Chapter 2
Proteomics Reveals A Potential Role of the Perisperm in Starch Remobilization During Sugarbeet Seed Germination

Julie Catusse, Claudette Job and Dominique Job

Abstract Sugarbeet is a crop of high economic importance because it is one of the two main sources of plant sugar, the other being sugarcane. The sugarbeet seeds have the peculiarity of containing at maturity a large starchy storage tissue, called the perisperm. In contrast to the well-documented cereal endosperm, the physiology of this perisperm is completely unknown. Here, we used proteomics of perisperm isolated either from dry mature or imbibed sugarbeet seeds to unravel the mechanisms of starch remobilization during germination. We also carried out a comparative proteomics analysis with the perisperm isolated from the dry mature sugarbeet seeds. We observed an accumulation of α-amylase in the perisperm isolated from imbibed whole seeds but not from the isolated imbibed perisperm alone, suggesting a role of the embryo in triggering the accumulation of this starch-mobilizing enzyme in the perisperm during germination. In this way, the mechanisms occurring in the sugarbeet seed perisperm during germination would appear to be similar to those documented for the endosperm of cereals. In contrast, an accumulation of β-amylase and α-glucosidase was observed in the isolated imbibed perisperm, suggesting that the embryo was not mandatory for induction of these enzymes in the perisperm during imbibition.

Keywords Amylases · Perisperm · Proteomics · Seed germination · Starch · Sugarbeet

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2.1 Introduction

2.1.1 Seed Physiology

In the botanical sense, the seed is the organ issued from the double fertilization of the ovule by the pollen grain. It contains a zygotic embryo that will form upon germination the new plant and a storage tissue, usually a triploid endosperm, but that may derive from the maternal nucellus in some species such as sugarbeet (*Beta vulgaris* L.), and that will contribute to successful seedling establishment following germination. In their mature form, most seeds ensure the dispersion of plant species in the environment (Bewley and Black 1994; Linkies et al. 2010; http://www.seedbiology.de/; accessed April 28th 2011). Embryos from seeds of most plants belonging to the orthodox family have the remarkable property of being able, through intense desiccation during maturation, to interrupt their metabolic activity on the mother plant and to restart this activity after a simple imbibition during germination (Angelovici et al. 2010; Boudet et al. 2006; Buitink et al. 2000).

The seed is thus one of the rare and remarkable examples in eukaryotes where there is disruption of development and preservation of the competence of an embryo in the dry state over long periods of time. Remarkably, radiocarbon dating allowed the determination of the age of date (*Phoenix dactylifera* L.) seeds at about 2000 years (Sallon et al. 2008), sacred lotus (*Nelumbo nucifera*) seeds at 1300 years (Shen-Miller 2002; Shen-Miller et al. 1995), or canna (*Canna compacta*) seeds at 600 years (Lerman and Cigliano 1971). This implies the existence of specific molecular and biochemical mechanisms to maintain the state of metabolic quiescence of mature dry seeds while preserving their viability (longevity), but also to ensure the restart of cellular metabolism during germination (Bewley and Black 1994; Catusse et al. 2011; Holdsworth et al. 2008a; Rajjou and Debeaujon 2008; Rajjou et al. 2008). These mechanisms determine the potential of seed germination, that is to say the success with which the new plant will be established. A better understanding of these mechanisms would allow the development of molecular and biochemical markers reflecting this potential, which could be used as quality markers in the seed sector for the marketing of high-vigor seed lots (Catusse et al. 2011). Knowing that our food depends on agricultural production, the concept of seed quality is central in a socio-economic context where agriculture is heavily industrialized, refocused on a few crops, and where it is expected that there will be 9 billion people to feed within the next 40 years (The U.S. Census Bureau: http://www.census.gov/ipc/www/idb/ (accessed April 28th 2011)).

In angiosperms, seed formation results from a double fertilization (Dumas and Rogoswki 2008). Initially, the male gametophyte (pollen grain) germinates on the stigma of the flower, thus forming a pollen tube carrying two sperm nuclei to the egg. There is then a double fertilization, one giving the zygotic embryo, the other a storage tissue (triploid endosperm) by fusion of a sperm nucleus and two polar nuclei from the female gametophyte. The embryo can be considered as a miniature plant with a root (radicle), a draft of stem (hypocotyl), and cotyledons. By definition,

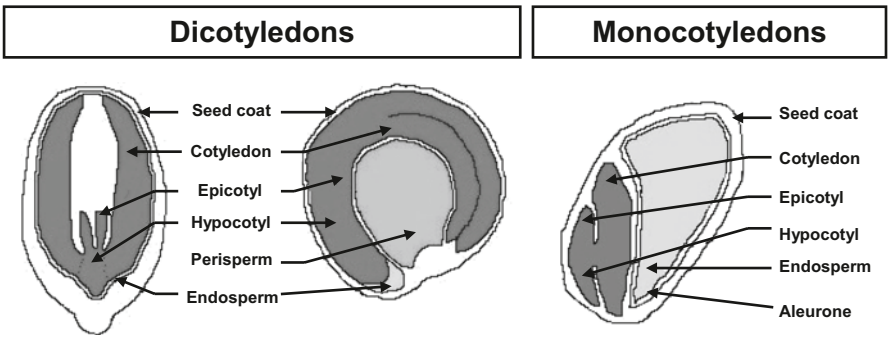

Fig. 2.1 Schematic structure of dicot and monocot seeds

embryos of seeds of dicotyledonous plants have two cotyledons (e.g., legumes and oilseeds), while there is only one cotyledon in seeds of monocotyledons (e.g., rice, maize, and wheat). Seeds can be classified by the presence or absence of a mature well-formed endosperm. In the latter case, the initial endosperm is readily consumed during development and other structures, as the cotyledons (e.g., *Arabidopsis*) but also perisperm (e.g., sugarbeet), becomes the main storage organs. In contrast, some seeds such as cereals contain at maturity a large dead starchy endosperm surrounded by a few living cell layers of endosperm, called the aleurone layer (Fig. 2.1). The death of the starchy endosperm cells is a developmental phenomenon (programmed cell death) rather than one induced by water loss (Bethke et al. 1991; Golovina et al. 2000). However, cells of the aleurone layer can express a specific developmental program allowing them to survive desiccation. The barley (*Hordeum vulgare*) aleurone layer can be separated from the other seed tissues and maintained in culture, allowing studying the effect of added signaling molecules in an isolated system (Bethke et al. 1997; Finnie et al. 2011).

Two main phytohormones, ABA and GAs control seed formation, dormancy, and germination; ABA being an inhibitor of germination involved in the development of the embryo, accumulation of seed reserves, and maintenance of dormancy, while GAs stimulate seed germination and mobilization of the stored reserves (Hilhorst and Karssen 1992; Ho et al. 2003; Koornneef and van der Veen 1980; Koornneef et al. 2002; Kucera et al. 2005; Lovegrove and Hooley 2000).

2.1.2 Proteomics in Addressing Biological Questions

With the completion of the genome sequence for several organisms and the accelerated development of analytical methods for protein characterization, proteomics has become an indispensable approach to functional genomics (Bradshaw and Burlingame 2005; Rabilloud et al. 2010; Walther and Mann 2010), notably in plants (Agrawal and Rakwal 2006, 2008, 2011; Agrawal et al. 2006, 2011a, b; Job et al. 2011; Wienkoop et al. 2010; http://www.inppo.com/; accessed April 28th 2011). In

particular, several proteomics studies on the development and germination of seeds have been published in recent years (Agrawal et al. 2008; Bourgeois et al. 2009; Bykova et al. 2011; Dam et al. 2009; Finnie et al. 2002, 2004; Gallardo et al. 2001, 2003, 2007; Hajduch et al. 2005, 2006, 2010; Irar et al. 2010; Miernyk and Hajduch 2011; Méchin et al. 2004; Müller et al. 2010; Nadaud et al. 2010; Nautrup-Pedersen et al. 2010; Rajjou et al. 2006; Sheoran et al. 2005; Wang et al. 2010; Yang et al. 2007). Moreover, the study of biosynthetic pathways responsible for the accumulation of the various seed storage compounds is of major importance for at least two reasons: (i) these reserves support the early growth of seedlings after germination, hence contributing to seedling vigor; and (ii) they are widely used as food and feed. Many biotechnological applications focus on improving the nutritional value of seeds and also to promote other non-nutritional uses (e.g., biofuel production) or the use of seeds as plants for the production/storage of recombinant proteins (Boothe et al. 2010; Gressel 2008; Job 2002).

2.1.3 Seed Proteins: A brief Historical Event

The systematic study of seed proteins dates from the nineteenth century. Particularly the work of Osborne (Osborne 1924) on proposed classification of proteins is still in use, according to their solubility properties in water (albumins), dilute salt solutions (globulins), alcohol-water mixtures (prolamins), or dilute acid or alkaline solutions (glutelins). Besides all these proteins, mature dry seeds also contain stored mRNA during maturation. Rajjou et al. (2004) showed that germination of *Arabidopsis* seeds could be observed even in the presence of an excess of a fungal toxin α-amanitin, which is a potent specific inhibitor of transcription elongation by eukaryotic DNA-dependent RNA polymerase II (Jendrisak 1980; de Mercoyrol et al. 1989). On the contrary, *Arabidopsis* seed germination (radicle protrusion) was completely inhibited in the presence of cycloheximide, an inhibitor of protein synthesis in eukaryotic cells that blocks translation elongation (Rajjou et al. 2004). These findings have been confirmed both in *Arabidopsis* (Kimura and Nambara 2010) and rice (He et al. 2011). These results disclosed that the germination potential is largely programmed during the process of seed maturation on the mother plant. They also highlighted the usefulness of a proteomics approach for studying the germination process since germination can be evidenced in the absence of *de novo* transcription, thus illustrating the role of stored proteins and of translation of mRNAs also stored in the seeds (Catusse et al. 2008a; He et al. 2011; Holdsworth et al. 2008b; Rajjou et al. 2004).

2.1.4 Sugarbeet and its Importance

Sugarbeet (*Beta vulgaris*), a dicotyledonous biennal plant belonging to the *Amaranthaceae*, can be grown commercially in a wide variety of temperate climates.

It has a high economic importance because it is one of the two main sources of plant sugar (the total world production in 2005 was about 250 million metric tons, http://www.sugarindustrybiotechcouncil.org/sugar-beet-faq/; accessed April 28th 2011), the other being sugarcane (*Saccharum officinarum* L.). The agronomic productivity of this direct seeded sugarbeet crop is determined significantly by the uniformity of seedling emergence in the field (de Los Reyes and McGrath 2003; Elamrani et al. 1992; Job et al. 1997; Mukasa et al. 2003). Besides food applications, its culture is attracting renewed interest, particularly in Europe, for the production of bioethanol (http://www.biofuelstp.eu/bioethanol.html; accessed April 28th 2011). The plants from which descend the current varieties and that were described as 'versatile garden plants', originated in the Middle East and their discovery dates back about 800 years BC. The high sugar content of their roots was noticed at the end of the sixteenth century by the French agronomist Olivier de Serres (years 1539–1619; http://www.bookrags.com/research/olivier-de-serres-scit-0312/; accessed April 28th 2011). Then, the German chemist Andreas Marggraf (1709–1782) used alcohol to extract the juices from several plants, including, in particular, sugarbeet. Andreas Marggraf is internationally recognized as the founding father of this technique (http://en.wikipedia.org/wiki/Andreas_Sigismund_Marggraf; accessed April 28th 2011). Fifty-four years later, in the year 1801, the world's first beet sugar factory opened at Cunern in Silesia (now Konary, Poland) and produced about 70 kg of sugar per day. Original forms contained only about 4 % sugar but careful selection and breeding have raised this to a content of 20 % (http://en.wikipedia.org/wiki/Sugar_beet; accessed July 11th 2011).

Sugarbeet seeds contain a large starchy storage tissue, called the perisperm. The perisperm is a dead tissue at maturity and consists of a network of thin-walled cells filled with starch grains in the form of globoids (Artschwager 1927). The perisperm exists in all seeds, but for the majority of them, it is consumed during embryogenesis or seed development. Only in certain cases (like sugarbeet), this tissue becomes the major storage tissue of the seed. In watermelon (*Cucumis melo*), the perisperm surrounds the embryo and forms an envelope that intervenes in the control of germination. This envelope is composed of two- to four-cell layers of perisperm and a single layer of endosperm. In the dry seed, the role of this envelope is to prevent the diffusion of solutes, but not water (Welbaum and Bradford 1990). Although in the case of the grain of coffee species the storage tissue was originally suggested to correspond to perisperm (Houk 1938), it is now identified as endosperm (Mendes 1941). Here, during development the grain is dominated by a well-developed maternal perisperm tissue up to approximately the halfway stage of maturation, following which the locular space is progressively filled with endosperm up to full grain maturity (Rogers et al. 1999). In contrast, among *Chenopodiaceae* and *Amaranthaceae*, the perisperm is located in a central position and is mainly dedicated to the accumulation of starch reserves (Fig. 2.1). This has been demonstrated in seeds of sugarbeet (Artschwager 1927), *Amaranthus hypochondriacus* (Coimbra and Salema 1994), *Chenopodium quinoa* (Prego et al. 1998), and some *Salicornioideae* (Shepherd et al. 2005), a subfamily of *Amaranthaceae*. In contrast to the well-documented cereal endosperm (Ritchie et al. 2000), the physiology of this perisperm is completely

unknown. In particular, it is presently unknown if the mechanisms of storage compound (starch, proteins) remobilization established for the cereal endosperm during germination can apply to the perisperm.

Starch can be degraded by hydrolysis in reactions catalyzed by the amylase family (Buchanan et al. 2000; Hills 2004; Machovic and Janecek 2006; Sun and Henson 1991; Yamasaki 2003). The α-amylases are endoenzymes that break α-(1,4) bonds at random within polysaccharide chains to produce shorter chains called limit dextrins, glucose, and maltose. The β-amylases are exohydrolases that hydrolyze α-(1,4) bonds in polysaccharides to remove successive units maltose (disaccharides of α-glucose) from nonreducing ends of α-1,4-linked poly- and oligoglucans until the first α-1,6-branching point along the substrate molecule is encountered. In germinating barley seeds, α-glucosidases can initiate the attack of starch granules and exert a marked synergistic effect on the initial stages of native starch granule degradation by α-amylases (Sun and Hanson 1990). Their roles are to degrade maltose and limit dextrin. All these three enzymes are particularly active during germination, and their expressions are induced by GAs secreted by the embryo (Bak-Jensen et al. 2007; Bethke et al. 1997; Buchanan et al. 2000; Fincher 1989; Gubler et al. 1987; Hills 2004; Jones and Jacobsen 1991; Laidman 1983; Sun and Gubler 2004; Zentella et al. 2002). In the presence of endogenous GAs, α-amylase is synthesized *de novo* in the aleurone layer surrounding the endosperm. In contrast, a precursor form of β-amylase is present in seeds in the dry state as a proenzyme devoid of enzyme activity, which is activated during germination after cleavage of a peptide sequence at the C-terminal part of the enzyme. Moreover, if the α-glucosidases are already partly accumulated in the dry seeds, they are for the most part newly synthesized in response to GAs in the aleurone layer and transported to the endosperm (Buchanan et al. 2000). Unlike for cereals, the mechanisms of regulation of starch degradation during germination are not known in species such as sugarbeet, which store starch reserves in a perisperm tissue. Also, the existence of an aleurone layer similar to that of cereals and *Arabidopsis* (Bethke et al. 1997; Finnie et al. 2011; Sun and Gubler 2004; Tasleem-Tahir et al. 2011) has not been described in these seed species, containing a large perisperm at maturity.

The α-glucosidases have previously been characterized in sugarbeet seeds (Chiba et al. 1978; Matsui et al. 1978; Yamasaki and Suzuki 1980). Also, the initiation of starch metabolism has already been documented during sugarbeet seed germination (Lawrence et al. 1990). This metabolism results in the transfer of sugars from the perisperm to the various tissues of the embryo upon imbibition and seedling growth (Lawrence et al. 1990). In particular, these authors reported a rise of α-glucosidase activity just before root extrusion. Although the work of Lawrence et al. (1990) firmly established an increase in certain enzyme activities (α-amylase, β-amylase, and α-glucosidase) in the sugarbeet seed perisperm during germination, the mechanisms responsible for such an induction were not addressed. At least two possibilities can be envisaged to account for the observed behavior. In the first one, the starch metabolizing enzymes would have been stored in an inactive precursor form in the perisperm during maturation and they became activated during the course of germination, for example owing to specific protein modifications occurring in the

precursor protein. In the second, as the mature perisperm is a dead tissue and hence presumably incapable of supporting protein synthesis, *de novo* synthesis of these enzymes could occur in the embryo upon seed imbibition followed by their transport in the perisperm. To address these questions, we presently studied the modifications occurring in the sugarbeet seed perisperm proteome during germination.

2.2 Experimental Strategies

2.2.1 Materials and Experimental Design

Fruits of a triploid monogerm sugarbeet seed lot (ref. 302–688C) were cleaned, polished, and calibrated after harvest according to commercial standards (KWS SAAT AG, Einbeck, Germany). They were previously used in proteomics (Catusse et al. 2008b, 2011) and transcriptomics (Pestsova et al. 2008) studies. Germination assays were carried out with triplicates of 400 seeds (true seeds plus pericarp) placed in covered plastic boxes (13 × 18 × 6 cm) on pleated filter paper wetted with 30 mL of distilled water and incubated at 10 °C in the dark. Germination started at about 60 h of imbibition and about 90 % of the imbibed seeds had germinated after 3 days imbibition.

Two protocols were used to characterize the role of perisperm during germination. In the first one, the whole seeds were incubated for 3 days as above, and the perisperm tissue was collected. In the second, the perisperm was first isolated from the dry mature seeds, and then imbibed for 3 days as above.

To isolate the perisperm from the dry mature seeds, the operculum, which is the ovary cap of the fruit at the upper part of the pericarp was removed from the fruit by prying it off with high precision reverse action tweezers and a scalpel, starting just in front of the position where is located the radicle (Hermann et al. 2007). Then, the botanical true seed appears as being layered in the remaining pericarp and it can be carefully removed from the pericarp. Tissues were then dissected using a scalpel under a binocular microscope. In particular, by the gentle action of the scalpel, the perisperm can be detached from the embryo, without contamination from adjacent tissues. The dissection of 100 seeds gave about 100 mg perisperm tissue. The isolation of the perisperm tissue from 3 days imbibed seeds was much easier, since at this imbibition time the tissue was fully hydrated and presented a viscous aspect.

2.2.2 Preparation of Protein Extracts, 2-DGE, Protein Staining, and Gel Analyses

Protein extracts were prepared from 100 seeds or perisperm tissue using a low ionic-strength buffer containing 50 mM HEPES (pH 8.0) and 1 mM EDTA (pH 9.0), yielding the albumin fraction as described (Catusse et al. 2008b). The 2-DGE was

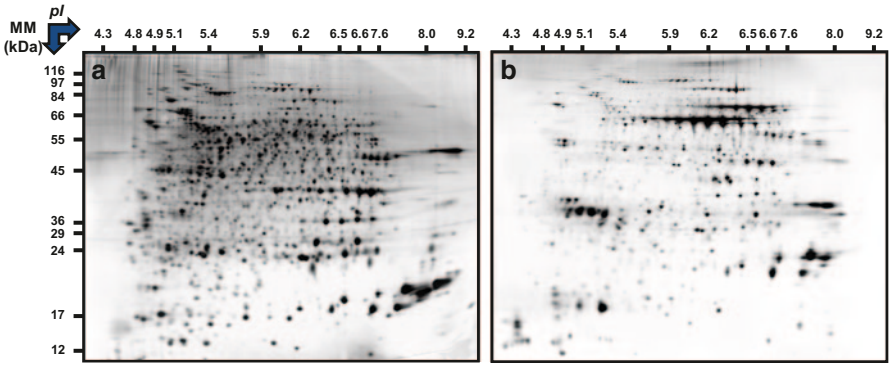

Fig. 2.2 Proteome of the dry mature sugar beet seed. **a** 2-DGE analysis of total soluble proteins (100 µg) from whole dry mature seeds (759 proteins identified; Catusse et al. 2008b). **b** 2-DGE analysis of total soluble perisperm proteins (100 µg) isolated from dry mature sugarbeet seeds (172 proteins identified; Catusse et al. 2008b)

carried out using an amount of 100 µg protein as described (Catusse et al. 2008b). Three gels loaded with biological replicates were run for each treatment. Following protein staining with silver nitrate, image analysis of the scanned 2-D gels was carried out using the ImageMaster 2-D Elite version 4.01 software (Amersham Biosciences; Catusse et al. 2008b).

2.3 Results and Discussion

Protein extracts from perisperm tissues of mature dry and imbibed seeds were prepared as described above and separated by 2-DGE (Fig. 2.2). Among the 182 proteins identified in the perisperm from dry mature seeds (Fig. 2.2b), 120 proteins are specifically expressed in this tissue, testifying of the purity of this tissue preparation (Catusse et al. 2008b).

We then characterized changes in protein accumulation levels occurring in the perisperm during imbibition using the two protocols described under EXPERIMENTAL STRATEGIES. A number of spots displayed varying accumulation patterns, as depicted in Fig. 2.3, in which either the accumulation pattern was similar or different when comparing the isolated imbibed perisperm or the perisperm isolated from whole imbibed seeds. Here, we concentrate on the spots related to starch metabolism as this metabolism has been very well detailed in the endosperm of cereal seeds during germination (Buchanan et al. 2000).

Highly abundant proteins (window b of Fig. 2.3) corresponding to soluble starch synthase dominated the proteome of the perisperm isolated from the dry mature sugarbeet seeds. As this enzyme catalyzes starch synthesis during seed maturation, it is therefore not surprising to observe its disappearance in the perisperm isolated

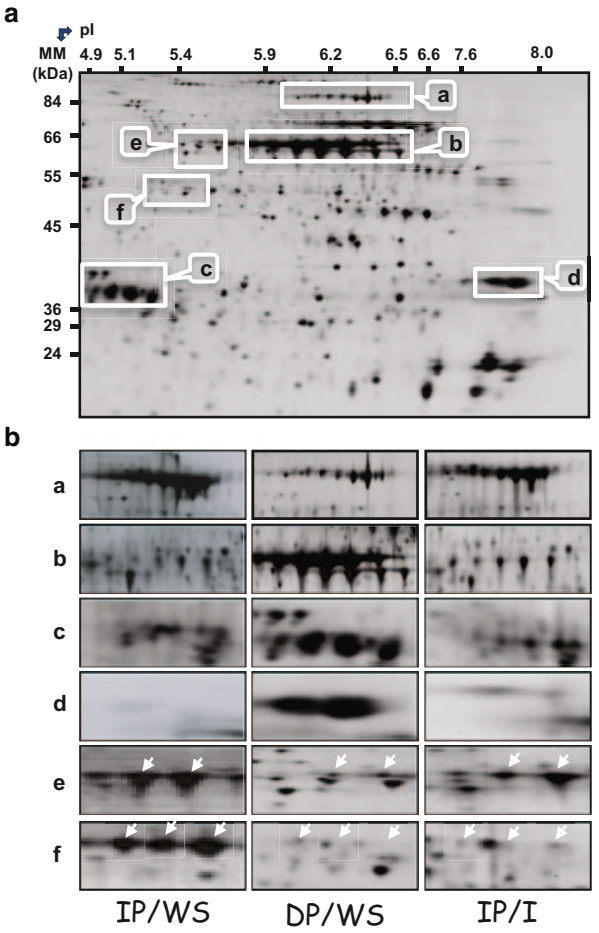

Fig. 2.3 Accumulation patterns of specific proteins in the sugarbeet perisperm during germination corresponding to 3 days imbibition on water. The perisperm was isolated from whole mature dry or imbibed seeds. For comparison the perisperm was also isolated from whole dry mature seeds then imbibed for 3 days on water. 2-DGE analysis of total soluble perisperm proteins (100 µg) was carried out as described under EXPERIMENTAL STRATEGIES. **a** Total soluble perisperm proteins isolated from dry mature sugarbeet seeds. The indicated portions of the gel (a, b, c, d, e and f) are reproduced in **b**. **b** Enlarged windows (a–f) of 2-D gels as shown in **a** for the perisperm isolated from imbibed whole seeds (left; IP/WS), the perisperm isolated from whole dry mature seeds (middle; DP/WS), or from the perisperm isolated from whole dry mature seeds then incubated for 3 days on water (right; IP/I). Window a, the train of spots corresponds to α-glucosidase; Window b, the train of spots corresponds to soluble starch synthase; Windows c and d; the major spots correspond to granule-bound starch synthase; Window e, the white arrows correspond to β-amylase; Window f, the white arrows correspond to α-amylase. Proteins were previously identified by mass spectrometry (Catusse et al. 2008b)

from the imbibed whole seeds (window b of Fig. 2.3). The same behavior was observed with the imbibed isolated perisperm only (window b of Fig. 2.3). In addition to the soluble starch synthases, we also found granule-bound starch synthases in several spots (window c of Fig. 2.3). All of them showed the same accumulation behavior than seen for soluble starch synthase in imbibed perisperm isolated from whole seeds and imbibed isolated perisperm. From these data, we conclude that the perisperm is autonomous for the specific degradation of such proteins, meaning that this degradation does not require the synergic action of the embryo. This finding is in agreement with our previous work documenting the existence of several proteinases and proteasome subunits in the mature dry perisperm (Catusse et al. 2008b).

In the perisperm isolated from imbibed whole seeds, we observed a strong accumulation of α-amylase containing spots (white arrows within window f of Fig. 2.3b). However, such an increased protein accumulation was not seen with the imbibed isolated perisperm (window f of Fig. 2.3).

Two spots containing β-amylases were detected in very low amounts in the perisperm isolated from the dry mature sugarbeet seeds (white arrows within window e of Fig. 2.3b). Their accumulation increased substantially both in the perisperm isolated from imbibed whole seeds and in the imbibed isolated perisperm (window e of Fig. 2.3).

The α-glucosidases were present in the perisperm of the mature dry seeds and their protein abundances increased both in the perisperm isolated from the imbibed whole seeds and the imbibed isolated perisperm (window a of Fig. 2.3).

As mentioned above, the enzymes involved in starch degradation could either be stored in the perisperm in the form of zymogens, or be newly synthesized in the embryo and then transported. In our study, it appears that the first assumption might not apply. Indeed, for α-amylases, β-amylases, and α-glucosidases, we did not observe on the 2-D gels the transition described for the β-amylase of barley endosperm, that is the transition from one form of the enzyme of high molecular weight in the proteome of the perisperm isolated from the dry seeds to a form of lower molecular weight in the proteome of imbibed perisperm. Moreover, *de novo* synthesis of the enzymes in the perisperm tissue is quite unlikely since it is a dead tissue (Artschwager 1927). The remaining possibility to explain the increase in volume of the spots of amylases and glucosidases in this tissue is *de novo* synthesis of these proteins in the embryo. This mechanism would be analogous to that established for starch mobilization in the endosperm of monocots.

2.4 Concluding Remarks

This study presents for the first time data on the accumulation of enzymes of starch mobilization (e.g., α-amylase, β-amylase, and α-glucosidase) in the perisperm of imbibed sugarbeet seeds. We noted important differences between the accumulation patterns of α-amylase in the perisperm isolated from imbibed whole seeds and the isolated imbibed perisperm alone. This observation suggests a positive role of the

embryo in triggering the accumulation of this starch-mobilizing enzyme (s) in the perisperm during germination. In this way, the mechanisms occurring in the sugarbeet seed perisperm during germination would appear to be similar to those documented for the endosperm of cereals (Bethke et al. 1997; Finnie et al. 2011; Sun and Gubler 2004). However, an accumulation of β-amylase and α-glucosidase was also observed in the isolated imbibed perisperm alone, suggesting that the existence of embryo was not mandatory for induction of these enzymes in the perisperm during imbibition. It is also noted that the catabolic processes leading to the disappearance of the highly-abundant soluble and granule-bound starch synthase can occur to the same extent in the perisperm isolated from imbibed whole seeds and the isolated imbibed perisperm alone, indicative of some autonomy of the perisperm concerning proteome changes during imbibition. Pending questions concern the role of GAs in the observed modifications in accumulation levels of the starch degradation enzymes and the existence of an equivalent of the cereal aleurone layer in sugarbeet seeds. Further work will allow addressing these questions.

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