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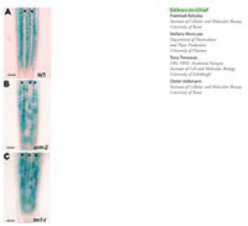

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On the role of a Lipid-Transfer Protein. Arabidopsis *ltp3* mutant is compromised in germination and seedling growth.

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Keywords: lipid transfer protein, oilseed, seed germination, seedling growth

Abbreviations: das, days after stratification; GLC, gas-liquid chromatography; LTP, lipid transfer protein; TAG, triacylglycerides; TTC, 2,3,5-triphenyltetrazolium chloride.

Plant Lipid-Transfer Proteins (LTPs) exhibit the ability to reversibly bind/transport lipids *in vitro*. LTPs have been involved in diverse physiological processes but conclusive evidence on their role has only been presented for a few members, none of them related to seed physiology. Arabidopsis seeds rely on storage oil breakdown to supply carbon skeletons and energy for seedling growth. Here, Arabidopsis *ltp3* mutant was analyzed for its ability to germinate and for seedling establishment. *Ltp3* showed delayed germination and reduced germination frequency. Seedling growth appeared reduced in the mutant but this growth restriction was rescued by the addition of an exogenous carbon supply, suggesting a defective oil mobilization. Lipid breakdown analysis during seedling growth revealed a differential profile in the mutant compared to the wild type. The involvement of LTP3 in germination and seedling growth and its relationship with the lipid transfer ability of this protein is discussed.

Introduction

Plant Lipid-Transfer Proteins (LTPs) are a family of peptides of usually 7–10 kDa in size, widely distributed among plants.[1-5] Unlike their animal counterparts, plant LTPs typically have broad substrate specificity and are also named nonspecific LTPs. Their affinity for lipids is presumed to be crucial for their biological function, but *in vivo* substrates remain largely unknown. LTPs were identified according to their ability to bind phospholipids and fatty acids and were originally proposed to participate in the intracellular transfer of lipids.[2] These peptides present a common structural feature which includes 8 cysteine residues involved in 4 disulfide bridges and a consensus amino acid signature (Prosite PS00597). Several LTP structures have been determined and revealed the presence of 4 α helices which define a central hydrophobic cavity that can accommodate lipids and justifies their lipid-binding properties. [6]

A plant LTP classification system has been updated [7-8] according to sequence similarities and the length of intervals among their 8 cysteine residues, identifying 9 different types. LTPs appear as multigenic families with complex expression patterns.[7,9] In *Arabidopsis thaliana* at least 15 genes were originally detected using a stringent criterion[10] but later the putative LTPs has been described to reach up to 50 in number.[7] Several roles have been proposed for LTPs [1] even if the conclusive demonstration of the function of individual isoforms is barely documented. They have been implicated in numerous biological processes including abiotic stress responses and pathogen defense.[11-15] In fact, LTPs have been recognized as pathogenesis-related (PR) proteins and constitute the PR-14 family.[16] This function may be related to their antimicrobial activity, initially observed in radish extracts[17] and later determined in several isoforms isolated from monocots as well as dicots.[1,18] Besides, the existence of a plant LTP sub-family involved in lipid signaling has been proposed.[19] The Arabidopsis LTP named DIR1 is implicated in defense signaling[20] and N5 from *Medicago truncata* is involved in the symbiotic interaction with rhizobia, probably regulating the competence of epidermal cells for rhizobial infection.[21] Other LTPs can also function in cuticle synthesis which correlates with a role in biotic and abiotic stress tolerance through reinforcement of this mechanical barrier which, in turn, prevents pathogen entry and reduces water losses.[22-23] On the other hand, a LTP from tobacco was shown to promote cell wall loosening and may play a role in cell expansion and consequently in plant growth.[24] Besides, the LTP SCA from lily styles is required for pollen tube adhesion.[25] Other LTPs have been functionally characterized in adult plants and appeared implicated in biotic stress,[26] pollen tube growth and fertilization[27] and freezing tolerance.[28] To our

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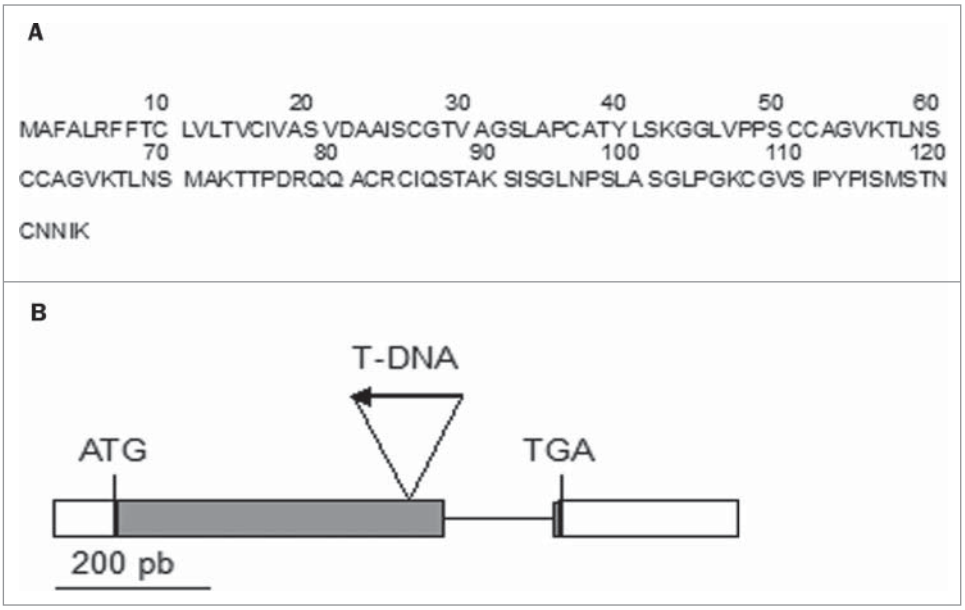

Figure 1. Amino acidic and genomic structure of *Arabidopsis thaliana* LTP3 (**A**) LTP3 amino acid sequence (Uniprot Q9LLR7). (**B**) Schematic representation of ltp3 insertion line genomic structure (SALK 095248). Exons, untranscribed regions, and intron, are indicated by gray boxes, white boxes, and lines, respectively. The T-DNA insertion in *ltp3* mutant is shown.

knowledge, no LTP function has yet been demonstrated related to seed physiology, although LTPs are frequently found in seeds and the fact that a castor bean isoform has been suggested to participate in lipid metabolism.[29]

Germination and post-germinative growth are essential processes in the life cycle of plants. Radicle emergence is considered as the completion of germination *sensu stricto* and then seedling establishment starts. Seedling growth is supported by storage reserves until the seedling becomes fully photoautotrophic. In oilseeds such as *A. thaliana*, seedling growth is accomplished by the catabolism of fatty acids.[30] Briefly, oils stored in the cotyledons are converted to fatty acids and glycerol and the former are next broken down by β-oxidation in the peroxisomes to produce succinate through the glyoxylate cycle. The resulting succinate is further converted to carbohydrates and used to sustain seedling growth. Despite the relevance of this pathway several aspects still remain unknown. Proteomic approaches have detected Lipid-Transfer Protein 3 (LTP3) in *A. thaliana* cotyledons (Proteomics Identifications Database) making it a suitable candidate to participate in oil mobilization. In this study Arabidopsis *ltp3* mutant was analyzed to investigate the putative involvement of this LTP in seed germination and seedling establishment.

Results and Discussion

Arabidopsis LTP3 (Uniprot Q9LLR7) is predicted to be a 115 amino acid protein with strict conservation of the critical cysteine residues of LTPs (**Fig. 1A**). A T-DNA insertion line disrupted in the first exon of the LTP3 gene is available (SALK 095248) and is depicted in **Fig. 1B**. This line, thereon *Atltp3*, has been analyzed elsewhere demonstrating that it is a loss-of-function mutant.[13] Here, the rate of germination was measured in *Atltp3* and compared to Col-0 wild type. **Fig. 2** reveals that *Atltp3* is compromised in its ability to germinate. It exhibits a delay in germination kinetics and lower germination frequency, attaining percentages of germination around a 50% of the wild type line. Nevertheless, evaluation of seed viability with 2,3,5-triphenyltetrazolium chloride (TTC) revealed that *Atltp3* and wild type seeds are equally viable (**Table 1**). So, the partial disability to germinate exhibited by *Atltp3* may be related to the control of this process rather than to cell death.

In oily plants the breakdown of stored triacylglycerides (TAG) following germination is essential to drive the initial phase of seedling growth and allow photosynthetic establishment.[30-31] So, *Atltp3* was further evaluated for its seedling phenotype. To that aim, mutant and wild type seeds were grown in darkness without any carbon supply addition to assess TAG dependent postgerminative growth. As shown in **Fig. 3A** (lower panel, minus sucrose), the mutant line exhibits shorter hypocotyls without any other evident difference compared to the wild type line. At 9 d after stratification (das) a significant reduction ($P < 0.001$) in hypocotyl length was observed in *Atltp3* compared to the wild type seedlings (**Fig. 3B**). Seedling establishment phenotypes can be frequently rescued by the provision of a carbon supply.[32] To elucidate this point we have analyzed wild type and *Atltp3* lines for seedling growth in darkness but in the presence of 1% sucrose (**Fig. 3 A-B**). Interestingly, the hypocotyl length reduction previously observed for *Atltp3* was reversed in sucrose supplemented medium. This behavior is similar to that exhibited by Arabidopsis mutants disrupted in lipid mobilization such as Ped3p.[32]

Accumulated evidence indicates that several mutants in oil mobilization pathways are compromised in seedling establishment[33-34] since the most important function of fatty acid β-oxidation is to support postgerminative growth.[35] In order to verify if the phenotype observed in *Atltp3* correlates with the inability to use seed oil reserves we have first analyzed the fatty acid profile of mutant and wild type seeds just before germination was triggered. Gas-liquid chromatography (GLC) determinations revealed a similar fatty acid composition for both lines (**Fig. 4A**), and this composition is in agreement with the relative levels determined in previous reports for wild type seeds.[36] It has been documented that eicosenoic acid (20:1) is specifically found in TAG in Arabidopsis. In fact, eicosenoic acid has been used as a convenient marker to monitor TAG breakdown.[34,37] **Figure 4B**

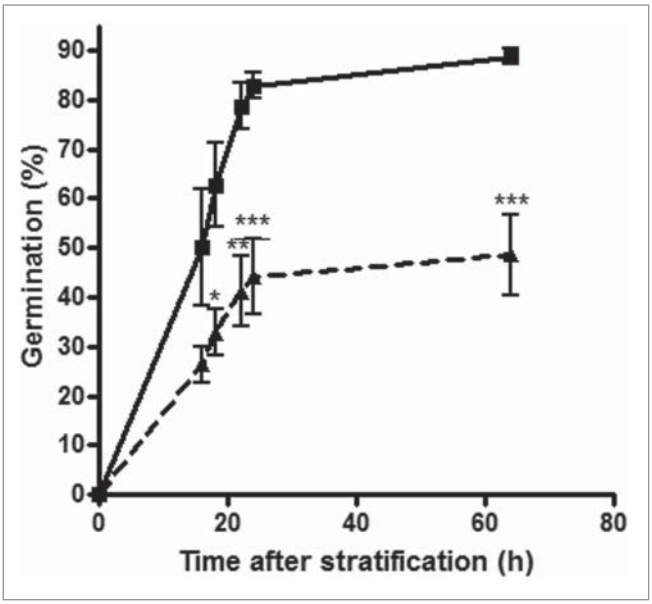

Figure 2. Germination is impaired in *Atltp3*. Seeds of wild type (black square) and *ltp3* (black triangle) lines were sown and allowed to germinate on MS media with vitamins in growth chamber with 16 h daily illumination at 25°C. Radicle protrusion was assessed in more than 40 seeds per batch, at different times after sowing. Error bars represent SD. A Two-way ANOVA and Bonferroni posttests was calculated at the probability of either 5% (*P < 0.05), 1% (**P < 0.01) or 0.1% (***P < 0.001).

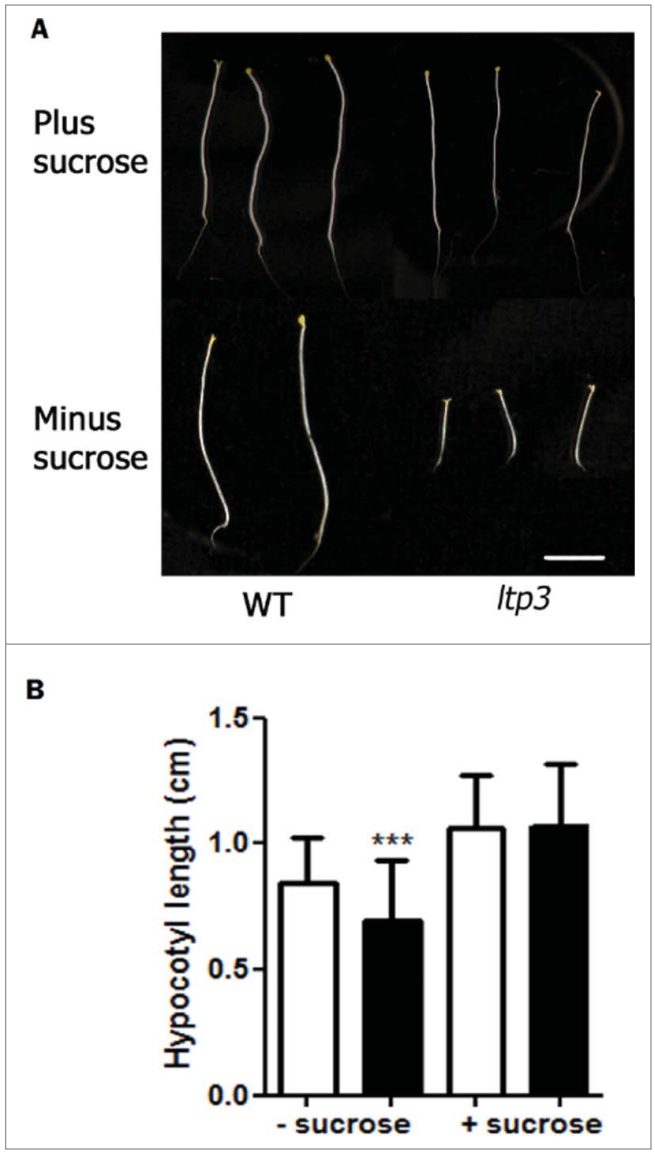

Figure 3. Postgerminative growth is reduced in *Atltp3*. (**A**) Wild-type and *ltp3* seedlings dark-grown on agar plates containing MS medium plus or minus 1% sucrose. Representative photograph taken 7 d after stratification. Bar: 1 cm. (**B**) Hypocotyl length in wild-type (white bars) and *ltp3* (black bars) seedlings. Error bars represent SD. A one-way ANOVA and Tukey's Multiple Comparison posttests was calculated at the probability of 0.1% (***P < 0.001).

shows the TAG-fatty acid composition of the wild type and *ltp3* mutant upon germination. It can be seen that after 5 d of germination the mutant exhibits a differential fatty acid profile compared to the wild type. As expected, eicosenoic acid is barely detected in the wild type but is still present in high levels in *Atltp3*. In fact, the profile observed in the mutant during seedling growth is similar to that observed before the germination start. This evidence confirms that *Atltp3* mutant is at least partially impaired in TAG mobilization.

TAG are packed in the oil bodies and these structures are in close proximity with peroxisomes. So, fatty acids must pass from one organelle to the other using not fully understood translocation mechanisms.[35] In *A. thaliana*, 3 independent experimental approaches have identified the same peroxisomal transporter, named CTS /PXA1/PED3, as responsible for the translocation of at least part of the fatty acids for β-oxidation. [31,38,39] It has been suggested that CTS /PXA1/PED3 might not be the only transporter for β-oxidation substrates into the peroxisome[34] and a LTP may be reasonable implicated in this process. A mayor concern to consider this hypothesis is the subcellular location of LTP3. Although LTP3 is predicted to be extracellular, a putative ortholog of LTP3 from sunflower seeds has been shown to change its localization from the apoplast to intracellular oil bodies when germination is triggered.[40] Thus, the bulk of evidence prompt us to hypothesize that LTP3 could play a direct role in oil mobilization. A previous report by Tsuboi et al.[29] supports this speculation since a LTP from castor bean cotyledons was detected in the glyoxysomal/peroxisomal fraction. Moreover,

Table 1. *Seed Viability is not affected in ltp3 lines*. Wild type and mutant seeds were stratified and submitted to TTC staining. Blue viable seeds were counted under magnifying glass and expressed as percentage of total seeds.

Line	Viability
Wild type col-0	96.81 ± 2.77a
ltp3	94.46 ± 5.55a

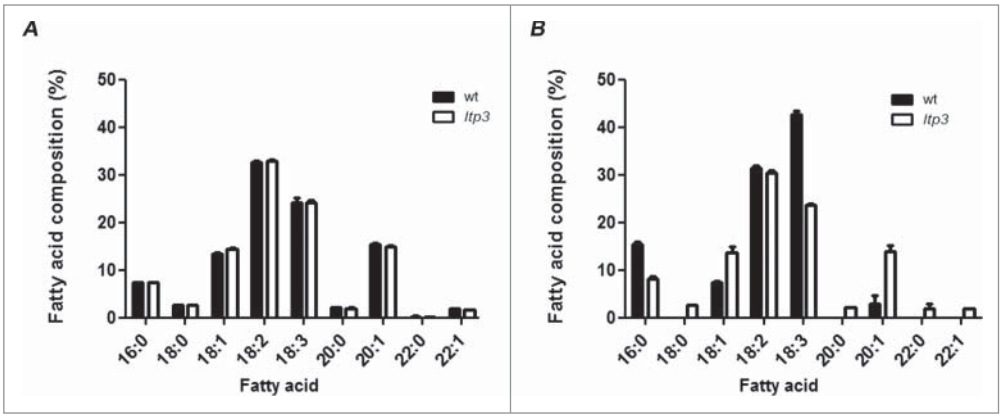

Figure 4. Fatty acid mobilization is altered in *Atltp3*. TAG-derived fatty acid profile in wild type (black bars) and *ltp3* (white bars) during germination. (**A**) Seeds, 0 das; (**B**) Seedlings , 5 das. Data are mean ± SD for n = 3 (wt) or 5 (*ltp3*).

that LTP was shown to bind oleyl-CoA and to enhance the activity of acyl-CoA oxidase *in vitro*.[29] Since this enzyme catalyzes the first step in the fatty acid β-oxidation pathway, the authors suggested a direct role of the LTP in lipid mobilization.

Guo et al.[13] have recently characterized the same *Atltp3* mutant used in this study (SALK_095248) regarding its involvement in abiotic stress in Arabidopsis adult plants. In fact, *Atltp3* was more sensitive to drought stress compared to wild type plants while overexpression of the gene resulted in enhanced freezing and drought tolerance. Consistent with our results, in that report seedlings were initially grown in MS medium containing 2% sucrose, so that the clear phenotype exhibited by the mutants in the absence of sucrose was prevented and probably resulted unnoticed by the authors. Interestingly, the ability of LTP3 to bind lipids was experimentally confirmed in that report [13] and complements our results. The phenotype exhibited by the *Atltp3* loss of function mutant regarding germination, together with the lipid-binding activity of LTP3 temps to speculate that it could participate in the transfer of lipids during seedling establishment. Data reported here reveal LTP3 involvement in seed germination and seedling growth in Arabidopsis and suggest the participation of this LTP in stored lipid mobilization during post-germinative growth. Future research will provide a more detailed understanding of the role of LTPs during this crucial process in the life cycle of plants.

Methods

Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as reference wild type. Seeds of the tDNA insertion line *ltp3* (At5g59320) were provided by NASC (SALK 095248) and primers shown in Supplementary **Table 1** were used to verify through PCR that *ltp3* was a homozygous line. All procedures used followed standard protocols for *A. thaliana.* Briefly, seeds were surface sterilized using 70% ethanol for 2 min, 50% bleach and 0.2% SDS for 10 min and then extensively rinsed with sterile water. They were stratified at 4°C for 4 d in darkness to break seed dormancy and allowed to germinate on agar plates containing Murashige and Skoog (MS) medium with vitamins. Plates were vertically positioned and placed in a growth chamber with 16 h daily illumination at 25°C. When indicated 1% sucrose was added to MS media for seedling growth.

Germination and seedling growth measurements

Germination was evaluated on 3 different seed batches of each genotype. Those batches were obtained from wt and *ltp3* plants germinated and grown at the same time under the same growth chamber conditions. Only seeds that were obtained from plants grown side-by-side were compared. For each assay at least 3 plates per time point per treatment were analyzed. Germination was evaluated under magnifying glass and defined as radicle protrusion. To analyze post-germinative growth etiolated seedlings 5 d after stratification were photographed and digitalized using a Nikon DS-FI 1 camera and hypocotyl length was determined from the original images using IMAGE J software (NIH, Bethesda, Md), according to Eastmond et al.[30]. Seed viability was determined using 1% TTC as vital dye.[41] Wild type and mutant seeds were stratified, submitted to TTC staining and blue viable seeds were counted under magnifying glass. Viability was expressed as percentage of total seeds.

Fatty acid determination

Oil extraction and TAG analysis by GLC were performed as described elsewhere.[42] To determine TAG derived-FA composition in mature seeds before starting germination (time 0), the seeds were stratified for 4 d at 4°C in the dark before extraction. A set of stratified seeds was transferred to germination conditions as described above and 5 das whole germinated seeds/seedling samples (15 mg) were collected, pooled and placed in screw-cap tubes (Teflon cap) containing 100 μl methanol. Each sample (by triplicate) was treated with 800 μl of methylation mix and incubated for 1 h at 80°C. Results are means of 3 independent repetitions for wild type seeds and 5 repetitions for *ltp3*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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