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Two cDNA clones (*FsDhn1* and *FsClo1*) up-regulated by ABA are involved in drought responses in *Fagus sylvatica* L. seeds

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Summary

Two abscisic acid (ABA)-responsive clones (*FsDhn1* and *FsClo1*) were isolated from a cDNA library of ABA-treated seeds of *Fagus sylvatica* L. *FsDhn1* codes for type-II late embryogenesis abundant (LEA) proteins, also known as dehydrins. The corresponding transcripts were ABA-induced and expressed when seeds were artificially dried. *FsClo1* codes for a caleosin, a calcium-binding protein. By expressing FsCLO1 as a fusion protein in *Escherichia coli*, we obtained biochemical evidence of its ability to bind calcium, a second messenger involved in ABA signaling. This cDNA clone is seed specific, shows a LEA expression pattern and is also induced by ABA and dehydration treatments. The relationship of both genes with seed responses to ABA is discussed.

Introduction

Seed development is an important phase of the life cycle of higher plants. This process requires complex regulation of several genes that must be coordinated and controlled in a spatial and temporal manner, beginning from the zygote stage and extending to the mature embryo in dormant seeds (Bewley, 1997). Abscisic acid (ABA) plays a key role in the regulation of this process by inducing reserve accumulation, desiccation tolerance and adaptation to environmental stresses (Bewley, 1997). ABA levels generally increase during early phases of seed development and decrease during embryo maturation, exhibiting two peaks throughout the process (Perata et al., 1990; Rock and Quatrano, 1995). In *Fagus sylvatica* seeds, the highest levels of ABA, both in the cotyledons and in the embryo

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Abbreviations: CaBP, calcium-binding protein; DHN, dehydrin; EGTA, ethylene-diamine-bis (β-amine-ethyl-ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid; mc, moisture content; PCB, paclobutrazol; PEG, polyethylene glycol; RAB, responsive to abscisic acid; W+d, warm pretreated and dried.

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axis, are detected after 13 weeks of embryogenesis. ABA levels then start to decrease, coincident with the start of drying during maturation (Nicolás et al., 1997). On the other hand, there is clear evidence that ABA is involved in both the induction and maintenance of dormancy. Seeds of ABA-deficient or ABA-insensitive mutants of *Arabidopsis thaliana* do not exhibit dormancy (Karssen et al., 1983). In *F. sylvatica*, Le Page-Degivry et al. (1997) reported that degradation of ABA in the axis exceeded its synthesis after cold pretreatment of the seeds, allowing them to germinate, while in dormant embryos ABA synthesis equilibrated its degradation. There is therefore evidence that the maintenance of dormancy may be associated with de novo biosynthesis of ABA.

Additionally, ABA accumulates rapidly under stress conditions such as water deficiency, inducing the expression of genes involved in stress tolerance. Among these, late embryogenesis abundant (LEA) proteins play an important role in these processes. These proteins are induced in immature embryos by ABA (Mundy et al., 1986), are related to the acquisition of desiccation tolerance (Bartels et al., 1988) and are expressed in response to several types of abiotic stress (Skriver and Mundy, 1990). Taken together, these data suggest that these LEA proteins are involved in the protection of cell membranes against damage caused by water loss during the desiccation process (Bray, 1993).

During the last decade, much progress has been made toward understanding the mechanism of ABA signaling. Several components involved in this signal transduction cascade have been identified, mainly in *Arabidopsis* (Finkelstein et al., 2002; Sáez et al., 2004; Reyes et al., 2006c) and in other species such as *F. sylvatica* L. (Lorenzo et al., 2001, 2002; González-García et al., 2003; Jiménez et al., 2006; Reyes et al., 2006a, b). Also, significant progress has been made in the characterization of second messengers involved in ABA action, such as cADPR (Wu et al., 1997) or calcium (Campalans et al., 1999).

It is well known that environmental and hormonal signals, such as ABA, induce changes in cytosolic calcium, regulating the expression of calmodulin and other calcium-binding proteins (CaBPs) (Poovaiah and Reddy, 1993). By binding calcium, these proteins undergo conformational changes that activate their corresponding targets, causing the physiological changes linked to the calcium stimulus and participating in signal transduction cascades (Rozwadowski et al., 1999; Grabarek, 2006).

CaBPs usually contain EF-hand paired calcium-binding motifs (i.e. calmodulin). However, in some cases, these proteins exhibit a single calcium-binding EF-hand, characteristic of CaBPs associated to cell membranes, such as oil bodies (in these cases, CaBPs are known as caleosins) (Naested et al., 2000) or vacuoles (Yuasa and Maeshima, 2000). Several CaBPs have been isolated in different plant species involved in ABA, seed development and/or stress responses (Nicolás et al., 1998; Lee et al., 2004; Liu et al., 2005; Jayasekaran et al., 2006).

In the present work, we isolated two ABA-induced cDNA clones from *F. sylvatica* seeds through two different approaches and studied their expression under different processes regulated by ABA, such as embryogenesis, the effects of dehydration or seed dormancy. The possible role of both *FsDhn1* (a dehydrin) and *FsClo1* (a caleosin) in these ABA-mediated processes in seeds is discussed.

Materials and methods

Plant material and germination conditions

F. sylvatica L. seeds (beechnuts) were obtained from the Danish State Forestry Tree Improvement Station. Seeds were dried to a moisture content (mc) of 10% (fresh weight basis) and stored at −4 °C in sealed jars. The pericarp was manually removed and seeds were sterilized in 1% sodium hypochlorite before soaking in sterile water or solutions containing 100 μM ABA, 100 μM GA_3, 1 mM NaCl, 30% polyethylene glycol (PEG4000) or 10 μM paclobutrazol (PCB). Seeds were maintained in the different media at 4 °C in the dark from 1 to 6 weeks.

For the experiments performed during embryogenesis, beechnuts were collected from a single tree in Lundtofte (Denmark) once a week early in seed development and once every 2 weeks later in development. The first harvest was on August 2, 8 weeks after flowering, and the latest took place on October 13, 1996, when the seeds were naturally shed, as previously described (Nicolás et al., 1997).

For the studies concerning loss of seed dormancy and responses to drought stress, seeds with an initial mc of 10% were soon after moistened to a target mc of 34% by adding a known volume of distilled deionized water directly to a known weight of seeds in a plastic bag (Mortensen et al., 2004). Whole seeds (including the pericarp) were pre-chilled (5 °C; treatment that released seed dormancy) or warm pretreated (15 °C; treatment that maintained the dormant state) for 0, 3, 5, 8, 11, 13, 15, 17, 19 and 26 weeks. After each period, one portion of the seeds was fully imbibed and germinated at 15 °C directly, while another portion received a drying treatment. Seeds were dried to a mc of around 10% (fresh weight basis) over a saturated NaCl solution in circulated air in an airtight chamber at 20 °C for 7 d, as described previously (Mortensen et al., 2004). Seed germination was considered when the radicle protruded 3–5 mm.