Proteins in development and germination of a desiccation sensitive (recalcitrant) seed species

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Abstract

In the recalcitrant seeds of Avicennia marina, protein content and the rates of protein synthesis increase during histodifferentiation. This is similar to the situation in desiccation tolerant seeds. During the stage of reserve accumulation the protein content and rates of synthesis remain constant and there is no de novo synthesis of proteins which might qualify as storage proteins. There is also no change in the nature of proteins present in either axis or cotyledonary tissues during development or germination. Similarly, fluorographs of axis proteins show only very limited changes in the patterns of protein synthesis during development and germination, at least until the onset of root growth. Heat-stable proteins are present from an early developmental stage. However, no late embryogenic abundant (LEA) proteins are synthesised during the late stages of development, indicating that seedling establishment is independent of such maturation proteins. It is suggested that the lack of desiccation tolerance of A. marina seeds might be related to the absence of desiccation-related LEAs. Although the rate of protein synthesis increases during germination, protein metabolism appears to remain qualitatively the same as that occurring during development. The present results suggest that in these desiccation sensitive seeds, protein metabolism characterising development changes imperceptibly into that of germination.

Introduction

The seeds of Avicennia marina (Forssk.) Vierh. are highly recalcitrant (desiccation sensitive) [30]. This paper presents the results of an aspect of a detailed study on the differences in development between recalcitrant and orthodox (desiccation tolerant) seeds.

In desiccation tolerant seeds, although there are proteins that are common to both development and germination [1, 11], there are also notable qualitative and quantitative differences among the proteins characteristic of these two phases. Some proteins are newly synthesised during germination whereas others are specific to development [5], the synthesis of storage proteins being confined to development. Generally only a few species constitute the storage proteins (for any one seed type) but they are made in large quantity. Although storage proteins do accumulate in some recalcitrant seed species, for example, Podocarpus henkelii [13], and Camellia sinensis [4], no protein bodies develop in Avicennia marina [19].

In addition, it has been demonstrated that in a number of desiccation tolerant seed species, a set of proteins is synthesised during the late stages of reserve accumulation. These proteins have been termed late embryonic abundant (LEA) proteins [14, 20], maturation proteins [6, 31] or dehydrin-like proteins [8]. LEA protein levels decline during early germination [6, 14, 32].

Although much of the evidence for the purported
functions of the LEAs is indirect, a survey of the literature indicates that different subsets of these proteins may have different roles. Some of the LEA proteins have been implicated in desiccation tolerance. In many species LEA proteins appear coincident with the acquisition of desiccation tolerance and/or desiccation per se, and decline with the onset of desiccation sensitivity during germination [2, 6, 7]. Some of the LEA proteins are heat-stable [6].

It appears that homology exists between a subset of LEAs from a variety of different species and that these proteins are hydrophilic [15]. In addition, some of these LEAs have been shown to accumulate in response to water stress in seedlings [27]. Proteins which accumulate during dehydration of seedling tissues have also been termed dehydrins [10]. As a consequence of their physical characteristics (extreme hydrophilicity and resistance to denaturation) these proteins have been suggested to protect tissues from dehydration damage [10]. As some LEAs act also as dehydrins, and as the two groups have the same physical characteristics, it has been suggested that LEAs have a similar protective role during development [15, 24].

There are no published data on the nature and pattern of protein synthesis during development and early germination in desiccation sensitive seeds. As these seeds are not tolerant of dehydration, it is unlikely that LEA proteins with a desiccation protective function are expressed. However, LEA proteins with a different function in maturation could occur.

Seeds of A. marina take about 85 days to develop. Histodifferentiation occurs over the first 50 days, with growth and reserve accumulation taking place over the remainder of the developmental period. The seed has no testa and the fruit consists of a pericarp enclosing the embryo which has two large cotyledons enrolling a well developed axis. On shedding the water content of the cotyledons and axis are both approximately 1.8 g per g dry mass. The pericarp is sloughed shortly after shedding [3] and germination, although not dependent upon pericarp sloughing, is initiated at this stage [30].

In the present contribution the following aspects of the development of seeds of A. marina have been investigated.

1. Does large-scale synthesis of storage proteins occur during development?
2. Embryos from prematurely harvested seeds of A. marina are capable of root protrusion at 55 days after fruit set (DAFS), but seedling establishment is possible only at 70 DAFS [18]. Are maturation proteins involved in the attainment of the ability to establish seedlings?
3. Are any other new proteins produced at a late stage of development? If so, do these have characteristics of the desiccation-related LEAs?
4. Is the initiation of germination associated with a change in pattern of protein synthesis in A. marina, which can be used as a marker for the onset of germination sensu stricto?

Materials and methods

Materials

The day of fruit set was determined by corolla abscission from some 10 000 flowers that had been individually tagged at the bud stage. The age of developing seeds could thus be accurately ascertained and collections made on the appropriate DAFS. To obtain germinating material, mature, newly-shed seeds were placed on moist vermiculite. As individual seed components could not be separated before 55 DAFS, rates of protein synthesis and total protein content were determined on entire seeds up to this stage. These measurements were made separately for pericarps, embryonic axes, and cotyledons at five day intervals from 55 DAFS until shedding, and daily for germinating seeds, until root growth had occurred. As a major interest was the possible production of LEA proteins, electrophoretic separations of proteins was performed on axes and cotyledons only from 55 DAFS onwards.

Methods

Determination of rate of protein synthesis and soluble protein content. The rate of protein synthesis was calculated as the rate of incorporation of \(^{1}H\) leucine (radioactive concentration 0.5 \(\mu\)Ci ml\(^{-1}\); specific activity 130 Ci \(\mu\)mol\(^{-1}\)) by triplicate 2 g samples of axis, cotyledons or pericarp [26]. Incubation was for 4 h at 25°C in a shaking water
bath. Control material was similarly incubated in a solution containing unlabelled leucine. Following incubation, material was ground in Tris-HCl buffer (pH 8.0) and 100 μl aliquots of the homogenate were applied to each of three Whatman 3 MM filter paper discs and the protein precipitated. After treatment to remove contaminants, the discs were dried and incorporation measured using a Beckman LS 75000 scintillation counter. Soluble protein content was determined on the remaining homogenate from each extraction after centrifugation to remove particulate matter. Triplicate 100 μl samples were taken from the supernatant in each case, and assayed for proteins [9].

**Protein extraction for electrophoresis.** Material was lyophilized and stored at −70°C (which did not cause any detectable changes in the proteins). All extractions were done in triplicate on unpoled samples. *A. marina* seeds have a polyphenolic content that ordinarily considerably impedes electrophoretic separation of proteins. To overcome the problem, the following extraction procedure was used. Frozen tissue (0.5 g) in liquid nitrogen was ground to a fine powder. One g of insoluble polyvinylpyrrolidone (Polyclar AT, Sigma) was mixed with the sample which was then suspended in 5 ml of extraction buffer (50 mM Tris-HCl [pH 7.0], 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 2% β-mercaptoethanol, 2 mM PMSF and 10 mM thiourea). After incubation on ice for 10 min, an equal volume of water-saturated phenol was added and the mixture shaken for 10 min at room temperature [21]. After centrifugation, five volumes of 0.1 M ammonium acetate in methanol were added and the proteins precipitated from the phenol phase at −20°C overnight. The pellet obtained after centrifugation was washed (four times with ammonium acetate in methanol and twice with acetone), dried under vacuum and solubilised in lysis buffer [29]. Total soluble protein was determined [9] for triplicate 100 μl samples and aliquots used for electrophoresis.

**Isolation and separation of heat-stable proteins** (modified from [6]). Protein was extracted from 20 mg of axis or cotyledonal tissue as described above, up to and including precipitation from the phenol phase. The pellet was then dissolved in 50 μl extraction buffer, heated for 10 min at 80°C, and the coagulated proteins removed by centrifugation. An equal volume of running buffer (50 mM Tris-HCl [pH 6.8], 20% glycerol, 10% β-mercaptoethanol and 4% SDS) was added to the supernatant. Protein content was determined as described above, and aliquots of the sample assayed electrophoretically as for the second dimension (below). Unheated, solubilized protein in equal volumes of extraction and running buffer, was used as a control.

**Electrophoresis.** Two-dimensional gel electrophoresis (first and second dimensions according to [29] and [25], respectively) followed by gel staining was used to map the pattern of proteins synthesised. Staining was for 4 h using 0.25% Page Blue (Sigma) in 50% methanol/10% acetic acid.

**Fluorography.** The wound sites resulting from cotyledon removal were sealed with petroleum jelly, after which axes were incubated for 4 h at 25°C (using a shaking water bath) in a solution containing 35S methionine (radioactive concentration 0.3 mCi ml⁻¹; specific activity 980–1000 Ci mmol⁻¹). The sealed wound sites were excised prior to processing for electrophoresis. Gels prepared from these samples were destained in a mixture of 5% methanol and 10% acetic acid and then soaked in Amplify (Amersham, UK) for 30 min. After they had been dried, the gels were exposed to X-ray film (Hyperfilm-MP, Amersham, UK) for 20 h at −70°C.

**Results**

Protein concentration of whole seeds increased during histodifferentiation (Figure 1). At 55 DAFS, when seed components could first be separated, the protein content of the pericarp was high and declined rapidly thereafter. The protein levels were similar in axes and cotyledons and changed very little during the period of embryo growth and reserve accumulation. The protein concentration of the embryo increased during the first day after shedding (DAS) if the seeds were placed on a moist substrate, and during the following three days further increases in protein occurred only in the embryonic axes.

Rates of protein synthesis in embryonic axes
changes in spot intensity have been recorded. From 55 to 70 DAFS only one new protein (labelled 8 on Figures 4 to 6) became apparent, and there was an increase in staining of protein 7 and 10. The staining of protein 5 declined in intensity and that labelled 2 at 55 DAFS could no longer be observed at 70 DAFS. With continued development of the seeds there was a decline in proteins 1, 4 and 5, the intensity of staining increased in proteins 7 and 10, and one new protein, labelled 9, was visualised. After seeds had germinated for four days, the new protein (9) was no longer apparent, protein 5 could no longer be observed, the increased staining in protein 7 was reversed, and there were declines in the intensity of proteins 3 and 6. The staining intensity of protein 8, which was first observed at 70 DAFS, increased.

It is possible that some proteins were synthesised only at early stages of development, but were always visualised by staining because of long lifetimes. Such a phenomenon would become apparent by fluorography of newly synthesised proteins that had incorporated a radioactive label. Fluorographs of proteins synthesised 55 and 70 DAFS and 2 DAS
showed no marked changes in patterns of protein synthesis with development. At 4 DAS, by which stage root growth had occurred, there were some qualitative and quantitative changes. Figures 7 and 8 are fluorographs of proteins synthesised at 70 DAFS and 4 DAS, respectively. These experiments were performed a season subsequent to the protein studies reported above, and a different batch of amphotoles was used. Because of this a somewhat different separation in the first dimension occurred and so the spots appearing in Figures 7 and 8 are not necessarily coincident with those in Figures 3 to 6. Thus the numbers allocated to stained spots in the two experiments do not coincide. Three new proteins were synthesised at 4 DAS (proteins 1 to 3 on Fig. 8), and the proteins 4 to 7 were more intensely labelled. There was a decrease in intensity of proteins 8, 9 and 10, to the extent that suggests that they were no longer synthesised at 4 DAS.

There were several heat-stable proteins in both axes and cotyledons of *A. marina*, although these are obviously not involved in desiccation tolerance. SDS-polyacrylamide gel electrophoresis of heat stable axis proteins at different stages of development are shown in Figure 9. As with all the other proteins, heat-stable proteins were present from the end of histodifferentiation, and showed few qualitative and quantitative changes during growth and germination.

**Discussion**

In common with most seeds, there were increases in rates of protein synthesis and protein content during histodifferentiation of *A. marina* seeds. However, storage proteins are not accumulated during the later stages of development. Although rates of protein synthesis remained relatively high during the period of reserve deposition, there were no increases in the rates of synthesis, there was no further protein accumulation, and no new proteins were synthesised, unlike the situation during this developmental stage in seeds which do accumulate storage proteins [5]. From the few studies that have
been made, protein contents as low as those in *A. marina* (30 mg g\(^{-1}\) dry mass) do not appear to be a general feature of desiccation sensitive seeds. *Podocarpus henkelii* embryos have a protein content of 100 mg g\(^{-1}\) dry mass [13] and *Shorea robusta* has approximately 300–400 mg g\(^{-1}\) dry mass [28].

The absence of storage proteins in the seeds of *A. marina* suggests that they have an alternative source of nitrogen for use during germination. Amino acids and oligopeptides can accumulate as storage reserves [12]. From the total protein content, estimated by organic nitrogen, of mature seeds of *A. marina* [33], organic nitrogen of 14 mg g\(^{-1}\) dry mass can be estimated. The soluble protein content determined in this study is equivalent to 5 mg g\(^{-1}\) nitrogen. The difference is presumably accounted for by insoluble protein and non-protein nitrogen. This would include any amino acids and amides which would be readily available for the nitrogen metabolism associated with germination.

The staining patterns of both the two-dimensional gels and the fluorographs indicate there was little qualitative change in the protein metabolism of either the axis or the cotyledons from completion of histodifferentiation at 55 DAFS until two days after the seeds were shed. Thus LEA proteins do not appear to occur in the embryos of *A. marina*. The ability of embryos to establish seedlings from 70 DAFS is apparently not related to the appearance of maturation proteins. In soybeans, heat-stable maturation proteins have been suggested to be implicated in some way in desiccation tolerance [6]. Heat-stable proteins do occur in the embryonic tissues of *A. marina*, but are present from an early stage. Furthermore, these seeds are never tolerant of desiccation so the presence of the heat-stable proteins must be suggested to have some alternative significance. The absence of LEA proteins from *A. marina* supports the proposal that truly desiccation-sensitive seeds will lack such proteins [8]. Thus their absence in *A. marina* may well be
related to the extreme sensitivity of these seeds to desiccation.

On seed abscission there was an increase in the rate of protein synthesis, and protein content increased. These processes are indicative of enhanced metabolism associated with the onset of germination. However, there was no apparent change in the nature of the proteins present or those synthesised up to the onset of root growth. As cell division is likely to have resumed by this stage [17, 19] some structural protein must have been required. Two possibilities exist. i. Such proteins are also involved during development and are thus not synthesised de novo for root protrusion. This would be possible, for example, if they were continuously required for maintenance and replacement of structural components. ii. New proteins are made, but were not detected. The fluorographs and protein gels presented here represent the protein spectrum from the entire axis. The root meristems (primordia) are very small relative to the hypocotyl tissue [3], thus protein synthesis occurring in the root primordia may have been masked and therefore not detected in the present study.

With the onset of root growth some qualitative and quantitative changes in protein metabolism were detected. Some proteins (presumably developmentally related) disappeared, and at least three new proteins became apparent in the fluorographs. The synthesis of several other proteins, already present, increased. It is not clear whether these changes were sufficient to produce structural elements (and the enzymes associated with their elaboration) which presumably accompany early root growth. In terms of the argument outlined in (ii) above, it is suggested that the proteins resolved may be indicative of only some of the metabolic events associated with root growth.

Irrespective of possible (but undetectable) changes in the root primordia, the fluorographs indicate that the protein metabolism of the majority of the axis tissue - that is, the hypocotyl - does not change qualitatively until at least two DAS. This can be explained, in part, by the nature of reserve accumulation occurring in these seeds. In desiccation tolerant seeds, many of the qualitative differences in protein synthesis during development and germination are a consequence of the different enzymic processes of reserve accumulation and degradation, respectively [11, 22, 23]. The seeds of _A. marina_ accumulate only starch and soluble sugars as reserves, and storage proteins and lipid are not elaborated [19]. Thus the enzymes associated with massive protein or lipid synthesis during development, or degradation of such reserves during germination, would not be required. The processes of starch accumulation and degradation occur during both development and germination in seeds of _A. marina_ [19]. Thus enzyme activity involved in both accumulation and degradation must occur during both development and germination. Unless they have long half-lives, these enzymes are presumably synthesised at all stages, as there is no apparent change in the patterns of protein synthesis accompanying the changes in starch levels in axes and cotyledons. Starch accumulation and degradation are ongoing processes in vegetative tissues, and the attendant enzymes will be present. A similar situation may exist in the seeds of _A. marina_ which remain metabolically active throughout their development.

The following conclusions may be drawn from the present study. During histodifferentiation protein metabolism is largely associated with the processes involved in formation of the embryonic tissues. After that stage (from 50 DAFS to maturity) protein metabolism remains qualitatively the same, is similar in both the axis and cotyledons, and is possibly equivalent to basal metabolism typical of vegetative tissues. The enzymic process associated with starch accumulation and degradation are suggested to be part of the basal metabolism. Ultrastructural studies have shown that apart from an increase in vacuolation and starch accumulation, there is little change in the subcellular organisation of axis or cotyledonary tissues during development [19]. In _A. marina_, maturation drying does not occur [19] and the mature seeds remain metabolically active: on shedding, the metabolism remains qualitatively the same.

However, the present, and previous, studies have shown that activity increases on shedding, suggesting amplification of metabolism associated with germination [16, 17, 30]. These initial events appear to occur without a qualitative change in protein metabolism of the embryo as a whole. It is suggested that structural and related enzymic changes may well accompany root protrusion and growth, but that such changes are localised and occur, at least initially, only in the root primordia. As these tissues
are insignificantly small relative to the rest of the axis [3], any such changes would not have been detected in the present study. The metabolism of the hypocotyl, and possibly also the cotyledons, remains at a basal level, at least up to four DAS, and possibly longer. It is suggested that further changes, both structural and metabolic, could occur on tissue differentiation associated with seedling establishment.

References

30. Rosenberg LA and Rinne RW (1988) Protein synthesis