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Research article

Vacuolar biogenesis and aquaporin expression at early germination of broad bean seeds

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ABSTRACT

A key event in seed germination is water uptake-mediated growth initiation in embryonic axes. *Vicia faba* var. *minor* (broad bean) seeds were used for studying cell growth, vacuolar biogenesis, expression and function of tonoplast water channel proteins (aquaporins) in embryonic axes during seed imbibition, radicle emergence and growth. Hypocotyl and radicle basal cells showed vacuole restoration from protein storage vacuoles, whereas *de novo* vacuole formation from provacuoles was observed in cells newly produced by root meristem. cDNA fragments of seven novel aquaporin isoforms including five Tonoplast Intrinsic Proteins (TIP) from three sub-types were amplified by PCR. The expression was probed using q-RT-PCR and when possible with isoform-specific antibodies. Decreased expression of TIP3s was associated to the transformation of protein storage vacuoles to vacuoles, whereas enhanced expression of a TIP2 homologue was closely linked to the fast cell elongation. Water channel functioning checked by inhibitory test with mercuric chloride showed closed water channels prior to growth initiation and active water transport into elongating cells. The data point to a crucial role of tonoplast aquaporins during germination, especially during growth of embryonic axes, due to accelerated water uptake and vacuole enlargement resulting in rapid cell elongation.

1. Introduction

Quiescent seeds are characterized by an absence of hormone-controlled dormancy and their germination can simply be triggered by imbibition (Obroucheva, 2010, 2012; Weitbrecht et al., 2011; Bewley et al., 2013). Progressive water inflow results in tissue passing through successive thresholds of water content (WC), at which main metabolic processes are activated. A primary metabolic activation is completed at a WC of about 60% fresh weight (FW). Yet, a further increase in water content is necessary for growth initiation in embryonic axes. This process requires the accumulation of endogenous osmotica as well as cell vacuolation. These events, together with cell wall loosening, determine the initiation of embryonic axis extension and radicle protrusion. They provide rapid and successful root contact with soil water, a prerequisite for advantageous germination.

Whereas cell elongation is a fundamental process during early seed germination, the initiation of cell division varies between species and can occur simultaneously with elongation or later on (Obroucheva, 1999). Broad bean (*Vicia faba* L. var. *minor*) seeds are typical orthodox seeds, that is, they are capable of drying during maturation without any loss of viability. Their germination occurs first by pure cell elongation, whereas cell divisions start in the root meristem 15 h after radicle emergence (Obroucheva, 1999). These seeds are therefore a suitable model for studying the physiological processes underlying the initiation of germination.

Previous work from our laboratory has provided an accurate description of growth initiation in germinating broad bean seeds (Obroucheva, 1999). These seeds exhibit hypogeal germination, during which cell elongation is initiated in the hypocotyl, to push the non-growing radicle tip and let it emerge through the seed coat. In terms of growth initiation, broad bean germination can be described as follows. In air-dry seeds, the embryonic axis consists of a radicle (2 mm) and a hypocotyl (2 mm), with a small plumula

Abbreviations: FW, fresh weight; NIP, nodulin-like intrinsic protein; PIP, plasmalemma intrinsic protein; PSV, protein storage vacuole; TIP, tonoplast intrinsic protein; WC, water content.

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above it. In imbibing seeds, cell elongation is initiated at a water content of 72–73% WC, firstly in the upper hypocotyl, and spreads gradually to its base, thereby protruding the radicle tip. No cell division occurs in the hypocotyl. At radicle emergence, this organ is 3-mm-long, with its upper cells elongated to 100–110 μm. Elongation then begins in the emerged roots, in cells adjacent to the hypocotyl, whereas cell proliferation commences in the root meristem, when embryonic axes are 1 cm-long. In growing embryonic axes, cell division and elongation proceed in roots while the hypocotyl has already reached its final size of 9–10 mm, with fully-elongated 150 μm-long cells.

Because of their role in protein storage and cell turgor, vacuoles potentially play a crucial role during seed maturation and germination. The biogenesis of vacuoles first as protein storage vacuoles and thereafter as large vegetative vacuoles has been described at least partially in Arabidopsis (Höfte et al., 1992; Bolte et al., 2011; Gattolin et al., 2011), pea (Robinson and Hinz, 1996) and pumpkin (Maeshima et al., 1994), but precise knowledge is still lacking in broad bean. Aquaporins, channel proteins that facilitate water transport across cell membranes, have emerged as important players in plant water relations (Maurel et al., 2008). In all plant species examined, aquaporins occur as numerous (>30) isoforms classified in at least 4 subfamilies. Members of the Plasma membrane Intrinsic Protein (PIP) and Tonoplast Intrinsic Protein (TIP) subfamilies represent the most abundant aquaporins in the plasma membrane and tonoplast, respectively. The PIP subfamily can be further divided in PIP1 and PIP2 sub-types. The Nodulin26-like Intrinsic Protein (NIP) and Small basic Intrinsic Protein subfamilies encode homologues involved in the transport of micronutrients or as yet unknown substrates. Because of the intrinsic link between water inflow and germination, the expression of aquaporins in germinating seeds was studied (Obroucheva, 2013), particularly in *Arabidopsis* (Willigen et al., 2006; Gattolin et al., 2011), rice (Liu et al., 2007, 2013; Li et al., 2008), *Brassica napus* (Gao et al., 1999), and their presence in embryonic axes of germinating broad bean seeds was preliminarily shown (Shijneva et al., 2007).

Some of these studies have addressed the expression of the whole aquaporin family. In *Arabidopsis,* for instance, expression profiling using macro-array hybridization and immuno-blotting revealed that in dry seeds three TIPs (of which, two belong to the TIP3 subtype) were abundantly expressed, whereas no PIP expression could be detected (Willigen van der et al., 2006). Aquaporin expression was, however, dramatically altered during seed germination, the expression of the former TIPs vanished, whereas expression of seven PIPs and three TIP isoforms was progressively taking over. In rice, TIP3 homologues are also predominant in dry seeds and their expression is markedly reduced during germination (Li et al., 2008).

Whereas genetic evidence is just emerging (Liu et al., 2007, 2011), the role of aquaporins during germination has been tentatively investigated using pharmacological inhibition. Mercurials, which are potent but unspecific and toxic aquaporin blockers, did not reveal any role for aquaporins during the early imbibition of pea seeds (Veselova and Veselovsky, 2006). In *Arabidopsis* as well, no inhibiting effect of mercury on seed water uptake was observed until expansion of embryonic tissues (Willigen van der et al., 2006). Whereas these data were obtained in intact seeds, studies in embryonic axes, in which the preparation and early initiation of growth take place, could provide a higher resolution. Therefore, we reasoned that broad bean seeds could represent a relevant system for studying germination and investigated the expression and function of plasma membrane and tonoplast aquaporins in embryonic axes during seed imbibition, radicle emergence and growth.

2. Materials and methods

2.1. Plant material

Seeds of *Vicia faba* var. *minor*, cv. Streletskie, were provided by the Institute of Leguminous plants (Orel, Russia). Seeds being placed with their micropile down imbibed in distilled water in the dark at 27 °C. Embryonic axes were excised from imbibing seeds, at radicle emergence, and during the post-germinative growth. Excised embryonic axes were fixed for light or electron microscopy or for protein and mRNA expression analyses.

Cell length was measured in longitudinal sections along the third row of cortical cells.

For electron microscopy, cross sections of embryonic axes were prepared according to standard procedure and examined under a Temscan-100 CX (Jeol, Japan) microscope. Cell vacuolation was estimated as the ratio of vacuole to cell area on electron micrographs.

2.2. Water content

Water content (WC) was routinely measured by weighing prior to and after oven-drying for 1 h at 105 °C and then at 80 °C for 3 days and expressed as % FW. Water absorption by embryonic axes from killed seeds was measured after seed exposure to 105° C for 2 h, and to 80° C for a week. Rate of water uptake was calculated from the gain of water amount in the axis during imbibition.

2.3. Protein amount

Protein amount was estimated using a BCA protein assay (Sigma, USA).

2.4. Isolation of membrane fractions

Excised embryonic axes were homogenized in 300 mM sucrose, 10 mM EDTA, 5 mM potassium *m*-bisulfite, 5 mM DTT (dithiothreitol), 5 mM PMSF, 0.6% polyvinylpyrrolidone, 100 mM Tris–HCl, pH 8.0. The homogenate was centrifuged at 10,000 ×*g* for 15 min; the supernatant was recovered and centrifuged at 100,000 ×*g* for 30 min. The resulting microsomal pellet was resuspended in 300 mM sucrose, 0.5 mM EDTA, 1 mM DTT, 10 mM MES-*bis*-TRIS propane, pH 7.2 and stored at −70 °C.

2.5. Immunodetection

Microsomal proteins were separated by SDS-PAGE followed by transfer onto Hybond C membranes (Amersham) as described by Towbin et al. (1979) with addition of 0.1% SDS. The blots were blocked in PBS containing 0.05% Tween 20 (PBST) and 5% nonfat dry milk, and incubated with appropriate primary antibodies at 4 °C overnight. Since TIPs show a high level of homology in their N-terminal parts, antibodies were raised against selected peptides derived from the most variable C-terminal regions. Primary antibodies were raised in rabbits against a 18-amino acid (TNNMRPSGFHVSPGVGVG) peptide corresponding to residues 162–179 of *Phaseolus vulgaris* *Pv*TIP3;1, and a 12-amino acid (NTTHEQLPTTDY) C-terminal peptide of *At*TIP1;1. Because of the conservation of TIP sub-classes between plant species, it was assumed that these antibodies would cross-react with TIP homologues in *V. faba* (see supplemental Figure S1). Both TIP3;1 and TIP1;1 sequences were designed according to http://mbclserver.rutgers.edu/CPGN/AquuaporinWeb/Aquaporin.group.html. Polypeptides were synthesized in the Institute of bioorganic chemistry (Moscow) and antibodies were home-produced. The membranes

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