in only one to three petals in the opened flowers (data not show). These phenotypes occurred not only in primary inflorescences but also in other branched inflorescences. In addition, bumpy sepals and siliques with all distributed longitudinal ridges on the surface, similarly to the bumpy phenotype of leaves, were observed in 35S::BraLTP1 transgenic B. napus lines (Fig. 9D and E). Cross-section of sepal and siliques did not show a difference in cell proliferation between transgenic plants and wild type (data not show). Microscopic examination of the ovules within the carpels revealed developing embryos and endosperm, indicating self-compatibility and good fertilization; pollen viability identification revealed that pollen production was also normal (data not show). Silique filling occurred effectively, thus BraLTP1 over-expression had few detrimental effects on plant fertility (Fig. 9E).
Co-regulated genes to cytokinin synthesis and flower organ development

The transcript abundance of 10 genes involved in the cytokinin synthesis pathway, and 15 genes involved in floral organ development, were investigated by real-time PCR in the wrinkled leaves of BraLTP1 overexpression lines as well as in wild type (Table 1).

In Arabidopsis, seven genes for adenine phosphates isopentenyl transferase (AtIPT1 and AtIPT3 to AtIPT8) have been identified as cytokinin biosynthesis genes [44,45]. IPT and CYP735A4, are responsible for the differential distribution of de novo synthesis pathways for isopentenyladenine (iP), trans zeatin (iZ) [46]. Recently, a novel uridine ribohydrolase, URH1, was characterized that degrades isopentenyladenosine in Arabidopsis [47]. In leaves of BraLTP1 overexpression lines, the cytokinin biosynthesis genes IPT3 were significantly increased (P<0.01), with 3.8 times of expression levels in wild type, while IPT7, IPT9 and CYP735A2 were decreased (P<0.01), and there was no significant change in IPT1, IPT2, IPT4, IPT5, IPT6, IPT8, CYP735A1 and URH1 (P>0.05) (Fig. 10A).

The ABC model of flower development describes how the combinatorial interaction of three classes of genes directs the development of four types of floral organs [48,49]. Here, in BraLTP1 overexpression lines, the expression level of genes involved in flower development including AP1, AP2, AP3, PI, CRC were significantly increased in the abnormally-developed flowers, while AG, SPT and LEUNIG were significantly increased. There was no significant change in AINTEGUMENTA (Fig. 10B).

The decreased transcript levels of AP1, AP2, AP3, PI are consistent with the phenotype of BraLTP1 overexpressing flowers in B. napus, with short and humpy sepals and outwardly bent stamens. Furthermore, the increased expression levels of AG, SPT and LEUNIG were consistent with the over-developed longer carpels. These results, plus phenotypic data suggest that BraLTP1 plays a role in flower development.

Discussion

Functional characterization of BraLTP1

In this study, we report the isolation and characterization of an nsLTP-like gene from B. rapa; BraLTP1. Sequence analysis showed that the BraLTP1 protein is a single copy gene in the ‘A’ genome of B. rapa, with a homologous gene in B. oleracea, ‘C’ genome and two corresponding ‘A’ and ‘C’ genome copies in the amphidiploid ‘AC’ genome of B. napus. BraLTP1 was first reported by Dong [50] as a B. napus seed specific gene named Br15D18B (genbank number: AY208878), which was differentially screened in a seed-cDNA library harvested 15 days after pollination (DAP). The amino acid similarity of Arabidopsis, B. rapa, B. oleracea and B. napus LTP1 copies is high and extends throughout the whole protein, with increasing divergence consistent with the older evolutionary relationship of Arabidopsis. The configuration of the 8CM domain and inter-cysteine amino acid residues places BraLTP1 in class VI of nsLTP, which in Arabidopsis is composed predominantly of uncharacterized proteins including AtI1g32280.1, At4g30880.1, At4g33550, and At5g56480.1 [7]. Until recently, all type VI nsLTP genes were less studied, with unknown functions, providing good opportunity to expose new physiological functions of this family in processes such as cell division, as shown herein.

In this study, we cloned and functionally analysed a type VI nsLTP in B. napus. Over-expression of the BraLTP1 gene caused growth defects in the seedling and reproductive organs of B. napus. These included a distinct green, disorganized leaf surface with curled edges and abnormally developed flower. Decreased levels of epicuticular wax accumulated on the leaf epidermis, and cell layering and cell density were increased in the mesophyll cell.
of *BraLTP1* overexpressing leaves. Increased cytokinin levels including of tZ, tZR, iP, and iPR and increased expression of the cytokinin-synthesis gene *IPT3* in *BraLTP1* overexpression lines correlated well with the enhanced cell proliferation phenotype. Overexpression of *BraLTP1* also led to the altered transcription of many important ABC model flower development genes, coinciding with the visible morphological and developmental perturbations in these lines. Overall, our experiments suggest that *BraLTP1* is an important *nsLTP* gene affecting wax deposition, cell proliferation, and leaf and flower morphology development in *B. napus.*

**Overexpressing *BraLTP1* leads to a reduction of wax load**

*nsLTPs* are proposed to play a role in the delivery of wax components during the assembly of the cuticle [26,51]. Previous studies suggested that in *nsLTP* mutants such as *ltpg1*, *ltpg2* and *ltpgltpg2*, wax load decreases with the reduced expression of *nsLTP* genes [10,11,12]. Therefore, we hypothesised that overexpressing *nsLTP* in *B. napus* might lead to wax enrichment. However, in *BraLTP1* overexpressing plants, wax accumulation was decreased in seedling leaves. The amount of wax on *BraLTP1-22* leaves was significantly reduced with proportional deficiencies in the component aldehydes, alkanes, alcohols and acids compared with wild type. This universal reduction, with no specific component alteration, in *35S::BraLTP1* transgenic plants suggested that the effect of *BraLTP1* overactivity was not substrate specific.

There are a few possible explanations for this contradicting observation: (1) the genes above belong to glycosylphosphatidylinositol-anchored *LTPs*, which are different from the type VI *BraLTP1* and exercise different mechanisms *in vivo*; (2) overexpression of *BraLTP1* may lead to disordered or destructive secretion of wax out of cells, which is subsequently lost from the surface; (3) overexpression of *BraLTP1* gene somehow feedback inhibits, or competitively inhibits, other *nsLTPs* with important complimentary lipid synthesis or transport abilities. Until recently, functional analysis of wax-synthesis-related *nsLTP* genes has focused on mutants, while transgenic overexpression is seldom reported. To our knowledge the only prior overexpression study was on *LTP3* in *Arabidopsis*, overexpression of *LTP3* enhanced freezing and drought tolerance in *Arabidopsis* but with no change of cuticular wax seen [15]. Further investigation of the molecular mechanism of *BraLTP1* action will shed more light on its function in wax metabolism and feedback regulation.

Of *BraLTP1* overexpressing leaves. Increased cytokinin levels including of tZ, tZR, iP, and iPR and increased expression of the cytokinin-synthesis gene *IPT3* in *BraLTP1* overexpression lines correlated well with the enhanced cell proliferation phenotype. Overexpression of *BraLTP1* also led to the altered transcription of many important ABC model flower development genes, coinciding with the visible morphological and developmental perturbations in these lines. Overall, our experiments suggest that *BraLTP1* is an important *nsLTP* gene affecting wax deposition, cell proliferation, and leaf and flower morphology development in *B. napus.*

**Overexpressing *BraLTP1* leads to cell overproliferation**

Besides wax load reduction, we also found that (1) *35S::BraLTP1* transgenic *B. napus* plants exhibit disorganized leaf patterning/morphology; (2) mesophyll cells were over proliferated, with increased cell layer number and cell density, and; (3) cytokinin levels were significantly increased and (4) the cytokinin-synthesis gene *IPT3* was increased 3.8 fold in transgenic leaves. It is known that wax is composed of VLCFA and their derivatives, thus our data is consistent with Nobusawa et al [31], wherein VLCFA synthesis in the epidermis confines cytokinin biosynthesis via *IPT3* to the vasculature and restricts cell proliferation. While it is also possible that *BraLTP1* itself plays a direct role in cell division by affecting related genes. However, the internal mechanism remains to be clarified.

The decreased expression of other cytokinin synthesis related genes seen here may be due to feedback downregulation through the pathway, or functionally differentiated roles of *IPTs* in response to environmental conditions. VLCVA, its derivatives, or VLCFA-related lipids, may function as signaling molecules to control cell division by affecting cytokinin related gene transcription [31,52,53,54]. Further studies are needed to examine such possibilities and explore the specific mediators or ligands which suppress cell proliferation in tissues. Mutant material in Nobusawa’s study was difficult to observe macroscopically because of severe growth defects and in our experiment, overexpressing line for *BraLTP1* gene analysis produced a moderate, morphologically observable phenotype that produced fertile seed for continued research, shedding important light on the system for study of plant-environment interaction.
Overexpressing BraLTP1 led to altered flower morphology

Some studies have reported that nsLTPs are involved in flower development. For example, multiple nsLTP genes were identified to be differentially expressed in petals during different developmental periods in carnation flowers, suggesting their contributions to petal development [55]. FIL1, a non-specific lipid-transfer protein with an nsLTP-like domain was reported to be important in petal and stamen formation in Antirrhinum [56]. The identification of Antirrhinum nsLTPs as target genes of the class B MADS box transcription factors DEFICIENS, suggested a function during late petal and stamen development [57]. Kotilainen et al [58] reported that the gltp1 gene in Gerbera hybrida var. Regina was expressed only in the corolla and carpels and was developmentally regulated during corolla development.

In the ABC model of flower development, the A-class genes APETALA1 (AP1) and AP2 confer sepal. Their activity overlaps with B-class genes APETALA3 (AP3) and PISTILLATA (PI), which develops into petals. B-class genes and the C-class gene AGAMOUS (AG) specify stamen, while AG promotes carpel development [48,49]. Two newly characterized genes, CRABS CLAW (CRC) and SPATULA (SPT), function similarly to AG to promote carpel differentiation. LEUNIG and AINTEGUMENTA are also putative genes affecting carpel development [59]. In our study, overexpressing BraLTP1 led to altered expression levels of class ABC genes, which control flower organogenesis, with AP1, AP2, AP3, PI decreased. This is consistent with the morphological defects of sepals and stamens seen. The expression level of AG, SPT and LEUNIG were increased, which may result in the early development of longer carpels (Fig. 10B). Combined with previous studies of nsLTP on

Figure 10. Transcript abundance of various cytokinin-related (A) and flowering-related (B) genes in 35S::BraLTP1 (BraLTP1-22) and wild type (WT) plants as determined by qRT-PCR. Data is the average of three plants with standard errors. Asterixes indicate significant differences to WT (** P<0.01).

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flower development, we speculate that BraLTP1 may affect flower development through the regulation of morphologically important cellular components like the cell wall and cuticle, to affect flower-related genes.

**Conclusion**

This study identifies a novel nsLTP gene BraLTP1 that influences wax deposition, cell proliferation and flower development when overexpressed in *B. napus*. Although the precise biological role is yet to be determined, we suggest that *BraLTP1* may link the metabolism of wax lipids and/or cell wall components in the epicuticular, or internal plant interfaces, to the coordinated execution of developmental programs, including cell division and flower development. Therefore, *BraLTP1* likely plays important roles in different developmental periods in plants.

**Author Contributions**

Conceived and designed the experiments: FL DF GW. Performed the experiments: XX LW XZ YC YW. Analyzed the data: FL AH. Contributed reagents/materials/analysis tools: YL. Wrote the paper: FL DF AH GW.
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