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High accumulation of legumin and *Lea*-like mRNAs during maturation is associated with increased conversion frequency of somatic embryos from pedunculate oak (*Quercus robur* L.)

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Summary. The expression patterns of the storage protein legumin gene and Em- and dehydrin-like homologues were investigated in somatic embryos from *Quercus robur* L. The effect of different maturation treatments (1% agar, 6% sorbitol, or 5% polyethylene glycol) and partial desiccation on transcript accumulations as well as conversion capacity of somatic embryos was also investigated. Differential expression of putative Em- and two dehydrin-like homologues (designated as Dhn1a and Dhn1b) was detected in somatic oak embryos with heterologous probes. Low expression levels of legumin, Em- and dehydrin-like mRNAs were detected in somatic embryos prior to maturation treatment. A high accumulation of these transcripts was found in embryos that had been cultured on media supplemented with 6% sorbitol or 1% agar. These embryos also showed a high conversion frequency into plantlets. In contrast, no improvement in plant conversion as well as a low accumulation of legumin, Em-like and Dhn1b-like transcripts was observed in embryos that were matured on polyethylene glycol medium. Partial-desiccation treatment significantly enhanced the plant conversion. Nevertheless, a decline in expression of legumin, Em-like and Dhn1a-like homologues was detected upon dehydration. In contrast, Dhn1b and oak homologues to peach dehydrin were also strongly expressed in desiccated embryos. In addition, the treatment of embryos with abscisic acid promoted the accumulation of all investigated transcripts. These results suggest that the regulation of the legumin storage protein gene and Lea-like homologues in somatic oak embryos is under developmental control and that their regulation can be influenced by manipulating the culture conditions.

Keywords: Abscisic acid; Dehydrin; Desiccation; *Quercus robur*; Somatic embryogenesis; Storage protein.

Abbreviations: ABA abscisic acid; PEG polyethylene glycol; SE somatic embryo.

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Introduction

Pedunculate oak (*Quercus robur* L.) is ecologically and economically one of the most important broadleaf forest tree species in central and western Europe. Oak seeds are considered recalcitrant in terms of seed storage. Therefore, interest in somatic-embryogenesis procedures for oak species has increased during recent years with the aim of integrating this plant propagation technique into tree improvement programs. At present, the major limitation in oak somatic embryogenesis seems to be the low conversion frequency and the resulting low plant production rates (Wilhelm 2000). The low conversion frequency may be caused by incomplete or deficient maturation, which might be a consequence of inappropriate culture conditions or the different nature of zygotic and somatic embryos. Increased conversion rates were obtained by the application of osmotic compounds or exposure to exogenous abscisic acid (ABA). Recently, attention has been given to somatic-embryo (SE) quality and parameters for controlling conversion capacity have been identified, such as high cytokinin levels and low ABA levels (Malá et al. 1999).

The maturation phase during embryogenesis is characterized by the synthesis of storage reserves such as starch, lipids, and proteins. Especially the accumulation of storage proteins followed by late-embryogenesis-abundant (LEA) proteins has been proposed as a useful marker for the maturation process and high-quality SEs of conifers (Flinn et al. 1993, Dong and Dunstan 2000). Seed storage proteins are abundant

and characteristic for the final phase of the maturation process and for embryo quality. They are generally classified according to their solubility as albumin, globulins, prolamins, and glutelins (Shewry et al. 1995).

Globulins such as 12S (legumin) and 7S (vicilin) are the major storage proteins in dicots. They are mainly utilised as a nitrogen supply in germinating embryos and plantlets (Leal et al. 1995, Dong and Dunstan 1996). LEA-like proteins and their corresponding transcripts become highly accumulated during the later stages of embryo development (Gaubier et al. 1993, Corre et al. 1996, van Damme and Peumans 1996, Vicient et al. 1998). They have been grouped according to their sequence and structural similarities to known cotton LEA proteins (Dure et al. 1989), from which group I and II have been most extensively studied. The *Em* (early-methionine-labelled) gene is a member of group I of the *Lea* genes and encodes one of the most abundant cytosolic proteins accumulating in embryos. The *Em* gene is expressed only during the later steps of seed maturation (Gaubier et al. 1993, van Damme and Peumans 1996, Vicient et al. 1998) or during SE development (Corre et al. 1996). The expression of the *Em* gene is embryo-specific and can be precociously induced by ABA as well as by osmotic, salt, or water stress (Morris et al. 1990, Bostock and Quatrano 1992, Gaubier et al. 1993).

Dehydrins belong to group II of the *Lea* genes (Dure et al. 1989) and so far more than 65 plant dehydrin sequences have been identified (Close 1997). They are believed to play a role in the acquisition of desiccation tolerance and they prevent embryos from damage due to drought stress and from precocious germination during seed development (Close 1997). They can be precociously induced in immature embryos or in vegetative tissues by the application of ABA or under conditions of water, salt, or cold stress as well as by wounding (Morris et al. 1990, Bostock and Quatrano 1992, Danyluk et al. 1994, Rouse et al. 1996, Close 1997, Richard et al. 2000).

In the present study, different maturation and desiccation treatments were tested with developing oak SE in order to investigate expression patterns of the storage protein legumin gene and Em-like and dehydrin-like homologues, with the goal of identifying molecular markers associated with increased conversion frequency.

Material and methods

Plant material

The embryogenic culture line 6QR5 was obtained from immature zygotic *Quercus robur* L. embryos following the protocol of Endemann and Wilhelm (1999). The culture line was maintained via repetitive embryogenesis on P_{24} medium (Teasdale 1992) supplemented with 3% sucrose, 0.89 µM N^6-benzyladenine and solidified with 0.8% agar (Daishin; Brunschwig Chemie, Amsterdam, the Netherlands) as described earlier (Endemann and Wilhelm 1999). Cotyledonary SEs (size, ≥5 mm) were manually separated prior to the maturation treatment, and were used as a control in Northern analyses. The abscisic acid [(+, −)-cis, trans ABA; Fluka Chemie, Buchs, Switzerland] was prepared as a 100 mM stock solution dissolved in 1 N NaOH and was added under sterile conditions after autoclaving as required.

Maturation treatment

Cotyledonary SE (size, ≥5 mm) were cultured on media containing 5% polyethylene glycol (PEG 4000), 6% D-sorbitol, or 1% agar for 5 weeks to achieve maturation. To clarify the effect of ABA on the regulation of gene expression, the SE were cultured in liquid P_{24} medium containing 50 or 100 µM ABA for two days.

Partial-desiccation treatment

SE that were derived from maturation media, as well as ABA-treated embryos, were dehydrated to approximately 50% of their initial fresh weight by putting them into empty petri dishes in the dark at 25 °C. In a separate experiment, the SE that were matured on medium containing 6% sorbitol were partially dehydrated as described above to approximately 85, 80, 75, 70, 65, 60, 50, and 45% of their initial fresh weight.

Germination

SE harvested after each treatment were placed onto P_{24} medium that was supplemented with 1 µM N^6-benzyladenine and 0.1 µM isobutyric acid and cultured in the light (16 h photoperiod) at 24 °C. The germination and conversion frequencies were scored after 8 weeks for complete germination (radicle elongation, <5 mm; shoot development, >3 mm) or incomplete germination (only root or shoot elongation).

Statistical analysis

The maturation experiments were repeated three times with 15 to 20 replicates. The data were analysed by the ANOVA-procedure and the Waller–Duncan k-ratio t-test on all means (Waller option of the same procedure) in SAS (SAS Institute, 1989). The analysis of data that were derived from the partial-desiccation experiments was performed by a Kruskal–Wallis and a Mann–Whitney test between the control and each desiccation treatment.

RNA extraction and Northern blot analysis

The total RNA from the SE derived from each treatment as well as cotyledonary SE prior to maturation (control) was isolated and purified according to Chang et al. (1993). Equal amounts of total RNA (20 µg) were denatured with formamide and formaldehyde and were fractionated on 1.2% agarose-formaldehyde gels at a constant voltage of 5 V/cm as described by Sambrook et al. (1989). The RNA was transferred to nylon membranes (Hybond N^+; Amersham, Little

Chalfont) by capillary blotting according to the instructions of the manufacturers, fixed by UV cross-linking, and baked at 80 °C for 2 h. The RNA blots were stained with methylene blue (Sambrook et al. 1989) and scanned to check the amount of the loaded RNA. The RNA blots were prehybridized in a 0.5 M $NaPO_4$ buffer, pH 7.2, containing 7% (w/v) sodium dodecyl sulfate (SDS), and 1 mM EDTA for 1 h prior to adding [α-^{32}P]dCTP-radiolabelled probes. cDNA probes (oak legumin precursor [Genbank accession number X99539], *Picea glauca* dehydrin *PgDhn1* [AF109916], *Prunus persica* dehydrin [AJ271620], *AtEm6* from *Arabidopsis thaliana* [Z11924], and *EMRP19* from *Robinia pseudoacacia* [U40821]) were labelled with [α-^{32}P]dCTP with the random oligonucleotide priming kit (Stratagene, La Jolla, Calif., U.S.A.) according to the instructions of the manufacturer. Northern hybridizations were carried out at medium-stringency conditions (55 °C) when heterologous probes were used, or at high-stringency conditions (65 °C) for the oak legumin probe, for 16 h. The blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (w/v) SDS, followed by washes in 1× SSC and 0.1% (w/v) SDS at room temperature (10 min each). Final washing was done in 0.5× SSC and 0.1% (w/v) SDS at hybridization temperatures (55 °C or 65 °C). The membranes were exposed to Kodak X-Omat films (Kodak, Rochester, N.Y., U.S.A.) with an intensifying screen at –80 °C for at least 5 h or longer, depending on the specific activity of the probe and the intensity of the autoradiograph signals. For reprobing, each blot was stripped with 250 ml of boiled 0.1% (w/v) SDS. The membranes were checked by overnight exposure to ensure complete probe removal.

Results and discussion

Conversion

The maturation of SE on media containing osmotic compounds promoted the rate of regeneration into plantlets. However, differences among the treatments were observed (Table 1). Media supplemented with 6% sorbitol or 1% agar both with subsequent partial-desiccation treatments significantly ($P > 0.05$) increased the conversion frequency (33.3% and 43.9%, respectively) in relation to the control (11.7%). The incomplete conversion rate was significantly ($P > 0.05$) enhanced on medium containing 1% agar with subsequent partial desiccation (31.3%) compared to the control (13.6%) and the 1% agar treatment (15.8%) (Table 1). To get a detailed insight into the possible effects of the desiccation treatment, SE that were matured on media containing 6% sorbitol were partially dehydrated until they lost 15–55% of their initial fresh weight in moisture (Fig. 1). Moisture losses of 20 and 25% resulted in a significant ($P > 0.05$) improvement in conversion rates (52.6% and 62.9%, respectively). Continued desiccation of SEs to a moisture loss of >25% caused a subsequent decline in conversion frequencies (Fig. 1). From this, it can be concluded that partial desiccation as a postmaturation treatment seems to be necessary to break the epicotyl dormancy of oak SE. The regeneration of ABA-treated SE failed due to necrosis. This gives rise to the conclusion that ABA treatment is not effective for promoting maturation and conversion in oak SE.

Table 1. Effect of osmotic compounds and partial desiccation on germination frequencies of *Q. robur* SE [a]

Treatment	% of embryos [b]	
	completely converted	incompletely converted
Control, 0.8% agar	11.7 ± 4.1 c	13.6 ± 4.4 b
1% agar	31.2 ± 6.7 ab	15.8 ± 5.2 b
1% agar + desiccation	43.9 ± 7.5 a	31.3 ± 7.0 a
5% PEG	16.3 ± 5.2 bc	21.7 ± 5.9 ab
5% PEG + desiccation	20.2 ± 5.7 bc	26.0 ± 6.3 ab
6% sorbitol	26.6 ± 6.7 bc	21.7 ± 6.3 ab
6% sorbitol + desiccation	33.3 ± 6.1 ab	21.7 ± 5.4 ab

[a] Secondary SEs (control) were matured on media with 1% agar, 5% PEG, or 6% sorbitol. After each treatment the SEs were partially dehydrated to 50% of their initial fresh weight. Germination (complete and incomplete) was evaluated after 8 weeks on germination medium

[b] Values are means with standard errors of three repeated experiments. Means with the same letters are not significantly different according to the Waller–Duncan k-ratio t-test

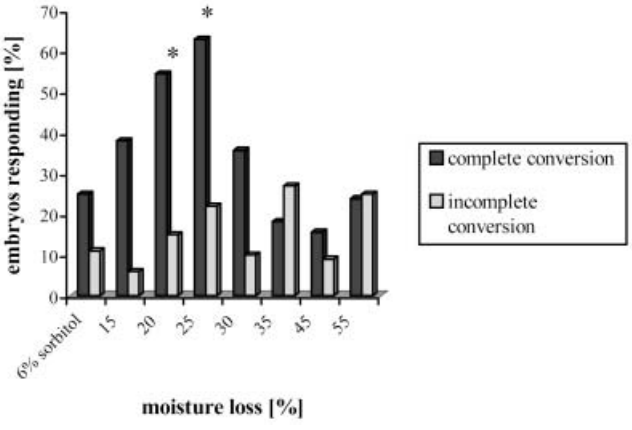

Fig. 1. Effect of partial-desiccation treatment on germination frequencies (complete and incomplete) in *Q. robur* SE. SE matured on 6% sorbitol medium were partially dehydrated until they lost 15–55% of their initial fresh weight in moisture. After 8 weeks on the germination medium, the germination rates were scored. *Means significantly different from other treatments according to the Mann–Whitney test

Expression analysis

The expression patterns of the storage protein legumin gene and Em- and dehydrin-like homologues were investigated in oak somatic embryogenesis during

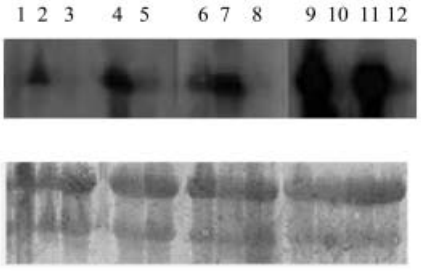

Fig. 2. Accumulation of legumin mRNA in oak SE after osmotic, ABA, or partially drying treatments. *1* Control SE prior to maturation treatment; *2* 5% PEG (6 weeks); *3* 5% PEG plus desiccation (5 days); *4* 1% agar (6 weeks); *5* 1% agar (6 weeks) plus desiccation (5 days); *6* 6% sorbitol (1 week); *7* 6% sorbitol (6 weeks); *8* 6% sorbitol (6 weeks) plus desiccation (5 days); *9* 50 µM ABA (48 h); *10* 50 µM ABA plus desiccation (5 days); *11* 100 µM ABA (48 h); *12* 100 µM ABA plus desiccation (5 days). The membrane was hybridized with oak legumin cDNA under high-stringency condition. In the lower panel, methylene blue-stained membrane after RNA transfer to visualise ribosomal bands (26S and 18S)

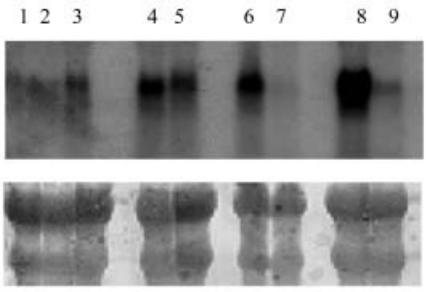

Fig. 3. Expression of putative Em-like homologue in oak SE after osmotic, ABA, or partially drying treatments. *1* Control SE prior to maturation; *2* 5% PEG (6 weeks); *3* PEG plus desiccation (5 days); *4* 1% agar (6 weeks); *5* 1% agar (6 weeks) plus desiccation (5 days); *6* 50 µM ABA (48 h); *7* 50 µM ABA (48 h) plus desiccation (5 days); *8* 100 µM ABA (48 h); *9* 100 µM ABA (48 h) plus desiccation (5 days). The membrane was hybridized with *EMRP19* cDNA from black locust under low-stringency condition. In the lower panel, methylene blue-stained membrane after RNA transfer to visualise ribosomal bands (26S and 18S)

maturation, ABA treatment, and desiccation. Northern hybridizations with *EMRP19* from *Robinia pseudoacacia* and *PgDhn1* from *Picea glauca* revealed that putative Em- and two dehydrin-like homologues (designated as Dhn1a and Dhn1b) were differentially expressed in oak SE.

Maturation

Negligible accumulations of legumin, Em and Dhn1 mRNAs were found in the SE prior to maturation treatment (Figs. 2–4, lanes 1). An increase in accumulation of all transcripts was found in SEs after being cultured on media containing 1% agar (Figs. 2–4, lanes 4). The legumin transcripts (Fig. 2, lane 6) started to accumulate after just one week of exposure to 6% sorbitol medium. After 6 weeks even higher mRNA levels were detected (Fig. 2, lane 7). Similar results were obtained with SE that were matured on medium containing 1% agar (Fig. 2, lane 4). The treatment with 5% PEG had no marked effect on legumin gene expression (Fig. 2, lane 2). The application of osmotic compounds during SE maturation was shown to enhance the accumulation of storage proteins and storage protein transcripts to an extent comparable to zygotic white spruce and Douglas fir embryos (Flinn et al. 1993, Misra et al. 1993). The maturation of SE cultured on medium with an increased agar content (1%) considerably promoted the accumulation of the Em-like and the Dhn1b-like transcripts (Figs. 3 and 4, lanes 4). In PEG-treated embryos, on the other hand, only a low

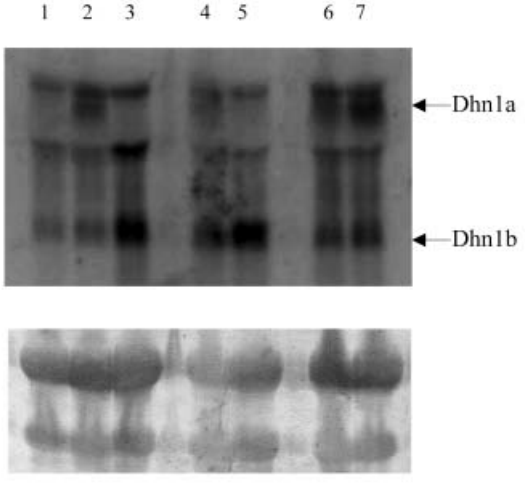

Fig. 4. Expression of putative Dhn1a- and Dhn1b-like homologues in oak SE after osmotic, ABA, or partially drying treatment. *1* Control SE prior to maturation; *2* 5% PEG (6 weeks); *3* PEG plus desiccation (5 days); *4* 1% agar (6 weeks); *5* 1% agar (6 weeks) plus desiccation (5 days); *6* 50 µM ABA (48 h); *7* 100 µM ABA (48 h). The membrane was hybridized with *PgDhn1* cDNA from white spruce under low-stringency conditions. In the lower panel, methylene blue-stained membrane after RNA transfer to visualise ribosomal bands (26S and 18S)

amount of transcripts was detected (Figs. 3 and 4, lanes 2). However, treating SE with PEG strongly induced the expression of the Dhn1a-like homologue, while 1% agar had only a very limited effect on the Dhn1a mRNA accumulation (Fig. 4, lanes 2 and 4). This all suggests, that the expression of legumin and Em- and Dhn1-like homologues is differentially regulated in oak somatic embryogenesis, strongly depending upon

the osmotic agents applied. Plasmolyzing osmotica, such as sorbitol, readily pass through the cell wall and cause temporary plasmolysis until their movement into the cytosol leads to osmotic recovery (Attree and Fowke 1993). In contrast, PEG molecules are too large to move through the cell wall and thus do not cause plasmolysis. Therefore, the latter may not be effective for promoting the maturation of oak SE.

Abscisic acid

The expression of storage protein genes and *Lea* genes can be precociously induced by the application of ABA to immature seeds (Flinn et al. 1993, Close 1997, Dong and Dunstan 2000). High transcript accumulations of legumin, Em-like homologues, and both Deh1-like homologues were observed after ABA treatment in our experiments (Fig. 2, lanes 9 and 11; Fig. 3, lanes 6 and 8; and Fig. 4, lanes 6 and 7, respectively). The levels of transcript accumulation positively correlated with the applied ABA concentrations being used. However, the detected mRNA levels were much higher than those found in SE that were exposed to osmotic agents. Moreover, the hybridization with an *AtEm6* cDNA probe from *A. thaliana* revealed that solely ABA could induce the expression of the oak *AtEm6*-like homologue, while no hybridization signal was detected in any other treatment (data not shown). Therefore we assume that osmotic compounds were not able to increase the endogenous ABA levels to the extent that is required to induce the *AtEm6*-like homologue. In contrast, Morris et al. (1990) and Bostock and Quatrano (1992) have reported that the addition of mannitol to culture media triggered high levels of *Em* mRNA accumulation without increasing the endogenous level of ABA in rice suspension cultures and wheat embryos. The authors proposed that the accumulation of Em transcripts was mediated via two pathways in response to osmotic stress, one involving ABA and another pathway acting synergistically with ABA (Bostock and Quatrano 1992). Em-like transcripts accumulated after ABA treatment in immature *A. thaliana* seeds at a level similar to that of mature seeds. Despite the strong mRNA expression, no accumulation of Em protein was detected (Gaubier et al. 1993, Bies et al. 1998). Therefore it is assumed that *Em* genes are highly regulated not only on a transcriptional but also on a posttranscriptional level. Bies et al. (1998) suggested that Em proteins cannot accumulate before a developmental signal is given. Although the expression of legumin and both Lea-like homologues was positively regulated by ABA in oak somatic embryogenesis, the pulse treatments with ABA (50 or 100 μM) led to tissue necrosis. This contradiction may be explained by the multiple roles of ABA in plants. Besides the fact that ABA is directly involved in the control of gene expression during seed maturation and stress response, it is also regarded as a plant growth inhibitor, particularly because it inhibits auxin-promoted cell wall acidification and loosening, which permit cell elongation (George 1993). These toxic effects were also observed in sandalwood SEs after treatment with ABA (Rao and Bapat 1995).

Desiccation

Desiccation is required to turn off maturation-associated gene expressions such as storage protein gene expression (Kermode and Bewley 1989, Jiang et al. 1996), which provides a switch-developmental program to germination. The partial dehydration of SE resulted in a rapid decrease of the legumin expression to an undetectable level (Fig. 2, lanes 3, 5, 8, 10, and 12). This concurs with Flinn et al. (1993), who reported a decline in expression of the storage protein albumin, vicilin, and legumin genes in both somatic and zygotic embryos of interior spruce after desiccation. A differential regulation of Em-like homologue was observed in oak SE upon drought stress. A lower expression level of Em-like homologue was found in desiccated SE, although this was still at a higher level than in control tissue (Fig. 3, lanes 1, 3, and 5). The desiccation of ABA-treated embryos caused a rapid decline in expression levels (Fig. 3, lanes 7 and 9). These results are in accordance with the observations of Gaubier et al. (1993), who reported a decline in the *AtEm1* mRNA levels during the later phases of desiccation in *A. thaliana* seeds. In soybean the premature desiccation of seeds resulted in a rapid decline of accumulated *Sle* transcripts (homologues to the *Em* gene), despite the fact that the expression of *Sle* mRNA was abundant in dry seeds (Calvo et al. 1997). A different regulation of the expression of two putative oak homologues to *PgDhn1* was observed upon drought stress. Desiccation repressed the expression of the *Dhn1a*-like homologue, while the accumulation of the *Dhn1b* mRNA was abundant in desiccated SEs (Fig. 4, lanes 3 and 5). The effect of desiccation treatment on the expression of the oak homologue to the peach dehydrin was analysed by controlled dehydra-

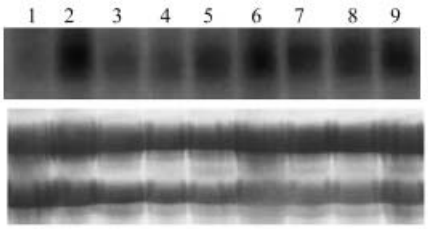

Fig. 5. Expression of oak homologue to peach dehydrin upon progressive dehydration of oak SE. Control SE prior to maturation treatments (*1*), matured on 6% sorbitol (*2*), and further dehydrated with moisture losses of 15% (*3*), 20% (*4*), 25% (*5*), 30% (*6*), 35% (*7*), 45% (*8*), and 55% (*9*). The membrane was probed with peach dehydrin cDNA under low-stringency conditions. In the lower panel, methylene blue-stained membrane after RNA transfer to visualise ribosomal bands (26S and 18S)

tion of SE (moisture loss of 15–55%). An increase in the accumulation of the dehydrin-like transcript in comparison to the control tissue was detected in SEs that were matured on sorbitol (Fig. 5, lanes 1 and 2, respectively). The abundance of the transcript level decreased slightly in SEs that lost 15–25% of moisture (Fig. 5, lanes 3–5). Afterwards, the transcript accumulation increased again as dehydration progressed (Fig. 5, lanes 6–9).

The reduced expression of Dhn1a-like homologue in oak SE upon drought stress is in contrast with results achieved in other plant species (Raynal et al. 1989, Still et al. 1994, Kermode 1997, Colmenero-Flores et al. 1999). A high accumulation of dehydrins was found in orthodox or desiccation-tolerant seeds. Recalcitrant seeds such as *Quercus* spp. are not capable of withstanding water loss and are presumed to be highly susceptible to desiccation injury (Kermode 1997). However, desiccation tolerance is a quantitative feature, the amount of dehydrins and LEAs or the rate at which proteins accumulate may determine the level of tolerance (Vertucci and Farrant 1995). The induction of dehydrin genes has been observed in stored *Q. robur* seeds by limited desiccation. During the development of *Q. robur* zygotic embryos, the increase in desiccation tolerance was accompanied by an increase in the amount of dehydrin protein (Finch-Savage et al. 1994). In our experiments, Dhn1b- and oak homologues to peach dehydrin were also strongly expressed in desiccated SEs. This may indicate their protective role during drought stress. In conclusion, complex expression patterns of legumin and Lea-like homologues were detected in oak SEs upon the application of various osmotic compounds and/or desiccation treatments. The accumulation of legumin and *Lea*-like mRNAs was strongly promoted during SE maturation. Differences in transcript abundance positively correlated with the regeneration capacity into plantlets. These results indicate that the regulation of storage protein genes and *Lea*-like genes during oak somatic embryogenesis is under developmental control and that the process of somatic embryogenesis can be modulated by the manipulation of culture conditions.

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