Effects of Abscisic Acid and High Osmoticum on Storage Protein Gene Expression in Microspore Embryos of *Brassica napus*¹

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ABSTRACT

Storage protein gene expression, characteristic of mid- to late embryogenesis, was investigated in microspore embryos of rapeseed (*Brassica napus*). These embryos, derived from the immature male gametophyte, accumulate little or no detectable napin or cruciferin mRNA when cultured on hormone-free medium containing 13% sucrose. The addition of abscisic acid (ABA) to the medium results in an increase in detectable transcripts encoding both these polypeptides. Storage protein mRNA is induced at 1 micromolar ABA with maximum stimulation occurring between 5 and 50 micromolar. This hormone induction results in a level of storage protein mRNA that is comparable to that observed in zygotic embryos of an equivalent morphological stage. Effects similar to that of ABA are noted when 12.5% sorbitol is added to the microspore embryo medium (osmotic potential = 25.5 bars). Time course experiments, to study the induction of napin and cruciferin gene expression demonstrated that the ABA effect occurred much more rapidly than the high osmotic effect, although after 48 hours, the levels of napin or cruciferin mRNA detected were similar in both treatments. This difference in the rates of induction is consistent with the idea that the osmotic effect may be mediated by ABA which is synthesized in response to the reduced water potential. Measurements of ABA (by gas chromatography-mass spectrometry using [*13C₆*]ABA as an internal standard) present in microspore embryos during sorbitol treatment and in embryos treated with 10 micromolar ABA were performed to investigate this possibility. Within 2 hours of culture on high osmoticum the level of ABA increased substantially and significantly above control and reached a maximum concentration within 24 hours. This elevated concentration was maintained for 48 hours after culturing and represents a sixfold increase over control embryos. The ABA-treated embryos accumulated the hormone very quickly, but ABA concentrations returned to basal levels within 72 hours after treatment. The possibility that embryo-synthesized ABA may be a mediator of effects of osmotic stress on gene expression in *Brassica* embryos is discussed.

Oilseed rape (*Brassica napus* L.) has been the subject of several studies on the regulation of gene expression in developing seeds (9, 10). In *B. napus* it has been shown that exogenous ABA treatment can have profound effects on the accumulation of transcripts and gene products of the major seed storage proteins (9), lipid profiles (8), and on levels of oil-body associated proteins (20). Many of the responses stimulated by ABA are also affected by water stress or elevated osmotica (7, 8).

The relationship between ABA effects and osmotic effects in developing seeds is not yet clear and has yielded results with contrasting interpretations (23). Bray and Beachy (2) suggested that the accumulation of soybean storage proteins, which is enhanced by culturing soybean embryos on high osmotica, is a consequence of elevated ABA content. In *B. napus*, on the other hand, Finkelstein and Crouch (7) concluded that storage protein accumulation could be affected by

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While recognizing that there will be physiological differences between zygotic and gametophytic embryos, it seemed possible that microspore embryos of rapeseed might offer some advantages in studying regulation of these genes. The embryos are already growing independently of any maternal tissue, seed coat or endosperm. Furthermore, it is possible to take samples at regular time intervals from the same culture. Finally, the handling of the embryos is minimal as they are maintained throughout their development in a defined liquid medium.

We, therefore, compared the effects of ABA and high osmoticum on the expression of storage protein genes. The sensitivity of microspore embryos to exogenous ABA was investigated at several developmental stages. Time courses of the responses to applied ABA and osmoticum were measured in order to estimate the relative rates of response to these stimuli. The effect of high osmoticum on ABA accumulation in these embryos was determined in order to elucidate the possible interactions between osmoticum- and ABA-mediated responses. Finally, ABA concentration in ABA-treated embryos was assessed over a 48 h time course.

**METHODS AND MATERIALS**

**Plant Material**

*Brassica napus* cv Topas (Agriculture Canada, Saskatoon) plants were raised in growth chambers at 20°C day (16 h photoperiod, 400 μmol m⁻²·s⁻¹) and 15°C night temperature regime. Plants grown for microspore isolation were transferred after six weeks to growth chambers with a 15°C day/10°C night temperature cycle until buds were harvested.

**Microspore Culture**

Buds (2–3 mm long) were collected from the cold treated plants and the microspores isolated by grinding in Lichter medium (16) with 13% sucrose, washed, pelleted and then suspended in 40% Percoll containing 13% sucrose. After overlaying with a hormone free Lichter medium (16) the tubes were spun (220g, 10 min) and the cells at the Lichter medium/Percoll interface were collected. After washing, these cells were pelleted and resuspended in the Lichter medium and 10 mL were plated at a density 3 × 10⁴ cells mL⁻¹ in Petri dishes (100 mm × 15 mm). The microspores were incubated in the dark for 4 d at 30°C and then transferred to 25°C. They were subcultured after 7 d with fresh medium with a fourfold dilution. For harvesting, the embryos are sieved through various size nylon meshes to isolate the morphological stages. After isolation, treatment with ABA was done by the addition of 10 μL of a 10 mM stock solution of ABA (mixed isomers, Sigma) dissolved in 70% ethanol to 10 mL of culture medium containing the embryos. Control embryos were treated with 10 μL of 70% ethanol. Treatment with sorbitol was done by plating isolated embryos directly in Lichter medium (16) containing both 13% sucrose and 12.5% sorbitol.

**RNA Extractions**

Total RNA was extracted by the method of either Natesan *et al.* (18) or Verwoerd *et al.* (21). The RNA was quantitated.
by A_{260} measurements and by ethidium bromide staining intensity, then stored as an ethanol precipitate at -20°C until use.

Northern Blotting and Hybridization

Total RNAs were separated by electrophoresis on formaldehyde gels (6% formaldehyde, 1 x MOPS, 1.2% agarose) by running at 30 V for 16 h to 19 h. Molecular weights were determined from the rRNA bands in B. napus leaf tissue which had been sized against a commercial RNA mol wt ladder (Bethesda Research Laboratories, No. 5620SA). The RNA was transferred to Gene Screen Plus (NEN-Dupont) membranes by capillary blotting with 20 x SSC (1 x SSC = 150 mm sodium chloride, 15 mm sodium citrate) for 24 h. The RNA was fixed onto the membranes by UV irradiation for 5 min and stored in Seal-a-Meal bags at -20°C. The filters were prehybridized in 25 ml of hybridization solution (50% formamide, 5 x SSPE (1 x SSPE = 150 mm sodium chloride, 10 mm sodium phosphate, 1 mm EDTA), 1% SDS, 10% dextran sulfate, 5 x Denhardt’s solution) with 5 mg yeast tRNA at 43°C for at least 6 h. Hybridization was carried out in fresh hybridization solution under the same conditions for at least 16 h with 5 mg of denatured yeast tRNA and 50 ng of labelled napin and/or cruciferin cDNA probes (kindly provided by Dr. M. L. Crouch, University of Indiana). The DNA was labeled by the random oligonucleotide priming method (6) to a specific activity of >5 x 10⁶ dpm µg⁻¹. The membranes were then washed twice in 2 x SSPE, 2% SDS at room temperature for 10 min per wash, followed by two washes with 0.1 x SSPE, 0.1% SDS at 65°C for 15 min per wash. The filters were exposed to Kodak XAR 5 film at -70°C for varying times.

ABA Extraction and GC-MS Analysis

Microspore embryos were lyophilized for 3 to 5 d prior to extraction of hormones. The weighed tissue was powdered in liquid N₂, and extracted with cold 80% aqueous methanol. [³H]ABA (100,000 dpm; specific activity, 69 Ci mmol⁻¹, Amersham) and [³H]ABA (50 ng, from Martial Saugy and Laurent Rivier, University of Lausanne, Switzerland) were added to the methanol extract. The extract was filtered through a Whatman No. 1 filter, and reduced in vacuo for 24 h. The dried residue was dissolved in 500 µL of 1% acetic acid (pH ~3.0) and then partitioned twice with equal volumes of ethyl acetate:hexane (95:5) saturated with formic acid. After drying with N₂, the residue was dissolved in 10% methanol and separated on a reversed-phase C₁₈ µBondapak HPLC column (14). The radioactive fraction and the fraction running just prior to the labeled ABA were collected to account for the differential chromatography of ABA and [³H]ABA (22), combined and reduced completely in vacuo. The samples were redissolved in 10 µL 100% methanol and methylated with 500 µL of diazomethane at room temperature. The samples were then analyzed by GC-MS-SIM² using the procedure of Ross et al. (19).

² Abbreviation: SIM, selected ion monitoring.

RESULTS

Storage Protein Gene Expression in Microspore Embryos

Microspore embryos produce mRNA transcripts for both napin and cruciferin storage protein gene families in B. napus. The level of expression in these gametophytic embryos when cultured on hormone-free Lichter medium (16) is minimal or undetectable by northern blot analysis. The addition of ABA (1–50 µM) to the medium for 48 h stimulates increases in the steady state level of napin and cruciferin mRNA substantially (Fig. 1). This figure also indicates that maximum induction of storage protein transcripts occurs when the concentration of exogenous ABA is greater than 5 µM.

When plated on medium containing 12.5% sorbitol, storage protein gene expression in these embryos is increased. Figure 2 shows the effect of 10 µM ABA (lane B) and culture on hormone-free Lichter medium (16) with 12.5% sorbitol (lane A) on napin expression. After 48 h of either treatment the level of storage protein mRNA detected is increased several-fold over untreated microspore embryos (lane C). The level of napin transcripts induced is approximately the same in the two treatments and similar effects are observed when a second osmoticum, mannitol, is used (data not shown). The same results were also observed for cruciferin mRNA accumulation (data not shown) and are consistent with previously reported osmotic effects on cultured zygotic embryos (7).

Developmental Expression of Storage Protein Genes in Microspore Embryos

A defined feature of zygotic embryogenesis is the appearance of storage protein mRNA at specific stages of development (3). During zygotic embryogenesis in B. napus, napin transcripts are first detected at 18 DAF (heart stage) and reach a peak at 27 DAF while in late torpedo/early cotyledonal morphological stage (9). Cruciferin mRNA accumulation lags behind that of napin by approximately 5 d (3). It accumulated in the microspore embryos only after they have developed into torpedo stage. Figure 3 is a Northern blot which illustrates the developmental profile of napin mRNA accumulation in microspore embryos cultured in the presence and absence of ABA. Napin transcripts in these embryos are very low or not detected until they reach late globular/heart morphological stage. At this stage they are detectable, but their level increases when ABA is added to the media (lane B). The mRNA is seen to accumulate with exogenous ABA as the embryos mature into torpedo shaped structures (lane C). When this blot was probed again using a cruciferin cDNA probe, no cruciferin mRNA was detected earlier than torpedo stage of development, and only in the presence of exogenous ABA (data not shown).

Time Course of ABA- and Sorbitol-Induced Storage Protein Gene Expression

The addition of 10 µM ABA to the culture medium elicits a rapid response on the expression of both napin and cruciferin (Fig. 4). Within 1 h of incubation with the hormone, a detectable increase in napin transcripts is observed (Fig. 4A). The mRNAs accumulate to a maximum within 24 h
and remain at elevated levels for at least 72 h after incubation with the ABA (data not shown). The effect on cruciferin is similar although the appearance of transcripts is slower than observed in napin (Fig. 4B).

The induction of storage protein gene expression by 12.5% sorbitol treatment (Fig. 4) is substantially slower than that seen on ABA treatment. With 12.5% sorbitol in the medium, an increase in napin transcript accumulation is not observed until 12 h after treatment. This accumulation of napin transcript plateaus after 48 h of culture in the high osmoticum (Fig. 4A). The induction of cruciferin is slower, and only after 24 h can its mRNA be detected (Fig. 4B).

**ABA Measurements during Sorbitol and ABA Treatment**

The differences observed in the rate of storage protein transcript accumulation between the two treatments, suggested that a relationship could exist between osmotic stress and ABA levels in the microspore embryos. To investigate this possibility, the sorbitol treated embryos were analyzed for ABA content by GC-MS-SIM (Fig. 5). The presence of ABA in all samples analyzed, demonstrated that microspore embryos can synthesize this phytohormone. The amount of ABA measured in the microspore embryos cultured for 2 h in the presence of 12.5% sorbitol was found to be approximately fivefold higher than observed in the untreated embryos. Maximum concentrations of ABA (sixfold higher than controls) were measured at 12 and 24 h after culture on the osmoticum. The level of hormone remained stable for an additional 24 h of treatment. Controls which were cultured in identical conditions except for the exclusion of sorbitol from the medium, did not show any variation in steady-state levels of free ABA during culture.

The endogenous concentration of ABA also increased rapidly in embryos treated with ABA (Table I). The concentration of ABA was approximately one order of magnitude higher than observed in the sorbitol-treated embryos after 4 h. However, this elevated level was transient and dropped by a factor of 10 after 48 h.

**DISCUSSION**

In this paper, we have investigated the relationship between osmotic stress and ABA in the regulation of expression of storage protein genes. By the use of microspore embryos for this study we have eliminated maternal tissue as a source of ABA.

We previously demonstrated (20) that ABA (10^{-5}M) treatment for 48 h stimulates the accumulation of napin and cruciferin mRNA in *B. napus* microspore or excised zygotic embryos. The accumulations were quantitatively similar in the two embryo types. Both embryo types were at the torpedo-stage of morphology. Figure 1 shows that mRNA encoding napin and cruciferin can be induced over a wide range of ABA concentrations, but that maximal induction occurs at about 10 μM ABA.

In the present study, we have demonstrated that a similar level of storage protein transcripts can be induced in microspore embryos by a treatment of 10 μM ABA or by 12.5% sorbitol added to the medium (Fig. 2). It is interesting to note that the normal culture medium for *Brassica* microspore embryos already contains 13% sucrose which creates an unusually low water potential for a plant tissue culture medium. Further reduction of this water potential by sorbitol to 25.5 bars elicits a dramatic response in storage protein transcript accumulation compared with the control grown in 13% sucrose. DeLisle and Crouch (5) recently demonstrated that in zygotic embryos the ABA-stimulated accumulation of storage protein mRNAs correlates well with enhanced rates of transcription. The equivalent in *vitro* transcription experiments have not yet been reported for treatments with high osmoticum.

The responsiveness of microspore embryos to applied ABA was investigated at three morphological stages—early globular, globular-heart, and torpedo. Not only is exogenous ABA necessary for storage protein mRNA accumulation in microspore embryos, but also the response to ABA is stage-dependent (Fig. 3). Thus, early globular embryos did not accumulate any detectable transcripts of napin even in the presence of ABA (10 μM). A small stimulation of napin transcript accumulation in response to ABA treatment was observed with globular-to-heart stage embryos. However, a dramatic response to ABA was only noted in torpedo-stage embryos. This may reflect a differential sensitivity to the applied hormone either by recognition or transduction of the hormonal signal.
However, it could also result from variations in stability of napin mRNA in the different stages of embryogenesis. These alternatives are now under investigation using in vitro transcriptional assays. If ABA were a mediator of osmotic effects on storage product gene expression, one would predict that osmotic stress would produce a response no more rapid than applied ABA. Most likely the osmotic effect would be somewhat slower than endogenous ABA accumulation would have to commence. Furthermore, we would predict that an osmotic shock of these embryos would result in an accumulation of ABA.

The first prediction is clearly fulfilled (Fig. 4). The time course of both napin and cruciferin mRNA accumulation in response to 10 μM ABA is quite rapid, with optimal levels being achieved within 24 h. In contrast, storage protein mRNA accumulation occurs quite slowly in response to 12.5% sorbitol treatment. Maximal levels of these transcripts are achieved only after 48 h.

The second prediction, that ABA accumulation should be stimulated by osmotic stress, is also satisfied. There is an approximately sixfold increase in ABA concentration within 4 h of sorbitol treatment (Fig. 5). This sequence of events is also consistent with the involvement of ABA in mediation of the osmotic response.

The ability of these microspore embryos to accumulate ABA in response to high osmoticum does not exclude the possibility that the import of ABA into the zygotic embryo from maternal tissue is also involved in the regulation of storage protein gene expression during seed development.
Table 1. Measurements of Endogenous ABA in Torpedo-Stage Microspore Embryos Treated with ABA (10 μM) at Various Times after Incubation

<table>
<thead>
<tr>
<th>ABA Treatment Time</th>
<th>ABA</th>
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<tbody>
<tr>
<td>h</td>
<td>ng/g dry wt</td>
</tr>
<tr>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>1</td>
<td>804</td>
</tr>
<tr>
<td>4</td>
<td>7978 ± 453</td>
</tr>
<tr>
<td>24</td>
<td>4494 ± 793</td>
</tr>
<tr>
<td>48</td>
<td>719</td>
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</table>

There is evidence that labelled ABA may be transported from leaves into the developing embryos during seed maturation (15). The maternal origin of ABA in crucifers is also supported by the application of ABA to ABA-deficient mutants of Arabidopsis (12). In Phaseolus vulgaris and Gossypium hirsutum migration of ABA takes place from the seed coat to the embryo in response to a pH gradient between these tissues (1, 11). Nevertheless, the capacity of these embryos to accumulate ABA in response to osmoticum implies that the embryo proper is able to augment its own ABA levels.

The 'physiological concentration' of ABA that accumulates in osmotically stressed microspore embryos is difficult to determine. However, free ABA concentrations of ~700 ng per g dry weight were found in embryos osmotically stressed for 24 h. As indicated by Table 1, embryos treated with 10 μM ABA may accumulate transiently ~8 μg per g dry weight of ABA. Thus, there is a 10-fold difference in the concentration of endogenous hormone between the ABA-treated and osmotically stressed embryos. Nonetheless, it would be difficult to exclude endogenous ABA as a component in the regulation of storage protein gene expression in osmotically stressed embryos. This is particularly evident on examination of Figure 1 where treatments of 1 μM ABA elicit a significant increase in both napin and cruciferin mRNA accumulation. Such apparent log-linear relationships between applied hormones and physiological or biochemical responses are common (4) and have been shown to occur in ABA-regulation of napin and cruciferin gene expression in zygotic embryos between concentrations of 0.1 and 10 μM of applied ABA (3).

The possibility that ABA acts as a mediator of osmotic stress responses in developing embryos has not yet been exhaustively investigated. Experiments with noncruciferous species suggest that accumulation of storage proteins in response to high osmotic stress can be mediated by ABA (2). However, two studies (9, 10) on cruciferous species, including one on B. napus, have suggested two independent pathways for ABA- and osmoticum-stimulated storage protein gene expression. Fischer et al. (10) used fluoridine, an inhibitor of carotenoid biosynthesis and the catabolic pathway of ABA production. They found that fluoridine applied to developing seeds of Sinapis alba did not greatly affect storage protein accumulation which had been triggered by osmotic stress (~22 bars). This result was interpreted as indicating a disjunction of the ABA and osmotic effects. However, no measurements of ABA levels in the tissues were reported, and only one concentration of fluoridine was used. Bray and Beachy (2) performed similar experiments on cultured soybean embryos and found a reduction in the accumulation of β-conglycinin transcripts in the cotyledons. Koornneef et al. (13) recently performed analyses on genetic crosses between double-recessive ABA-deficient and seed-specific ABA-insensitive mutants (designated abi 3) of Arabidopsis. These workers were able to show that dormancy in these seeds was linked primarily to the embryo genotype and did not depend on either maternal ABA or ABA applied to the mother plant. In ABA-deficient single mutants, storage protein accumulation was depressed, but not eliminated. Interestingly, double mutants, which were ABA-deficient and also ABA-insensitive, lost their ability to accumulate storage proteins. This may indicate leakiness of the ABA-deficient mutants or the involvement of other regulators of storage protein accumulation genetically unlinked to the aba locus. The dramatic effects of abi 3 on storage protein accumulation might indicate that these other regulators utilize the same signal transduction pathway as ABA.

Our work does not completely resolve these different interpretations. It does however, present two particularly relevant findings. First, in microspore embryos of Brassica, high osmoticum (~25 bars) promotes the accumulation of free ABA levels to approximately sixfold higher levels than the controls. The levels of ABA that accumulate appear to be high enough to provoke a physiological effect, as indicated by responses to exogenous ABA treatment (Fig. 1). The importance of an embryo-synthesized pool of ABA in regulation of dormancy-related events is also indicated by the genetic experiments of Koornneef et al. (13). Second, the timing of events is also compatible with ABA as a mediator of the osmotic effect, as ABA stimulates storage protein transcript accumulation rapidly while there is a lag of up to 12 h before major increases in these transcripts are detected after osmotic stress. Endogenous ABA accumulates during this lag period. In agreement with the results of Koornneef et al. (13), these data cannot be interpreted as indicating that ABA is the only mediator of the osmotic effect in this system. In fact, we have recently detected, using GC-MS, jasmonic acid as a natural product of microspore and zygotic embryos. Jasmonic acid, when supplied exogenously at 10 μM also induces napin and cruciferin transcript accumulation (RW Wilen, G. van Rooijen, D Pearce, R Phasis, L Holbrook, M Moloney, in preparation). It is therefore possible that other endogenous regulators which are produced by independent biosynthetic pathways, and therefore are genetically unlinked to ABA accumulation, may be involved in this mediation. We are now seeking to clarify the role of ABA as a mediator of osmotically regulated storage product accumulation by the use of inhibitors of ABA biosynthesis and by the use of structural analogs of ABA which can act as antagonists.

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