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Medicago marina (L.) seed: unravelling mechanisms controlling germination and dormancy

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Contents

1. Development and structure of seed

Seeds are the typical propagation units of the flowering plants. They represent the delivery system for the transfer of genetic materials from one generation to the next, trough the sexual reproduction in vascular plant. The seed-producing organisms of the plant kingdom belong to the division *Spermatophyta*, further classified into 2 sub-divisions: *Gymnospermae* (gymnosperms) and *Angiospermae* (angiosperms). The angiosperms are divided into 2 classes: *Monocotyledoneae* and *Dicotyledoneae* (Cantino et al., 2007, Linkies et al., 2010).

In angiosperm and gymnosperms seeds develop from ovules, which can be defined as unfertilized, immature seed precursors (Gasser et al, 1998, Finch-Savage & Leubner-Metzger, 2006; Frohlich & Chase, 2007).

During its development, the ovule differentiates into one or two covering layers that envelop the nucellus. In the gymnosperms, the nucellus is enveloped by one covering layer, while in the angiosperms, it is enveloped by two covering layers, called integuments (Linkies et al, 2010). Then, during the seed growth, the external covering layer develop into the testa (seed coat), which, in mature seeds is usually constituted by a layer of dead cells, while the inner integument, that develop into the tegmen, is formed by alive cells (Debeaujon et al., 2000; Windsor et al., 2000; Haughna & Chaudhuryb, 2005).

Within the nucellus, a megaspore develops into a haploid megagametophyte (female gametophyte), that in most gymnosperm species is multicellular, and usually several archegonia develop within the megagametophyte, forming only one egg in each archegonium (Floyd & Friedman, 2000; Baroux et al., 2002). On the contrary, the angiosperm megagametophyte, that in its mature state is also called embryo sac, is seven-celled and eight-nucleate (Polygonum-type) (Floyd & Friedman, 2000; Baroux et al., 2002; Friedman & Williams, 2004; Berger et al., 2008; Friedman & Ryderson, 2009). Occurrence, the mature megagametophyte is four-celled and four-nucleate, also called Nuphar/Schisandra-type, because can be found in the basal angiosperms, namely Nymphaeales and Austrobaileyales (Floyd & Friedman, 2000; Baroux et al., 2002; Friedman & Williams, 2004; Berger et al., 2008; Friedman & Ryderson, 2009).

After pollination, in all angiosperms and most gymnosperms a pollen tube is formed through which the sperm can reaches and fertilizes the egg cell, leading to development of the diploid embryo. The micropylar synergid cells help attract the pollen tube to the female gametophyte.

The egg cell, that is the progenitor of the embryo, lies adjacent to the synergid cells, while the large central cell, the progenitor of the endosperm, contains two polar nuclei, that fuse together forming a diploid central cell nucleus, at the time of fertilization (Higashiyama et al., 2001).

Angiosperms seed development begins upon double fertilization (Dumas & Rogowsky, 2008). In contrast of gymnosperm, the hallmark of angiosperm reproduction is the double fertilization mechanism, in which two haploid sperm nuclei are transported trough the pollen tube. Indeed, the double fertilization is characterized by two fertilization events. The first event involves the egg fertilization and occurs when one haploid sperm is transported to the ovule through the pollen tube, leading to the development of diploid embryo. The second fertilization event occurs when an other haploid sperm nucleus fuses with the central cell, which develops into the endosperm (Floyd & Friedman, 2000; Baroux et al., 2002; Friedman & Williams, 2004; Berger et al., 2008; Dumas & Rogowsky, 2008, Friedman et al., 2008; Friedman & Ryderson, 2009). The resulting embryo and endosperm are genetically identical except for ploidy level, as the embryo is diploid and the endosperm is triploid: since the central cell of most angiosperm species has either one (Nuphar/Schisandra-type) or two nuclei (Polygonum-type), the fertilized endosperm is either diploid or triploid (Gehring et al., 2004).

From this point onwards, seed development is characterized by the rapid development and growth of the endosperm and the embryo, until seed maturation.

Embryonic development proceeds within the protective maternal tissue of the ovule (which becomes the seed coat surrounding the developing embryo and endosperm) and, in higher plants, the embryogenesis can be divided conceptually into three distinct phases, partially overlapping (West & Harada, 1993; Dumas & Rogowsky, 2008). The first phase is characterized by morphogenesis events, during which the polar axis of the plant body is defined with the specification of the shoot and root apices, and the embryonic tissue and organ systems are formed. Generally, in many angiosperms, the initial division of the zygote is transverse and asymmetric and generates a small, chalazally oriented apical cell and a large basal cell (Mansfield & Briarty, 1990). From the apical cell originates the bulk of the embryo, including the cotyledons, shoot apex, and hypocotyls. First, the apical cell of embryo undergoes two longitudinal divisions to produce a four-celled embryo (Mansfield & Briarty, 1990, West & Harada, 1993), and then, a transverse division follows to produce two layers of cells in an octant stage embryo. The next division of the octant stage embryo is periclinal or parallel to the embryo surface and leads to the formation of first histologically detectable tissue, the protoderm, which is the precursor of the epidermis. The globular stage embryo is established following the delineation of the protoderm, and during this phase, the embryo increases in size and cell number

by anticlinal cell divisions of the protoderm and by longitudinal and, later, transverse divisions of interior cells (Mansfield & Briarty, 1990, West & Harada, 1993).

A part of the root apex and the suspensor originate from the basal cell, which again, undergoes a series of transverse divisions, resulting in the formation of the hypophysis and the suspensor (West & Harada, 1993). In many plants, the hypophysis, the uppermost derivative of the basal cell, serves as the precursor to the root cortex initials and the central region of the root cap (Mansfield & Briarty, 1990), while the suspensor is an ephemeral embryonic structure, formed by a various number of cell layers depending of plant species (Marsden & Meinke, 1985; Mansfield & Briarty, 1990). During embryogenesis, this structure functions as conduit for nutrients from the endosperm and for growth factors from the maternal tissue to the embryo (Yeung & Meinke, 1993).

During the transition from the globular to the heart stage, embryo undergoes a significant transformation of its morphology: trough the cell divisions that occur at specific regions of the lateral margins of the globular stage embryo, the two lobes of the cotyledons emergence. Then, the shift in embryo symmetry from radial, at the globular stage, to bilateral, at the heart stage, represents the initial delineation of the cotyledons and axis. Following their formation, the cotyledons and axis elongate rapidly as a result of cell division and cell expansion, and at this stage of embryogenesis are also visible other tissues and structures such as the ground meristem and the procambium, which is the precursor of the vascular tissue (Mansfield & Briarty, 1990). Thus, the cells that will form the root apex and the shoot apex can be distinguished from this stage of embryogenesis. After the heart stage, morphogenesis of embryos is interrupted and the second phase of embryogenesis takes place.

The second phase is characterized by a period of embryo maturation during which initiates the storage accumulation of reserve macromolecules: proteins, lipids and carbohydrates. These reserves are particularly prevalent in the embryonic cotyledons of plants that do not store substantial reserves in their endosperm, otherwise these reserves accumulates in developing endosperm and they are responsible for a rapid increase in embryo mass and size (Mansfield & Briarty, 1992). Therefore, during this second phase, also the endosperm developments and it grows much more rapidly than the embryo, initially through nuclear divisions and then by cellularization of each nucleus. In detail, during nuclear division, endosperm nuclei continue to divide without cytokinesis to create a syncytium of nuclei (Brown et al., 1999; Dumas & Rogowsky, 2008). Each nucleus is surrounded by dense cytoplasm and organelles that compose nuclear cytoplasmic domains (Brown et al., 1999). Then, after cellularization of each nucleus, three distinct domains are formed: the micropylar endosperm, the peripheral endosperm and the

chalazal endosperm (Boisnard-Lorig et al., 2001, Baroux et al., 2002; Olsen, 2004; Dumas & Rogowsky, 2008; Friedman & Ryderson, 2009).

The growth of the seed is coupled with the growth of the endosperm that constitutes the major contribution to the volume of the mature seed (Sundaresan, 2005).

However, when seed maturation is completed, the relative abundance of the endosperm can differ considerably. In some *Brassicaceae* species and many other dicots (like as many legume seeds), the endosperm is finally consumed, being replaced by the growing embryo, which then constitutes most of the mature seed (Forbis et al., 2002; Sundaresan, 2005, Finch-Savage and Leubner-Metzger, 2006). In other species, belonging for examples to *Solanaceae* family, the endosperm is not lost and constitutes a large portion of the mature seed. In some other cases the endosperm is just partially lost and is present as a thin layer, often formed by a single cell layer, as happens for *Lactuca sativa* or *Arabidopsis thaliana* seeds (Pritchard et al., 2002; Liu et al., 2005) and *Lepidium sativum* seeds (Müller et al., 2006). If, after seed maturation, the endosperm persists as the primary food storage tissue, seeds are described as endospermic; on the contrary, if the cotyledons become the site of food storage because the endosperm is consumed during embryo development and is absent, or exists only as a very thin layer of tissue in mature seeds, these species are designated as non-endospermic (Lopes & Larkins, 1993). When the second phase is completed, the embryo is differentiated and exhibits developmental polarity that is divided into the radicle (embryonic root) and the shoot with the cotyledons (only one in *Monocotyledoneae*, and two in *Dicotyledoneae*), that represent the embryonic leaves (Linkies et al., 2010).

The third stages of embryogenesis are concerned primarily with preparing the embryo for developmental arrest and germination (Kermode, 1990; Galau et al., 1991; Thomas, 1993). Indeed, during this final stage of embryogenesis, embryo acquires the desiccation tolerance and eventually enters a period of metabolic quiescence (Kermode, 1990). The mature embryo, if not affected by dormancy, remains quiescent until it encounters conditions appropriate for germination.

Fertilization also initiates changes in maternal tissues. The ovary develops into a fruit and the ovule integuments differentiate to form the protective seed coats, including testa and tegmen (Moïse et al., 2005). Before fertilization, cells of the integuments are relatively undifferentiated, then, after fertilization, during seed coats development, the undifferentiated cell layers can differentiate into specialized cell types. However, some of these cell layers not undergo any significant differentiation remaining parenchymatous, and often, are crushed upon the completion of seed maturity; other cells undergo a slight thickening of the cell wall, becoming collenchymatous. In addition, some cell layers can undergo an extensive secondary thickening of

various parts of the cell walls, becoming sclerotic: if these cells also elongates in the radial plane, these layers are called palisade layers (Moïse et al., 2005).

Although the seed coats of different species vary greatly in structure and composition, they undergo similar phases of development in relation to the embryo and endosperm. For example, in legume seed development the seed coat and endosperm develop first, followed by the development of the embryo, maturation of the seed coat, and maturation of the embryo (Weber et al., 2005). The coordination of these events is governed by communication among the tissues of the seed organs, such as communication between the seed coat and endosperm reported in *Arabidopsis* seeds (Weijers et al., 2003).

Seed coats accomplish multiple tasks in the mature seed. They can contribute to overall seed morphology thanks to the accumulation of large quantities of mucilage or pigments into some cell layers. During the early embryo development and differentiation that is controlled by the maternal tissues, seed coats are able to transmit growth signals to the embryo. For example, the cell wall invertases of legumes seed coats play a central role in the maternal control of seed development, facilitating the sucrose assimilation and accumulation by increasing the sucrose gradient (Weber et al., 1995). The increasing of sucrose concentration into seed coats, and its further transport to the embryo, promotes embryo growth by enhancing cell division (Borisjuk et al., 1998). Seed coats separate one generation of plants from the next and ensure the survival of the offspring, giving the resistance to biotic and abiotic stress (Moïse et al., 2005). Again, strong impermeable seed coats protect the embryos during quiescence or dormancy and maintain an environment around the embryo that is conducive for these conditions (Bewley, 1997). By governing seed dormancy and germination the seed coat plays an important role in determining the optimal environmental conditions for the viability and growth of the next plant generation, and during germination, they provide components that contribute to biotic and abiotic stress resistance (Bewley, 1997). Also, in a recent work by Weber et al., (2005), has been shown that the seed coat can contribute to the direct nutrient supply to the embryo during seed development. For example, some of their specialized cell types, called transfer cells, facilitate the transfer of nutrients within the seed (Wobus & Weber, 1999; Thompson et al., 2001; Weber et al., 2005). Moreover, seeds coat are able to integrate a variety of other signaling pathways that involve phytohormones (Bewley, 1997), hypoxia (Rolletschek et al., 2002) and carbon dioxide recycling (Furbank et al., 2004), that finally, converge in seed development (Gibson, 2004).

Thus, seeds consist of three genetically distinct components: seed coats, whit protective function for the embryo, embryo and the endosperm, that stores starch, lipids and proteins and acts as a source of nourishment during germination and early seedling development (Lopes & Larkins, 1993).

While gymnosperms seeds are naked because they are not enclosed by an ovary, and usually the embryo is enveloped by two covering layers, a typical angiosperm seed is covered, as it is enclosed inside the ovary. A mature ovary, called fruit, contains one or more mature seeds and both seeds and fruits can be the dispersal units of angiosperms (Linkies et al., 2010).

1.2 Legumes seed development and features

Legumes represent the third largest and most diverse family (*Leguminosae* or *Fabaceae*) of flowering plants, with approximately 20,000 species classified (Doyle & Luckow, 2003). Traditionally, the legume family comprises three subfamilies (*Caesalpinioideae*, *Mimosoideae* and *Papilionoideae*) and the largest, *Papilionoideae*, contains most of the model species, in which different aspects of plant biology, including seed and embryo development, have been studied (Goldberg et al., 1989; Johnson et al., 1994; Coste et al., 2001; Weterings et al., 2001; VandenBosch & Stacey, 2003; Gepts et al., 2005; Gonzales et al., 2005, Weber et al., 2005; Zhu et al., 2005). The most common legumes used as models are peanut (*Arachis hypogaea*), *Lotus* (*Lotus japonicus*), *Medicago* (*Medicago truncatula*), soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and broad bean (*Vicia faba*). Many legumes, such as soybean and peanut, are food crops of major economic importance for human nutrition and animal feed, because of their high nutritional value (Duranti & Gius, 1997; Graham & Vance, 2003).

One of the most advantages of legumes is that they produce a large range of seed sizes. For example, some legume seeds are giants and are excellent models for developmental studies because the manipulation of seeds and embryos at early stages of development resulting facilitated thanks to legume large size (Weterings et al., 2001). A second important advantage of legumes is that their embryos show a wide range of morphological forms. The variety in size and shape of legume seeds and embryos makes them excellent models for comparative morphological studies (Le et al., 2007).

Seed development in legume is highly related to nutrient metabolism and its transport and the phases of seed development are well established in many legume species (Weber et al., 2005), for example in *Phaseolus vulgaris* (Coelho & Benedito, 2008), *Lotus japonicus* (Dam et al, 2009); *Medicago truncatula* (Gallardo et al; 2003). Cross talk among various pathways must play a major role in the control of seed development and provide mechanisms for communication among the various seed organs, which undergo coordinated development (Olszewski et al., 2002; Gibson, 2004; Weber et al., 2005).

A typical mature legume seed consist mainly of the embryo. Seed development proceeds through three distinct phases: histodifferentiation (embryogenesis), seed filling, and desiccation (Weber et al., 2005).

Initially, legume seed development is characterized by a relative slow mass accumulation during histodifferentiation; the maturation phase is followed by a continuous and fast increase of dry matter, until reaching the maximum content of dry matter at physiological maturity. Seed dehydration is characteristic of the third phase, together with biological mechanisms that leads to embryo desiccation resistance and germination viability. Sugars and nitrogen are signals regulating seed development (Wobus & Weber, 1999) and metabolic exchange are intermediated by seed coats (Borisjuk et al., 2003). Also, many hormones play fundamental roles during seed development (Koornneef et al., 2002; Ali-Rachedi et al., 2004).

During the first phase, growth of the embryo is characterized by cell division and differentiation that will generate the embryo tissue and the endosperm. Thus, histodifferentiation can be divided into two phases, where cell division is confined to endosperm and seed coat in the first phase and to the embryo in the second phase (Wang & Hedley, 1993). The endosperm first develops into a syncytium and then cellularizes, while, cell division of the zygote (fertilized egg cell) initiates when a small portion of the endosperm is already formed and also, the polarity lines are established in the embryo sac, in preparation for future division and growth (Wang & Hedley, 1993; Coelho & Benedito, 2008). The embryo, which develops in the interior of the ovule from the zygote, initially assumes a claviform or cylindrical shape, then, its distal portion becomes the active site of cellular division, increasing in volume, assuming a spherical shape. At this level it is possible distinguishes the body of embryo and the suspensor. In the following events, the embryo changes its symmetry: from the spherical shape with radial symmetry it develops acquiring bilateral symmetry. At this level, the suspensor is hereby degenerated by programmed cell death events (Lombardi et al., 2007). The cotyledons also are defined at this level, initially like small protrusions, which, after a series of cell divisions and expansion, acquire their characteristic aspect similar to leaves, while the terminal portion of the axis below the cotyledons differentiate into root meristem, forming the hypocotyls-radicle axis (Wang & Hedley, 1993; Coelho & Benedito, 2008). At the advanced developmental stage of the embryo, all structures that formed it can be easily identified: the apical meristem, both cotyledons (in the case of dicots, or just one in the opposite case of monocots), the hypocotyls located below the cotyledons, the radical meristems and the embryonic root

Whereas the embryo and the endosperm are developing, also the teguments go through visible modification, especially increasing in thickness, and the funiculus usually suffer abscission, leaving a scar that represents the hilum (Wang & Hedley, 1993; Coelho & Benedito, 2008).

Schematic picture of embryo morphogenesis and seed development events of soybean, used as a common model legume, is represented in figure 1.

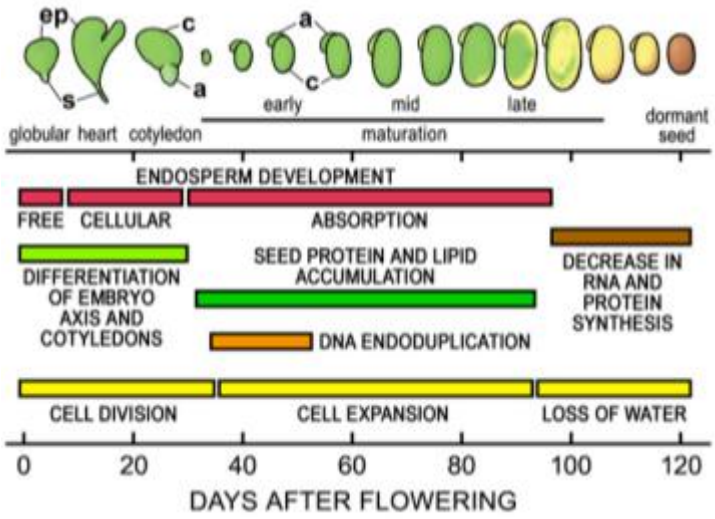

Figure 1 Schematic representation of soybean seed development. Embryo morphologies and developmental events; a: axis; c: cotyledon; ep: embryo proper; s: suspensor (Brandon et al., 2007).

The mitotic activity of the seed in this first phase is controlled by hormones, environmental factors, and carbon and nitrogen supply (Egli et al., 1989; Munier-Jolain & Ney, 1998; Ozga et al., 2002). In the recent literature it has been demonstrated that cytokinins, auxin and gibberellins are involved in early embryogenesis. Cytokinins are present in the beginning of seed development, regulating growth trough cell division and sugar metabolism, increasing at rapid speed after fecundation and diminishing as the seed develops. As extensively studied during pea seed development (Quesnelle & Emery, 2007), cytokinins reach a peak of concentration during the heart-shape stage of embryo developmement. Gibberellins are associated whit cell expansion and, in association whit the auxins, whit driving reserve synthesis. The expression of genes involved in the last step of active gibberellins synthesis (GA-oxidases) was localized in different tissues and a at different stages of embryogenesis during seed development of *Phaseolus coccineus*: between the late globular and heart stages, in the embryo epidermal cells, in the endosperm during the transition from globular to heart stage, and during cotyledons and inner tegument development (Solfanelli et al., 2005). Auxins are synthesized from the aminoacid tryptophan, and in seeds it is present since the initial phases of development and it's responsible of compound assimilation from the mother plant. It has been shown that the auxins are important in controlling seed development of common bean: in fact, a functional enzyme involved in

auxins conjugation, during the auxins homeostatis control, was defected during the rapid growth period of seed development in common beans (Walz et al., 2002; Walz et al., 2008).

During the seed-filling phase (second phase of development), the endosperm contents are hydrolyzed and transported to the embryo. Seed nitrogen accumulation and protein composition depend on both symbiotic N_2 fixation and nitrogen from the soil, while carbon storage derives mainly from recent photosynthate rather than from remobilized sources (Domoney et al., 2006). Proteins involved in cell division that were abundant during early stages of seed development, undergoes a decreases of their level, before the accumulation of the major storage proteins during seed filling (Gallardo et al., 2003).

Thus, during seed-filling the embryo stops growing, mitosis is less intense, teguments tissue differentiate, storage compound starts to accumulate, and seed develops tolerance to desiccation (third phase of development) (Gutierrez et al., 2007).

In general, at the end of the third phase of development, the maximum accumulation of dry matter in the seed is observed, as well as the rupture of trophic connection with the mother plant, representing the physiological maturity. At this level, although the metabolism decreases drastically, the embryo remains viable thanks to the high concentration of abscissic acid that guaranties dry matter flow and enzyme activity acting on anabolic process (Pammenter & Berjak, 1999). This metabolic profiling indicates that the preparation for germination starts during seed desiccation (Fait et al., 2006). At this stage gibberellins and brassinosteroid synthesis are locally active and exert important functions during maturation (Radchuck et al., 2006), while the high accumulation of abscissic acid is essential for embryo tolerance to desiccation (Buitink et al., 2006).

The accumulation of reserve compounds in the seeds serves to feed the embryo during development and guaranties the completion of germination and the early seedling development. Among the main seed reserve compounds are carbohydrates, proteins, lipids and phytic acid (phytin). The main reserve substances derives from carbon fixation by leaf mesophyll cells into sucrose that is transported via phloem from the mother plant to the seed cells and later incorporated into the seed trough seed coats (Weber et al., 1997; Patrick & Offler, 2001). Then, during the maturation phase, when all trophic connection with the mother plant is interrupted, the sucrose arrive to the seed trough a ventral vascular system localized along the pod, in a region delimited around the hilum, which forms a vascular net around the seed teguments (Borisjuk et al., 2003). Besides the sucrose, also other simple molecules are transported to the developing seed such as aminoacids, especially asparagine and glutamine, and mineral ions (Golombek et al., 2001). Seed maturation genes are controlled by specific regulatory transcription factor that in legumes may be regulated by sugar (Tsukagoshi et al., 2007) and by abscissic acid (ABA).

In particular, as shown for common bean (*Phaseolus vulgaris* L.) a seed specific transcriptor factor (PvALF,) is induced by ABA and it is involved in chromatin remodeling and activation of protein storage genes, such as phaseolin, during the seed filling phase of development (Bobb et al., 1995, 1997). Thus, the metabolic profiling of seed during maturation shows reduction of sugar, organic acid and aminoacids towards incorporation into storage compounds when compared with the embryogenesis phase of development (Fait et al., 2006; Udvardi et al., 2007).

During the desiccation phase (third phase of development), specific proteins and carbohydrate are synthesized, such as LEA (Late Embryogenesis Abundant) proteins and sucrose. LEA proteins are associated with the embryo capability to withstanding dehydration; structurally they are characterized by a hydrophilic aminoacids composition, highly soluble in water and resistant to high heat, and functionally they act to protect the embryo from dehydration and others environmental stresses, such as high salts concentration and heat (Wang et al., 2003b). Also the sucrose where found associated with stress tolerance, thanks to its capability to confer a low cellular osmotic potential to the mature seed, increasing seed longevity (Gurusinghe & Bradford, 2001; Pinheiro et al., 2005).

When the legume seed maturation is completed, the large majority of the storage proteins are globulins. There are two main groups of globulins that evolved from a common single domain ancestor and have sedimentation coefficients of 7S and 11S (Shutov et al., 1995). In pea, 7S and 11S globulins are called vicilins and legumins, respectively, and are often referred to as vicilin - and legumin like globulins in other legumes (Dam et al, 2009). Most of the stored nitrogen and sulfur and some of the carbon is found in the protein fraction, mainly in the storage proteins. These proteins accumulate in protein bodies in the cotyledonary parenchyma cells until they undergo hydrolysis upon germination (Tabe et al., 2002). Carbon is also stored in lipids and starch, and generally, the levels of protein, lipids, and starch differ considerably between legume species. For example, soybean (*Glycine max*) and peanut (*Arachis hypogaea*) are oil crops, because of their high content of lipids, whereas common beans (*Phaseolus vulgaris*), lentils *(Lens culinaris*), peas (*Pisum sativum*), as well as chickpeas (*Cicer arietinum*) are mostly composed of proteins (Domoney et al., 2006).

Others important structures of legume seed are the seed coats that take part in many physiological process, both during the seed development/maturation and germination. Their function is essential for the physiological and biochemical processes that govern embryo nutrition and composition (Rochat & Boutin, 1991; Zeng et al., 2004; Wang & Grusak, 2005) or for the protection of the embryo against abiotic stress and diseases (Ndakidemi & Dakora, 2003); the control of the aqueous and gaseous environment around the embryo also depends on the presence of the seed coats (Gijzen et al., 1999b; Souza & Marcos-Filho, 2001).

The seed coats of legumes are relatively large and complex and generally, the mature seed coat is structurally characterized by an outer integument which consists of a single layer of palisade cells (macrosclereids) and hourglass cell (osteosclereids), and an inner integument consisting of parenchymatous cells (Miller et al., 1999) (Fig. 2).

The outermost layer of the legume seed coat is the cuticle, which is variable in thickness and represents the first barrier to imbibitions. As suggested for soybean seeds (Ragus, 1987; Souza & Marcos-Filho, 2001), the cuticle is formed by two layers of waxy deposits, one very stable and the other environmentally labile. The epidermis (macrosclereids) is located below the cuticle and originates from the outer cell layer of the outer integument (Zeng et al., 2004). It is composed of a layer of thick-walled cells, called palisade cell. In general, only one palisade layer is present, except under the hilum, where two layers can be present (Souza and Marcos-Filho, 2001). It has been proposed that the cell walls of the palisade layer contribute to the mechanical strength of the seed coat (Algan & Büyükkartal, 2000).

Below the epidermis is located the hypodermis (osteosclereids), which is formed from a single layer of cells. Because of their shape, the cells that formed the osteosclereids are called hourglass cell. They arise from the outer-cell layer of the inner integument (Zeng et al., 2004) and are usually larger than adjacent cell layers and are separated by wide intercellular spaces (Souza and Marcos-Filho, 2001). Often, hourglass cells contain numerous starch grains during embryogenesis: this indicates that the seed coat could synthesize nutrients, for the developing embryo. Besides that, the hourglass cells can serve as a reserve of proteins, for the developing embryo, as shown for soybean seed coat (Gillikin & Graham, 1991; Gijzen et al., 1993). In addition, like palisade cells, hourglass cell walls, play a role in the mechanical strength of the seed coat (Algan & Büyükkartal, 2000; Wang & Grusak, 2005).

Adjacent to the hourglass cells is the interior parenchyma, formed by 6-8 layers of thin-walled, tangentially elongated parenchyma cells (Moïse et al., 2005), uniformly distributed throughout the seed coat. It has been proposed, that an important function of the parenchyma cells layers is to deliver nutrients to the embryo, thanks to the presence of a vascular systems, which are responsible for transporting the nutrients from the maternal organs to the embryo (Algan & Büyükkartal, 2000; van Dongen et al., 2003). Then, in mature seed coats, the interior parenchyma is often crushed or partially crushed (Miller et al., 1999) as the embryo expands.

The endosperm cell layers close to the embryo degenerate and eventually appears as compressed wall materials at seed maturity. However, the outermost endosperm layer remains intact and differentiates into the aleurone layer (Miller et al., 1999), in which, during germination, occurs the enzymatic mobilization of seed reserves, such as carbohydrates (Ma et al., 2004b).

Miller et al., (1999), showed that a mature soybean seed contains endosperm remnants inside the aleurone and, by convention, it is considered to be part of the seed coat and a nourishment source for the embryo.

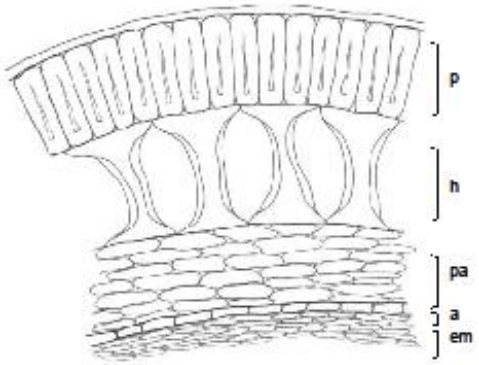

Figure 2 Schematic diagrams illustrating the general organization of the legume mature seed coat; p: palisade layer; h: hourglass cells; pa: partially crushed parenchyma; a: aleurone; em: crushed endosperm. The palisade layer and the hourglass cells comprise the outer integument while the inner integument consists of parenchymatous cells (Moïse et al., 2005).

Besides that, the seed coat structure (Wang & Grusak, 2005) and the seed development (Djemel et al., 2005) of the model legume *Medicago truncatula* have been characterized. *Medicago truncatula* seed coat are composed of an outer tegument constituted by an epidermal layer of macrosclereids, (epidermis), a subepidermal layer of osteosclereids (hypodermis) and two to five row of internal parenchyma cells. The parenchymal layer is thinnest at the end of the seed coat opposite the hilum (Wang & Grusak, 2005). These coat features, that are similar to soybean seed coats, are founded also in other *Medicago* species (Small & Brookes, 1990; Jha & Pal, 1992). However, *Medicago truncatula* seeds coat are relatively thin, in comparison to other legume such as *Phaseolus vulgaris* and *Pisum sativum*, whose seed coats posses more that twelve cell layers at full maturity (Offler & Patrick, 1984; van Dongen et al., 2003; Wang & Grusak, 2005). Embryo protection in *Medicago truncatula* is provided also by the seed coats, which confer the hard-seededness feature that as studied also for several *Medicago* species, is able to delay the germination (Crawford et al., 1989). Djemel et al. (2005) showed that the seed development of *Medicago truncatula* is very similar to that of other legumes, except for the presence, in the mature seed, of an endosperm layer that is absent in grain legumes. During early embryogenesis and until mid-maturation, transient storage of starch occurs in the seed coat and embryo, contributing to the early development of the embryo and reserve synthesis. Then, during maturation the synthesis and accumulation of proteins and oil takes place at constant rates, while oligosaccharides are synthesized only during late maturation and at the beginning of desiccation.

The composition in the major class of storage compounds of *Medicago truncatula* seeds is similar to other grain legumes, although the major difference resides in the nature of their carbon storage, which in *M. truncatula* is represented by triacylglycerides, and in other grain legumes by starch (Djemel et al., 2005).

2. Seed dormancy

Seed dormancy is described as the inability of an intact viable seed to complete germination under favorable conditions (Hilhorst, 1995; Bewley, 1997; Li & Foley, 1997). This block to germination has evolved differently across species through adaptation to the environment, so that germination occurs when conditions for establishing a new plant generation are appropriate (Hilhorst, 1995; Vleeshouwers et al, 1995; Bewley, 1997; Li & Foley, 1997; Baskin & Baskin, 2004; Fenner & Thompson, 2005). A more sophisticated definition of dormancy has been proposed by Baskin & Baskin (2004): "A dormant seed is one that does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors (temperature, light/dark, etc.) that are otherwise favorable for its germination, i.e. after the seed becomes non-dormant". On the other hand, a non-dormant seed has the capacity to germinate over the widest range of normal physical environmental factors (temperature, light/dark, etc.). A non-dormant seed does not germinate if a certain combination of physical environmental factors (temperature, light/dark, etc.) is absent. In that case seed enters in a state of quiescence, also called enforced dormancy by Harper (1957, 1977) and pseudodormancy by Hilhorst & Karssen (1992), Koornneef & Karssen (1994) and Karssen (1995). A quiescent seed germinates when the appropriate set of environmental conditions, required for radicle emergence, is restored (Baskin & Baskin, 2004).

Thus, dormancy is a mechanism of the plant to adapt germination timing to environmental conditions, to prevent germination when the conditions for seedling establishment and plant growth are unfavorable. Dormancy can have different origins and several schemes for its classification have been published (Harper, 1957, 1977; Nikolaeva, 1977, 1999; Lang et al., 1985, 1987; Lang 1987).

On the basis of the classification system provided by Nikolaeva (1977, 1999, 2004), that devised dormancy types studying both morphological and physiological properties of the seed, Baskin & Baskin (1998, 2004) have proposed a complete classification system which includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD).

Physiological dormancy (PD) is the most abundant form and is found in seeds of gymnosperms and all major angiosperm clades.

Physiological dormancy (PD) is established and regulated by the phytohormone abscissic acid (ABA). It is generally accepted that the mechanisms of seed dormancy and germination involve the plant growth regulators abscisic acid and gibberellins (GA). It has been proposed a hormone-balance model, in which ABA (inhibitor) and GA (promoter) simultaneously and antagonistically regulate the onset, maintenance and termination of dormancy (Amen, 1968; Wareing & Saunders, 1971). ABA, produced by the embryo, induces dormancy during seed development, when its concentration within the developing embryo increases respect to the GA concentration, and GA promotes germination upon dormancy overcoming, when, on the contrary, the levels of GA increases (Karssen & Lacka, 1986; Karssen & Groot, 1987; Groot & Karssen, 1992; Hilhorst & Karssen, 1992; Karssen, 1995; LePage-Degivry et al., 1996; Fennimore & Foley, 1998). More recently, evidences has been presented for the involvement of both ABA and GA in dormancy-break in seeds of *Fagus sylvatica* (Nicolás et al., 1996; Lorenzo et al., 2002), *Arabidopsis* (Debeaujon & Koornneef, 2000), potato (Alvarado *et al.*, 2000) and *Nicotiana plumbaginifolia* (Grappin et al., 2000; Jullien et al., 2000). All these models for control of dormancy and germination show antagonistic interactions of ABA and GA by decreasing and increasing, respectively, embryo growth potential. In addition to ABA and GA, a third plant hormone, ethylene, is involved in the regulation of seed dormancy and germination. Ethylene breaks dormancy and stimulates germination in the seeds of many species (Kępczyński & Kępczyńska, 1997; Matilla, 2000), apparently by decreasing the sensitivity of the seed to endogenous ABA. Thus, ethylene may promote germination by interfering with the action of ABA (Beaudoin et al., 2000; Ghassemian et al., 2000, Matilla & Matilla-Vázquez, 2008). At the molecular level, several studies have shown that specific ABA-responsive mRNAs and heat-stable proteins are present in embryos of dormant seeds. Amounts of dormancy-associated transcripts remained high in embryos of dormant seeds and completely disappeared during germination (Morris et al., 1991; Goldmark et al., 1992; Dyer, 1993; Li & Foley, 1994, 1995, 1996, 1997; Johnson et al., 1995; Holdsworth et al., 1999). Thus, the continuous presence of specific mRNAs and proteins seems to be required to maintain dormancy, that consequently it is regulated at the level of gene expression (Bewely, 1997; Li & Foley, 1997; Holdsworth et al., 1999; Garello et al., 2000; Koornneef et al., 2002).

Physiological dormancy can be divided into three levels: deep, intermediate and non-deep (Baskin & Baskin, 2004). In deep physiological dormancy embryos excised from the seeds do not grow or produce abnormal seedlings. Also, GA treatment does not break their dormancy, and

several months of cold or warm stratification are required before germination can takes place (Baskin & Baskin, 2004; Baskin et al., 2005).

Embryo excised from seed affected by intermediate physical dormancy produce normal seedlings; GA can promotes germination in some species, although seeds require two or three months of cold stratification or a dry storage period for dormancy break (Baskin & Baskin, 2004).

The non-deep physiological dormancy is the most common type of seed physiological dormancy (Baskin & Baskin, 2004). Embryos excised from these seeds produce normal seedlings; GA treatment promotes germination and, depending on species, dormancy can also be broken by scarification, after-ripening in dry storage, and cold or warm stratification. Also, based on patterns of change in physiological responses to temperature, it's possible to distinguish five types of non-deep physiological dormancy. Most seeds belong to type one or two, in which the temperature range at which seed germination can occur, increases gradually during the progression of non-deep dormancy release from low to higher (Type 1), or from high to lower temperature (Type 2). Only few seed have Type 3, seeds with Type 4 or 5 appear to be even more uncommon than those with Type 3 (Baskin & Baskin, 2004).

In seeds affected by morphological dormancy (MD), the embryo is underdeveloped in term of size, but differentiated (cotyledons and hypocotyls-radicle can be distinguished) (Baskin and Baskin, 1998, 2004). These embryos are not physiologically dormant, and does not require a dormancy-breaking pretreatment in order to overcoming dormancy, but need time to grow to full size and then germinate. Thus, the dormancy period is just the time elapsed between incubation of fresh seeds and radical emergence; consequently, under appropriate conditions, embryos begin to elongate within a period of a few days to some weeks, and then completes the germination.

Besides that, several studies show that the morphological dormancy is also the most primitive dormancy class. The thick endosperm layer, that characterizes most of the angiosperms seeds affected by this type of dormancy, is able to inhibit the germination of the embryo. Thus, morphologically dormant seeds germination timing is regulated by the time the embryo requires to elongate and, finally to protrude the surrounding tissues, including the endosperm. Because of that, was observed a general trend of reducing endosperm abundance during the higher plant evolution, that caused a gradually decrease in morphological dormancy and, at the same time, the appearance of physiological dormancy (Finch-Savage & Leubner-Metzger, 2006; Linkies et al., 2010). On the basis of these remarks, it has been proposed that the morphological dormancy is the ancestral type of dormancy among seed plants (Baskin & Baskin, 1998; Forbis et al., 2002; Linkies et al., 2010).

Seeds with morphophysiological dormancy (MPD) have an underdeveloped embryo with a physiological component of dormancy. Thus, in order to germinate, these seeds require a dormancy-breaking pretreatments, such as a defined combination of warm and/or cold stratification otherwise, is some cases, GA application. In seeds with morphophysiological dormancy, the embryo growth and therefore, the radicle emergence requires a considerably longer period of time than in seeds with morphological dormancy. There are eight known levels of morphophysiological dormancy, based on the protocol for seed dormancy break and germination (Baskin & Baskin, 2004).

In seeds whit physical dormancy (PY), prevention of water uptake causes the seed to remain dormant until some factors, such as, in nature, high temperatures, fire, drying, freezing/thawing or the passage through the digestive tracts of animals, render the covering layers permeable to the water (Baskin & Baskin, 1998; Baskin et al., 2000). Once the permeability to the water is restored, seeds can germinate over a wide range of temperatures in both light and darkness and, generally, coat of seeds cannot revert to impermeability (Baskin & Baskin, 1998; Baskin et al., 2000). Thus, physical dormancy is associated with the main mechanical layers of the seed coat which in most hardseeded species are represented by one or more water-impermeable layers of palisade cells (Baskin et al., 2000, Baskin & Baskin, 2004). This kind of dormancy can be removed under both natural and artificial conditions, that allow the formation of an opening, called “water gap”, in a specialized anatomical structure on the seed coat, through which water moves to the embryo (Baskin et al., 2000). In *Leguminosae* seeds, the water-impermeable layers become permeable after the lens (strophiole) is disrupted. In most legume seeds, stress, such as heating, causes disruption of the thin-wall cells of the lens, or disruption occurs via a pop-up lens (Baskin & Baskin, 1998; Baskin et al., 2000).

However, Morrison et al., (1998) have presented evidence that, in some *taxa* of *Fabaceae*, dormancy break by heating may occur through the disruption of the seed coat in a region other than the lens. In particular, these authors showed that in some legumes, an area on the seed coat, in addition to lens region, was disrupted by dry-heating pretreatment. Seed disrupted only at the lens generally had a thinner testa, thicker palisade layer, a thinner mesophyll layer and became permeable only at the lens (Morrison et al., 1998).

Mechanical or chemical scarification can promote germination in seeds with physical dormancy (Baskin & Baskin, 2004).

Combinational dormancy (PY + PD) refers to a condition in which seeds coat is water impermeable and, in addition, the embryo is physiologically dormant. Generally, the physiological component appears to be at the non-deep level, and embryos will come out of dormancy in dry storage or in the field within a few weeks after maturity, even while the seed coat remains impermeable to water (Baskin & Baskin, 1998, 2004). However, embryos in such *genera* as *Cercis* (*Fabaceae*) and *Ceanothus* (*Rhamnaceae*) are more deeply dormant and therefore, require a few weeks of cold stratification: after physical dormancy is broken and seeds imbibe water, the germination can be completed (Baskin & Baskin, 2004).

Moreover, as proposed for some species, seeds have a delaying mechanism to prevent germination when moisture is not sufficient, during late summer and early autumn, for seedling establishment and growth (Thanos & Georghiou, 1988; Thanos et al., 1992; Abeles, 1986; Gallardo et al., 1991; Yoshioka et al., 1998). The suppression of germination at supraoptimal high temperatures is often called thermoinhibition or thermodormancy (Reynolds & Thompson, 1971; Abeles, 1986; Gallardo et al., 1991; Yoshioka et al., 1998). It has been shown that seed responsiveness to temperature is closely related to the level of dormancy in soil-buried seeds of winter and summer annuals (Baskin and Baskin, 1998). Thus, the change in seed sensitivity to temperature plays an ecologically important role in the detection of the appropriate seasonal timing for germination (Baskin and Baskin, 1998; Yoshioka et al., 1998, 2003).

Thermodormancy condition can be overcome by various phytohormones, such as ethylene, as showed for thermoinhibited *Cicer arietinum* seeds (Gallardo, 1991), in which small quantities of this phytohormone are produced when seeds started to germinate. Other studies, performed on *Lactuca sativa* seeds (Abeles, 1986; Dutta & Bradford, 1994; Yoshioka et al., 1998), demonstrate that thermoinhibition can also be prevented by removing the endosperm or weakening it. These authors suggest that the effect of supraoptimal high temperatures inhibit the ability of the embryo to develop sufficient force to penetrate the barrier endosperm, and when the endosperm is removed or punctured the embryo germinates readily.

Phytohormones abscisic acid (ABA) and giberellic acid (GA), that are well known to be involved in germination control, are also important in establishing and removing thermodormancy condition. In seeds of lettuce and chickweed the reduction of ABA content and de novo ABA biosynthesis is required for thermoinhibition (Yoshioka et al., 1998, 2003), while, although it is not completely clarified, the application of exogenous GA can suppress thermoinhibition on several plant species (Madakadze et al., 1993; Dutta et al., 1994; Carter & Stevens, 1998; Gonai et al., 2004; Tho et al., 2008).

In a more recent work by Tho et al., (2008) carried out on thermodormant *Arabidopsis thaliana* seeds, it has been demonstrated that ABA synthesis de novo, after the start of imbibitions, is essential for germination inhibition at supraoptimal temperature in the light. These authors reported genetic evidence that shows that ABA has a critical function in thermoinhibition of *Arabidopsis seeds* and that a subset of ABA signaling components, including ABI1, ABI3 and ABI2, are required for germination inhibition at high temperature. The presence of these components suggests that seeds have a specific mechanism to modulate ABA content in response to high temperature and use a specific ABA-signaling pathway for germination inhibition (Tamura et al., 2006; Tho et al, 2008). ABA synthesized in the endosperm is also involved in thermoinhibition of the seeds (Lefebvre et al., 2006; Tho et al, 2008).

Alleviation of thermoinhibition by exogenous GA_3 in lettuce and *Arabidopsis* seeds suggests that suppression of active GA synthesis at high temperature, through the action of ABA is required for thermoinhibition (Gonai et al., 2004; Tho et al., 2008). Thus, as been proposed from these authors, ABA are able to suppresses the expression of GA biosynthesis genes, *GA3ox1*, *GA3ox2*, *GA20ox2*, and *GA20ox3*, at high temperature in the light, avoiding the completion of germination. Therefore, ABA may inhibit GA action by suppressing GA biosynthesis and also by suppressing GA signaling (Gonai et al., 2004; Tho et al., 2008).

Thus, dormancy condition can be consider as a complex seed trait to regulate germination timing and, also the endosperm tissue, can contribute to dormancy and germination timing (Finch-Savage & Leubner-Metzger, 2006). The role of endosperm in regulating germination is described in the second chapter of this thesis.

When, under natural or artificial conditions, dormancy is released germination can takes place.

3. Seed germination

Germination is the process that start whit the imbibition of a mature seed (uptake of water by the quiescent dry seed or non-dormant seed) and terminates whit the the elongation of the embryonic axis (Bewley & Black, 1994; Bewley, 1997). The visible sign that germination is complete is the penetration of the covering layers around the embryo by the radicle. All the other events that take place after the radicle protrusion, including the mobilization of the major storage reserves, are associated with growth of the seedling, and because of that, are part of post-germination process (Bewley & Black, 1994; Bewley, 1997).

Thus, the first event occurring during germination is the imbibition and resumption of metabolism by dry mature seed.

Water uptake in germinating seed is triphasic process, with a rapid initial uptake during the first phase (imbibition phase), followed by a plateau phase (phase II). A further increase in water uptake occurs only after germination is completed, during radicle protrusion (Schopfer & Plachy, 1984; Bewley, 1997; Manz et al., 2005).

During the phase I, the influx of water into the cells of dry seeds causes a temporary structural perturbation to membranes, which rapidly discharge solutes and low molecular weight metabolites into the surrounding imbibition solution. This is symptomatic of a transition of the membrane phospholipid components from the gel phase, achieved during maturation drying, to the hydrated liquid-crystalline state (Crowe & Crowe, 1992). Then, within a short time of rehydration, the membranes return to their more stable configuration, and the solute leakage is reduced. These desiccation and rehydration events can induce damage to membranes and organelles, thus repair mechanisms are activated during the imbibitions phase. It has been demonstrated that, the amount of phospholipid with membrane-stabilizing properties, such as N-acetylphosphatidylethanolamine, increases during the imbibition of dry seed: these molecules may be involved in maintaining or enhancing membrane integrity (Sandoval et al., 1995).

Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. The structures and enzymes necessary for this initial resumption of metabolic activity are present, partially intact within the dry seed, having survived the desiccation phase. After the water uptake, turnover or replacement of components occurs until the full metabolic status is achieved (Bewley, 1997). One of the first changes upon imbibition is the resumption of respiratory activity, detecting within few minutes. After an initial steep increase in oxygen consumption, the rate declines until the radicle penetrates the surrounding structures, when, another increase of respiratory activity occurs (Botha et al., 1992; Bewley and Black, 1994). The glycolytic and oxidative pentose phosphate pathways both resume during phase I, and the Kreb's cycle enzymes

become activated (Salon et al., 1988). Tissues of the mature dry seed contain poorly differentiated mitochondria as a consequence of maturation drying. Nevertheless, these mitochondria are able to provide adequate amounts of ATP to support metabolism for several hours after imbibition (Ehrenshaft & Brambl, 1990; Attucci et al., 1991).

During germination of embryos, there appear to be two distinct patterns of mitochondrial development. These patterns, which are particularly obvious in cotyledons, depend on the nature of the stored reserves. In starch-storing seeds, repair and activation of preexisting organelles predominate, whereas oil-storing seeds typically produce new mitochondria (Morohashi & Bewley, 1980; Morohashi, 1986; Ehrenshaft & Brambl, 1990).

Although polysomes are absent, all of the components necessary for the resumption of protein synthesis upon imbibitions are present within the cells of mature dry embryos. In fact, within minutes of rehydration, polysomal protein-synthesizing complexes were assembled and the synthesis of necessary proteins can be started (Dommes & Van der Walle, 1990). Preformed mRNAs are also present within the dry embryo and some of these are residual messages associated with previous developmental processes that may be used transiently during early germination (Comai & Harada, 1990; Lane, 1991). Messages encoding proteins that are important during seed maturation and drying, such as Late Embryogenesis Abundant (LEA) proteins, are likely to be degraded rapidly upon imbibition (Jiang & Kermode, 1994; Han et al., 1996). Conversely, those encoding proteins required during early germination, for example ribosomal protein messages, are replaced by identical messages at later times, when protein synthesis becomes more dependent on the new transcripts (Beltrán-Peňa et al., 1995). New mRNAs are transcribed as germination proceeds. The majority of these are likely to encode proteins essential for the support of normal cellular metabolism (Bewley & Marcus, 1990), like the enzymes for to the mobilization and conversion of the major stored reserves.

Radicle extension, through the covering layers surrounding the embryo, is the event that terminates germination. This extension may or may not be accompanied and by cell division (Bewley, 1997), but cell elongation is essential for radicle extension and protrusion (Barroco et al., 2005; Kucera et al., 2005). Two distinct phases of DNA synthesis occur in the radicle cells after imbibitions. The first takes place soon after imbibition and probably involves the repair of DNA damaged during maturation drying and rehydration, as well as the synthesis of mitochondrial DNA. The second phase of DNA synthesis is mainly associated with postgerminative cell division (Zlatnova et al., 1987; Osborne & Boubriak, 1994).

The extension of the radicle is a turgor-driven process (Cosgrove, 1997), and, besides that, it requires the activation of many enzymes that allow the radical elongation, such as xyloglucan endotransglycosylase (XET), (Wu et al., 1994), or expansin (McQueen-Mason & Cosgrove,

1995; Cosgrove, 1997). Xyloglucan-endotransglycosylase enhances cell wall loosening trough the cleavage and rejoining of xyloglucan molecules that tether adjacent cellulose microfibrils, permitting expansion by microfibril separation. Conversely, expansin allows the radical cell expansion thanks to its ability to disrupt the hydrogen bonds between cell wall polymers, like as matrix polysaccharides and cellulose microfibrils (Wu et al., 1994; McQueen-Mason & Cosgrove, 1995; Cosgrove, 1997).

Moreover, there is a severe constraint on radicle cell growth imposed by the surrounding structures, as the external covering layers or the endosperm. In particular, the endosperm acts as a mechanical barrier to the germination of seeds in several angiosperm clades. A decline in this mechanical resistance of the micropylar endosperm (the endosperm layer covering the radicle tip) appears to be a prerequisite for radicle protrusion during seed germination and is called endosperm weakening (Ni & Bradford, 1993, Hilhorst, 1995; Bewley, 1997; Leubner-Metzger, 2003; Sanchez & Mella, 2004; Kucera et al., 2005; Finch-Savage & Leubner-Metzeger, 2006). The endosperm weakening mechanism, that can be promoted by gibberellic acid and, at least in part, inhibited by ABA, is part of the seed germination process of endospermic species, like *Arabidopsis thaliana* and *Lepidium sativum* (*Brassicaceae*) or *Nicotiana tabacum* (*Solanaceae*). For these species the germination consists of two visible steps: testa rupture and endosperm rupture. Testa rupture takes place after a certain time of imbibition and then, endosperm rupture event occurs after several hours or even days, depending on the species (Leubner-Metzger, 2003; Liu et al., 2005; Muller et al., 2006).

Germination is regulated by several environmental factors, such as temperature, light, oxygen, moisture, and nutrients. Further, hormones play a key role in seed dormancy and germination.

Generally, ABA is a negative regulator of seed germination, while GA, cytokinins, ethylene and brassinosteroids promote germination (Kucera et al., 2005). ABA and GA and their antagonism during germination are extensively described in literature, but the mechanism of interaction between other hormones during germination needs further clarification.

4. Hormones involved in the control of seed dormancy and germination

The two most important hormones involved in the control of seed dormancy and germination are, respectively, Abscissic Acid (ABA) and Gibberellin Acid (GA). ABA is a positive regulator of dormancy and a negative regulator of germination, while, in contrast to ABA, GA regulates negatively seed dormancy and positively the germination (Koornneef, 2002; Peng & Harberd, 2002; Leubner-Metzger, 2003; Kermode, 2005; Kucera et al., 2005; Finkelstein et al., 2008; Holdsworth et al., 2008b). ABA is also a key regulator of seed development, and adaptive

responses to abiotic stresses (Zeevaart & Creelman, 1988). Endogenous ABA content is a determinant of these physiological processes, and ABA-deficient mutants exhibit reduced seed dormancy and reduced drought tolerance (McCarty, 1995). ABA is synthesized from carotenoids through the indirect pathway (Zeevaart & Creelman, 1988; Nambara & Marion-Poll, 2005) and the first compound formed is zeaxanthin. Afterwards, zeaxanthin is converted to all-trans-violaxanthin by two-step epoxidation catalyzed by zeaxanthin epoxidase (ZEP) in plastids (Marin et al., 1996; Thompson et al., 2000a; Agrawal et al., 2001; Audran et al., 2001). Then, 9-cis-epoxycarotenoid dioxygenase (NCED) catalyzes oxidative cleavage of the 9-cis isomer of violaxanthin or neoxanthin, producing produces a C_{15} product called xanthoxin and a C_{25} metabolite (Tan et al., 1997; Burbidge et al., 1999; Qin & Zeevaart, 1999; Chernys & Zeevaart, 2000; Iuchi et al., 2000, 2001). NCED is considered to be a key regulatory enzyme in ABA biosynthesis (Tan et al., 1997; Qin and Zeevaart, 1999, 2002; Thompson et al., 2000b; Iuchi et al., 2001). NCED enzymes are encoded by a multigene family (Tan et al., 1997; Burbidge et al., 1999; Qin & Zeevaart, 1999; Chernys & Zeevaart, 2000; Iuchi et al., 2000, 2001) and each member of the NCED family plays a unique regulatory role in specific environmental responses and developmental processes, ad also in dormancy establishment (Lefebvre et al., 2006).

The reactions following production of xanthoxin occur in the cytosol. Xanthoxin is converted to abscisic aldehyde by dehydrogenase/reductase (SDR) (Cheng et al., 2002), and finally, the abscisic aldehyde is oxidized to ABA by abscisic aldehyde oxidase (Seo et al., 2000a).

On the other hand, the inactivation of ABA is obtained through hydroxylation and conjugation reactions (Zeevaart & Creelman, 1988; Nambara & Marion-Poll, 2005). However, among these pathways, the ABA hydroxylation pathway is shown to be the regulatory step in a variety of physiological processes, and has a major role in the rapid decrease in ABA content during seed germination (Kushiro et al., 2004; Saito et al., 2004).

As mentioned before, GA is a positive regulator of germination and it is also essential for stem elongation and flowering. Within the plant cells, GA responses are regulated by the modulation of GA levels and by altering the ability of cells to respond to the hormone (Richards et al., 2001). Biologically active GAs, such as GA_1 and GA_4, are tetracyclic diterpenoids synthesized from geranylgeranyl diphosphate. The biosynthesis pathway of biologically active GA is divided into three stages (Hedden & Kamiya, 1997; Olszewski et al., 2002). Briefly, first, geranylgeranyl diphosphate is cyclized to entkaurene in plastids, then ent-kaurene is oxidized to GA_{12} by microsomal cytochrome P450 monooxygenases, and, finally, GA_{12} is converted into active GAs in cytosol by two 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase and GA 3-oxidase (Hedden & Kamiya, 1997; Olszewski et al., 2002).

Conversely, bioactive GAs are deactivated by a 2-oxoglutarate-dependent dioxygenase, GA 2-oxidase, which also catabolizes immediate precursors of active GAs (Hedden & Kamiya, 1997; Olszewski et al., 2002). Also, additional deactivation mechanisms by a cytochrome P450 monooxygenase and methyltransferases were reported (Zhu et al., 2006; Varbanova et al., 2007).

Environmental signals regulate GA level through modulation of the late steps of GA biosynthesis and catabolism (Olszewski et al., 2002). Light signals mediated by phytochromes are critical environmental determinants for photoblastic seed germination (Shinomura et al., 1996; Toyomasu et al., 1998; Yamaguchi et al., 1998), while also the exposure to low temperature can promotes germination trough GA signaling pathway, as shown for *Arabidopsis* seeds, in which the expression of GA 20-oxidase genes, *GA20ox1* and *GA20ox2*, and *GA3ox1* was up-regulated by low temperature in darkness, while the germination of *Arabidopsis GA3ox1* mutant seeds are not stimulated by low temperature (Yamauchi et al., 2004). Genetic studies have identified several GA-signaling components in *Arabidopsis*, some of which play a role in GA-induced seed germination, as shown by their loss-of-function mutant phenotypes (Olszewski et al., 2002). The DELLA subfamily of GRAS proteins and SPINDLY (SPY), a Ser/ThrO-linkedN-acetylglucosamine transferase, acts as negative regulators of GA pathways, inhibiting all the process, including germination, that depend on GA signaling (Jacobsen & Olszewski, 1993; Jacobsen et al., 1996; Lee et al., 2002; Wen & Chang, 2002).

SPY is thought to increase the activity of DELLA proteins by N-acetylglucosamine modification (Silverstone et al., 2007) and, also acts as a positive regulator of cytokinin signaling (Greenboim-Wainberg et al., 2005) and as a negative regulator of brassinosteroid biosynthesis (Shimada et al., 2006). One of the five DELLA protein genes isolated in *Arabidopsis*, RGL2, plays a major role on seed germination (Lee et al., 2002; Tyler et al., 2004), and has been documented that others DELLA proteins GAI, RGA, and RGL1 enhance the function of RGL2 (Cao et al., 2005).

Besides the antagonistic action of GA and ABA in the control of dormancy and germination, ethylene (ET) is also known to promote the germination of non-dormant seeds (Matilla & Matilla-Vàzquez, 2008) and an ABA-ethylene antagonism has been shown for the germination of several species, such as *Arabidopsis thaliana* and *Lepidium sativum* (Linkies et al., 2009).

4.1 Molecular factors and hormonal cross-talk in regulating seed dormancy and germination

Seed germination is considered to be determined by the balance of the negative and positive effects of ABA and GA, respectively (Amen, 1968; Wareing & Saunders, 1971; Karssen & Lacka, 1986; Karssen & Groot, 1987; Groot & Karssen, 1992; Hilhorst & Karssen, 1992; Karssen, 1995; LePage-Degivry et al., 1996; Fennimore & Foley, 1998; Alvarado et al., 2000; Debeaujon & Koornneef, 2000; Grappin et al., 2000; Jullien et al., 2000).

Interaction between ABA and GA signaling has been well studied and recent reports shed light on the cross-talk pathway between ABA, GA and also ethylene, during dormancy release and germination.

Dormancy of developing seeds is dependent on ABA that is synthesized in the embryo and not on maternal sources of ABA (Frey et al., 2004). However, the surrounding seed tissues and mother plant play an important role in maintaining or encouraging ABA biosynthesis in developing embryos (Kermode et al., 2005)

The various ABA mutants are important model in explain ABA induction dormancy. ABA-deficient mutants of maize, *Arabidopsis*, and tobacco exhibit reduced dormancy (Tan et al., 1997; Frey et al., 1999; White et al., 2000). Severe mutants of *Arabidopsis* exhibiting relative ABA insensitivity and the characterization of these mutants and also the ABA-insensitive mutants of maize led to the identification of genes involved in the ABA signaling, some of which are transcription factors (Kermode et al., 2005). Thus, six classes of transcription factors have been identified as essential for ABA-or seed-specific gene expression: ABI3/VP1, ABI4, ABI5, LEC1, LEC2 and FUS3 (Finkelstein et al., 2002).

The proteins belong to the transcription factors family ABI3/VP1 accomplishes important tasks during seed development and dormancy induction; in addition to that, this family of transcription factors are described as “ancient” dormancy regulators because there are widely spread and therefore evolutionary old (Holdsworth et al., 2008b; Romanel et al., 2009). The ABA insensitive mutant *abi3* in *Arabidopsis thaliana* (Nambara et al., 2000; Bassel et al., 2006) show reduced dormancy and premature germination. Thus, the ABI3/VP1 family of proteins is thought to perform at least three important functions within seeds. First, they mediate the action of ABA in promoting storage reserve accumulation and synthesis of desiccation/stress protective factors, such as LEA proteins and small heat shock proteins, during seed maturation (Kermode & Finch-Savage 2002; Zeng et al., 2003; Zeng & Kermode 2004). ABI3/VP1 regulates seed storage-protein gene expression by acting synergistically with other transcription factors FUS3 and LEC1, LEC2, as shown for *Arabidopsis* seed (Finkelstein et al., 2002; Kroj et al., 2003).

Second, ABI3/VP1 transcription factors repress gene expression, particularly the genes involved in post-germinative phase, for example they repress the expression of α-amylase gene in aleurone layer cells of developing seeds (Hoecker et al., 1995; McCarty 1995; Nambara et al., 1995, 2000; Paek et al., 1998; Hoecker et al., 1995, 1999). The repression of these genes leads to the repression of germination, in fact, when ABI3 genes are defective, the mutants seeds show a premature germination. Third, ABI3/VP1 transcription factors play a role in dormancy inception during development of seeds and further, maintain a dormant state in mature dispersed seeds, as shown for yellow-cedar seeds (Zeng et al., 2003). These functions of VP1 and ABI3 proteins are achieved by interactions with the other transcription factors ABI5 and ABI4. In fact, ABI3/VP1 trough the interaction whit ABI4 and ABI5, forms a regulatory complex mediating seed-specific and/or ABA-inducible expression gene trough the binding to the ABA Responsive Elements (ABREs) located on the ABA-inducible gene promoter (Finkelstein et al., 2002). In that way ABI3/VP1 play a key role in the activation or repression of target genes, including those important for maintaining/terminating the dormant state.

Mutations in the ABI4 and ABI5 gene loci have effects on seed development and ABA sensitivity that are similar to those associated with the *abi3* mutant in which seeds show reduced dormancy and germinate precociously (Finkelstein et al., 2002; Kermode, 2005).

Dormancy release is accomplished by diverse mechanisms that include complex interactions with the environment mediated of course by phytohormones (ABA,GA and ethylene) but also by other small molecules, namely brassinosteroids, reactive oxygen species, and nitrogen-containing compounds, such as nitrate and nitric oxide (NO) (Finkelstein, 2008).

Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues such as the endosperm or seed coat, allowing mobilization of seed storage reserves, and stimulating expansion of the embryo (Bewley & Black, 1994). Also, GA stimulates germination via the transition from embryonic to vegetative development, in part mediated by the chromatin remodeling factor PICKLE (PKL) (Henderson et al., 2004). GA stimulation of this transition is suggested by enhancement of the *pkl* embryonic root phenotype by GA biosynthesis inhibitors, and by GA-stimulated disappearance of the embryonic identity protein FUSCA 3 (FUS3), which positively regulates ABA synthesis and negatively regulates GA synthesis (Gazzarini et al., 2004; Henderson et al., 2004). The role of gibberellins in dormancy release is controversial. GA synthesis is regulated developmentally and in response to environmental signals.

Light promotes GA synthesis, and light and GA together promote the degradation of ABA in imbibing seeds (Toyomasu et al., 1998). In fact, the accumulation of the GA biosynthetic gene *GA3ox2* (*GIBBERELLIN 3 OXIDASE*) transcript, strongly increases in germinating seeds whereas the GA-deactivating enzyme *GA2ox1* (*GIBBERELLIN 2 OXIDASE)* was expressed at

the highest levels in dormant seeds (Finch-Savage et al., 2007). Changes in expression of GA biosynthetic genes in response to light and cold are regulated by two basic helix-loop-helix (bHLH) transcription factors, SPATULA (SPT) and PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5 (PIL5) (Oh et al., 2004; Oh et al., 2006; Penfield et al., 2005). SPT inhibits expression of two *GA3ox* genes (*GA3ox1* and *GA3ox2*) during seed imbibitions in the cold, and mutation in gene that encodes this transcription factor generates *spt* mutants. *Spt* mutants show reduced dormancy more pronounced in the light, suggesting a SPT-mediated cross talk between light and cold in regulation of dormancy. On the other hand, PIL5 prevents seed germination in the dark at low temperature by repressing expression of *GA3ox1* and *GA3ox2* genes, whereas it induces expression of the *GA2ox2* catabolic gene during imbibition in the dark (Oh et al., 2006; Penfield et al., 2005). When PIL5 protein disappears, in the light, due to phytochrome-stimulated degradation by the 26S proteasome, the germination can be completed, thanks to the accumulation of bioactive GA (Oh et al., 2006). PIL5 also repress GA responses by stimulating expression of the DELLA repressors of GA response, *REPRESSOR OF GA1-3* (*RGA*) and *GA-INSENSITIVE* (*GAI*), through direct binding to their promoters (Oh et al., 2007). This suggests that also DELLA proteins are involved in the control of seed dormancy, mediating the signaling of GA.

The DELLA family is defined by an N-terminal DELLA domain required for GA regulation and a C-terminal GRAS [GAI (GA-INSENSITIVE), RGA (REPRESSOR OF GA 1-3), and SCARECROW] domain required for function of these putative transcription factors (Peng et al., 1999; Pysh et al., 1999). Mutations in the DELLA domain result in gain-of-function GA signaling phenotypes resembling GA deficiency, whereas loss-of-function mutations in the GRAS domain typically result in an enhanced or constitutive GA response (Thomas et al., 2005).

DELLA proteins are negative regulators of a variety of GA responses (Sun et al., 2004) and generally, when DELLA proteins are degraded, GA becomes active and explains its effect, including the stimulation of germination (Ariizumi et al., 2007; Tyler et al., 2004). GA relieves DELLA repression of seed germination through proteolysis of the DELLA protein by the 26S proteasome, triggered via polyubiquitination reaction by the SCF $^{SLY1/GID2}$ E3 ubiquitin ligase complex (Ariizumi et al., 2007). Upon binding to the GA receptor GDI (GIBBERELLIN INSENSITIVE DWARF 1) the complex of GA and the receptor binds to DELLA factors that act as negative regulators in the GA signalling pathway. Then, thanks to the binding of GA-GID1-DELLA, DELLA protein is recognized by the F-box proteins SLY1 and GA INSENSITIVE DWARF 2 (GID2) that are F-box subunits of an SCF E3 ubiquitin ligase complex. These F-box proteins target ubiquitinates DELLA protein for degradation by the 26S proteasome, which in turn leads to a de-repression of the GA signalling pathway (Dill et al., 2004; Fu et al., 2004;

McGinnis et al., 2003; Sasaki et al., 2003). The GA signal is received by the GID1GA receptor (Griffiths et al., 2006; Iuchi et al., 2007; Nakajima et al., 2006; Ueguchi-Tanaka et al., 2005; Willige et al., 2007). The GID1 protein undergoes a GA-dependent interaction with DELLA proteins, which promotes the interaction of DELLA with the F-box protein (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2007).

Among the identified DELLA proteins, *RGL* (*RGA-LIKE 2)* appears to be the major DELLA protein regulating seed germination. In particular, RGL2 is a potent repressor of seed germination, because loss of *RGL2* function partly restores germination in GA-deficient seeds (*ga1-3mutants*) (Lee et al., 2002; Tyler et al., 2004). Thus, DELLA-mutants show increased germination, while GA-insensitive mutants of the GA-receptor or the F-box protein show impaired germination (Steber et al., 1998; Griffiths et al., 2006; Iuchi et al., 2007).

ABA may interfere whit GA signaling trough the stabilization of RGA/GA complex, avoiding the proteasome mediating degradation of RGA and inhibits, finally, the completion of germination (Penfield et al., 2006). Moreover, recent evidences in literature, suggests that DELLA proteins promote seed dormancy through inhibition of cotyledon expansion prior to germination (Penfield et al., 2006), by repressing expression of hydrolytic and wall-modifying enzymes.

On the other hand, the loss of dormancy of many seeds is directly related to the increase in sensitivity to GA and the ABA/GA ratio is important in the maintenance of seed dormancy. Thus, a dynamic balance between synthesis and catabolism of these two antagonistic hormones controls the equilibrium between dormancy and germination processes, by regulating signaling pathways that modify seed sensitivity to the ambient germination environment (Nambara et al., 1991; Achard et al. 2006; Penfield et al. 2006 a,b; Seo et al. 2006; Zentella et al. 2007; Piskurewicz et al. 2008; del Carmen Rodríguez-Gacio et al., 2009; Penfield & King, 2009).

ABA acts directly and negatively on GA synthesis (Seo et al., 2006). Key evidence for this is that ABA-deficient mutants show elevated expression of the *GA3OXs* gene (Seo et al., 2006). Thus, once ABA levels fall below a certain threshold, GA synthesis can begin and germination follows.

Light is the most important environmental factor that controls the integration of ABA and GA signaling (Seo et al., 2009). Light-regulation of GA metabolism has been studied in detail under PhyB-dependent or related germination conditions in *Arabidopsis* and lettuce seeds, where endogenous levels of bioactive GAs increase after irradiation with a R-light (Red-light) pulse and the increase is suppressed by a FR-light (Far-red light) pulse given immediately after the R-light pulse (Toyomasu et al. 1993; Oh et al. 2006; Seo et al. 2006;) (Fig.3). Consequently, with

the change in GA levels, expression of GA biosynthetic genes encoding GA3ox (*GA3ox1* and *GA3ox2*) is induced by R light and this induction is lost by FR light (Toyomasu et al. 1998; Yamaguchi et al. 1998). In contrast, transcript levels of a GA deactivating gene GA2ox (*GA2ox2*) are decreased by R light treatment (Nakaminami et al. 2003; Oh et al. 2006; Seo et al. 2006; Yamauchi et al. 2007). Light-regulation of *GA3ox* and *GA2ox* genes are also observed during PhyA-dependent germination (Oh et al. 2006), demonstrating that the bioactive GA levels are regulated by phytochromes through reciprocal regulation of *GA3ox* and *GA2ox* genes.

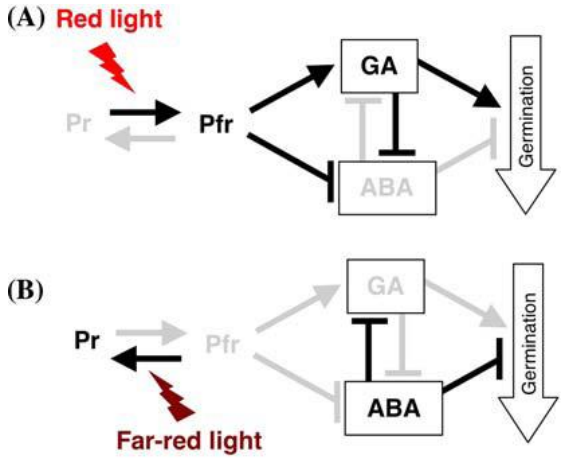

Figure 3 Interaction of ABA and GA metabolism. **A**: Pfr formed by R light increases GA levels and decreases ABA levels. R-light induces GA accumulation and also contributes to reducing ABA levels. **B**: In the absence of Pfr, ABA levels are higher because of the loss of negative regulation by Pfr. Increased ABA levels are required to reduce GA levels. Arrows indicate positive regulation and T-bars indicate negative regulation. Grey elements are indicative of inactive regulation or low abundance in each condition (Seo et al., 2009).

Light also regulate the metabolism of endogenous ABA. Endogenous ABA levels decreases in presence of R light and increase in presence of FR light (Seo et al., 2006; Oh et al., 2007; Seo et al., 2009) (Fig.3). Consistent with the change in ABA levels, expression levels of the two key enzymes involved in ABA biosynthetic pathway ZEP (zeaxanthin epoxidase) and NCED (9-cis-epoxycarotenoid dioxygenase) are decreased by R light treatment (Seo et al. 2006; Oh et al. 2007). In contrast, transcript levels of ABA deactivating genes encoding ABA8ox (ABA 80-hydroxylase, that inhibits the ABA action trough its hydroxylation) are elevated by R light treatment (Seo et al., 2006; Oh et al., 2007; Seo et al., 2009). These evidences indicate that ABA concentrations in seeds are regulated by phytochrome in a manner opposite to GA concentrations. In fact, the PhyB-mediated induction of GA biosynthetic genes (*AtGA3ox1* and *AtGA3ox2*) and consequently, the levels of bioactive GA is partly suppressed in response to the high accumulation of transcripts of ABA8ox encoding genes, indicating that GA levels are

negatively regulated by ABA during seed germination (Seo et al., 2006). There is also evidence that GA negatively regulates endogenous ABA concentrations. In *Arabidopsis*, higher ABA levels, activation of ABA biosynthetic genes and suppression of an ABA deactivating gene are observed in the GA deficient mutant *ga1* in comparison with wild type (Oh et al., 2007).

Moreover, Zentella et al., (2007) reported that XERICO, one of the early GA and DELLA-responsive genes, regulated ABA levels in seeds. XERICO encodes a protein containing a RING-H2 type zinc finger motif. Over-expression of this gene causes accumulation of ABA through an increased ABA biosynthesis. Conversely, DELLA proteins repress the expression of XERICO by directly associating with its promoter (Zentella et al., 2007). Thus, also the repression of XERICO by DELLA proteins is an important mechanism by which GA regulates ABA metabolism, in a mutual negative regulation manner.

Recently, Penfield & King (2009), proposed a new model to explain the integration of the signaling underlining these two hormones. They suggest that the stabilization of ABI3 by ABA and the destabilization (or the stimulation of GID-binding and deactivation) of DELLAs by GA are central processes in hormone balance. In this model, they assumes that germination is promoted by a complex containing two proteins PIFs (PHYTOCHROME-INTERACTING FACTORS), and PRRs (PSEUDORESPONSE REGULATOR), that can interact with each other, and each with one of either ABI3 or DELLA. In a dormant seed, the concentration of the germination-promoting PIFs-PRRs complex is low because PIFs remains bound to DELLA and PRRs to ABI3. In a germinating seed, free DELLA levels are low (because DELLAs are either turned over or are prevented from binding PIFs by GIDs), as are those of ABI3. This allows the formation of PIFs- PRRs complex and subsequent germination, thanks to the degradation of DELLA protein via proteasome 26S.

Besides GAs, ethylene (ET) is also known to promote the germination of non-dormant seeds of several species (Matilla & Matilla-Vàzquez, 2008). This hormonal involvement is demonstrated by the use of inhibitors of ethylene synthesis and action, for example, 1-methylcyclopropene, 2,5-norbornadiene and silver thiosulfate. When non-dormant seeds are subjected to the application of these inhibitors, they are incapable to complete the germination (Gallardo et al., 1994; Nascimento et al., 2004; Kozarewa et al., 2006). Different possibilities can explain the action of ethylene in the germination process, including the promotion of radial-cell expansion, the increase of the water potential in the embryonic axis, and the increase in seed respiration (Kucera et al., 2005). Recently, Achard et al., (2003) reported that part of the growth-regulatory action of ethylene is mediated via its effects on the DELLA proteins, which repress growth in response to ethylene, auxin and GAs signals.

Also, thermoinhibition (or thermodormancy) can be overcome by ethylene, as shown for lettuce seeds (Nascimento, 2003) and chick-pea (Gallardo et al., 1994; Gallardo et al., 1996; Matilla, 2000). As proposed by these authors, ethylene is needed for the germination of thermosensitive lettuce genotypes at high temperatures, while the germination of chick-pea under optimal conditions depends on ethylene production by the embryonic axis.

Moreover, ethylene promotes dormancy breaking and germination trough the interaction whit ABA signalling. Linkies et al., (2009) demonstrated that ethylene biosynthesis in germinating *L.sativum* and *A. thaliana* seeds, promotes endosperm rupture by promoting endosperm weakening, counteracting the ABA inhibition of this process. Furthermore, in different works, Beaudoin et al., (2000), Ghassemian et al., (2000), Chibani et al. (2006) reported that seeds whit mutations in receptors of ethylene signal (such as *ethylene resistant 1 (etr*) *1* receptor and *ethylene insensitive* (*ein*) *2/enhanced response to aba* (*era*) *3* mutants) display increased dormancy correlated with increased sensitivity to ABA and increased ABA biosynthesis. In comparison with wild-type, these seeds showed increased dormancy due to ABA overaccumulation.

Thus, the balance between plant hormone signaling pathway controls the induction and the release of seed dormancy regulating finally, the germination timing.

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Objectives

The general aim of this thesis is to understand the mechanisms involved in seed dormancy and germination which are part of the life cycle of higher plant. Seeds are the vital dispersal unit of the angiosperms and gymnosperms and the evolution of the seed structure and its proprieties have ensured the propagation of the flowering plants in a wide variety of habitats. The evolution of dormancy trait has considerably contributed to the dominance of the angiosperms in the terrestrial ecosystems, since seed dormancy, regulating the germination time, is considered as an ecological mechanism that allows the adaption of the plant to environmental changes and specialized life habitats (Baskin & Baskin, 2004; Linkies et al., 2010), like the dunes.

Because of that, seeds of *Medicago marina*, a dune plant species, distributed along the whole coasts of Italian peninsula, were chosen to analyze the complex and unknown mechanisms underlying dormancy and germination of this species, adapted to living in a hardly stressed environmental condition.

Results of these studies are reported in two main chapters: the first is focused on the dormancy habit of these seeds and on their ability to tolerate stress conditions; the second reports data related with the specific involvement of micropylar endosperm and the role of hormones as abscissic acid (ABA), gibberellic acid (GAs) and ethylene in seed dormancy. Moreover by using a proteomic approach integrated with biostatistic analyses, key factors controlling dormancy and germination are investigated.

Chapter I: Dormancy of *Medicago marina* (L.) seed

Results obtained during the first stage of PhD program are published in a paper by Scippa et al., (2011). Seeds are first subjected to three different pretreatments (hand mechanical scarification, freezing at -20°C and heating at 100°C) in order to break dormancy condition and then to the germination test. Further, the effects of these treatments in dormancy overcoming and germination were analyzed by a combination of physiologic (abscissic acid assay) and proteomic approaches (study of proteome and phosphoproteome maps and immunoblotting analysis). The data reported in this paper showed that *Medicago marina* seeds adopt a dormancy strategy based on hard seed coats that prevent water uptake and germination, known as hardseededness, which is a common strategy present also in seed of other legumes. The three pretreatments used to overcome dormancy condition yielded different results on germination percentage and rate, indicating that dormancy is regulated by other complex mechanisms beside seed coat, such as

thermoinhibition, a delaying mechanisms of germination adopted by different species when, during late summer or early autumn, the environmental condition are not favorable for seedling establishment and growth. Also, the three pretreatments are perceived by the seeds as an environmental stress condition that evoke an increase of abscissic acid level and of the expression of proteins that protect important cellular component, like chromatin and the cell membranes. Thus, abscissic acid regulates *Medicago marina* seed dormancy/germination and seed response to environmental stress through different signaling pathways. Finally, the analysis of the proteome maps revealed the presence of 46 differentially expressed spots among treated and untreated seed, 14 of which were subsequently identified and collocated in four different functional classes. Some of these identified spots resulted also phosporylated indicating, besides the altered protein pattern, the presence of post-translational modifications in seeds subjected to various types of treatment.

Chapter II: Dormancy of *Medicago marina* (L.) seed: the role of the micropylar endosperm

Starting from the results obtained during the first period of PhD program, the research proceeded in further investigations of the role of the endosperm, and also of the micropylar endosperm, in dormancy establishment and in regulation of the germination timing.

In order to accomplish this aim, the weakening of the endosperm was analyzed trough the puncture force technique in seeds subjected to mechanical scarification and abscissic acid (ABA) treatment, along a 22 hours time course of imbibition. In addition to that, the effect of abscissic acid and gibberellic acid (GAs) treatments on germination rate and speed, were analyzed by performing germination test. Results obtained from the puncture force measurements and germination test for scarified seeds and ABA treated scarified seeds, revealed that the physiological phenomena of endosperm weakening occurs in *Medicago marina* seeds before the germination and that the ABA is able to delay the weakening and also the germination of treated seeds. Then, the using of proteomic analysis coupled whit the multivariate statistical analysis allowed the individuation of 95 spots differentially and significant expressed among the scarified seeds (treated and not treated whit ABA) collected along the 22 hours time course, scarified germinated seeds not subjected to the ABA treatment and scarified not germinated seeds subjected to the ABA treatment. In addition to that, the role of ABA, GAs and ethylene in seed dormancy and germination, was investigated by RT-PCR approach trough the evaluation of the expression of genes encoding the key enzymes involved in the final step of these hormones biosynthesis.

Preliminary results, reported in a paper in preparation, suggest that, also the endosperm, and in particular the micropylar endosperm, can contribute to the establishment of *Medicago marina* physical dormancy, that once again, it can be removed by mechanical scarification. Moreover, abscisic acid seems to be strongly involved in regulation of germination timing, by its action on micropylar endosperm weakening.

Chapter I

Dormancy of *Medicago marina* (L.) seed

All the experiments carried out whit the purpose to investigate the dormancy features in *Medicago marina* seeds and the obtained results, are reported in the following paper.

Dormancy of *Medicago marina* (L.) seed

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Keywords:
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Medicago marina
Physical dormancy
Proteomics

A B S T R A C T

Medicago marina (L.) is a Mediterranean species whose seeds show strong dormancy that prevents germination. We used an integrated approach of physiological analyses and proteomics to investigate the mechanisms that control *M. marina* dormancy/germination and that underlie stress tolerance. First, we evaluated the effects on dormancy breaking of the following treatments: mechanical scarification, freezing at −20 °C, storage for 4 months and heating at 100 °C for 1 h. Mechanical scarification and freezing were the most effective treatments in overcoming dormancy. The role of abscisic acid (ABA) in *M. marina* dormancy was studied by ELISA immuno-enzymatic assay. The ABA content of germinated and non-germinated mature (control) and treated seeds was determined. The level of ABA was higher in treated seeds than in control seeds; the most significant increase occurred in the heated seeds. A comparison of the ABA level in the germinated, control and treated seeds suggests that different mechanisms modulate ABA content in response to different stresses, and that a specific ABA-signalling pathway regulates germination. Proteomic analysis revealed 46 proteins differentially expressed between treated and untreated seeds; 14 of these proteins were subsequently identified by mass spectrometry. Several of the proteins identified are important factors in the stress response, and are involved in such diverse functions as lipid metabolism, protein folding and chromatin protection. Lastly, an analysis of the phosphoproteome maps showed that the function of many proteins in seeds subjected to temperature treatment is modulated through post-translational modifications.

1. Introduction

Upon physiological maturity, the seeds of many plant species may enter a state of deep dormancy, and, upon sowing, they will not germinate or germinate very slowly compared to the corresponding non-dormant seeds (Chibani et al., 2006). It is generally accepted that dormancy is an adaptive trait that enables seeds to remain quiescent until the conditions for germination become favourable (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). Dormancy, is determined by physiological and/or morphological properties of the seed and, according to Baskin and Baskin (2004), it can be classified in five major categories: physiological, physical, morphological, morphophysiological and combinational (physical and physiological). Generally, physical dormancy is caused by one or more water-impermeable layers of palisade cells (macrosclereids) in the seed coat (Baskin et al., 2000; Baskin and Baskin, 2004). Indeed, the outer layers, cuticle and epidermal, are the first barrier to imbibitions, followed by the hypodermis and interior parenchyma that, like palisade cells, play an important role in the mechanical strength of the seed coat (Moïse et al., 2005). In many legume seeds, for example of the *Medicago* species, the hardness of the tegument prevents water uptake and gaseous exchange thereby resulting in a low germination rate when seeds are subjected to i
(Crawford et al., 1989). This coat-imposed dormancy, known as hardseededness, has been interpreted as an ecological mechanism that prevents seed from imbibing and germinating until the environmental conditions are favourable for the development of young seedlings (Loi et al., 2005).

Several studies on seed dormancy have revealed a highly complex interaction between environmental conditions and the sensitivity of seeds to both the environment and the plant growth regulators abscisic acid (ABA) and gibberellic acid (Finkelstein et al., 2008 and references therein). ABA plays a crucial role in embryo development, seed maturation, abiotic stress tolerance, dormancy and germination. Radicle emergence and mobilization

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of reserves are two distinct programmes influenced by ABA and are thought to control germination, although how this hormone controls seed germination is still poorly understood. It was recently suggested that, by regulating the expression of genes encoding enzymes of cell-wall biosynthesis and architecture modification and structural proteins, ABA inhibits cell-wall loosening and expansion, and consequently inhibits radicle emergence and germination (Gimeno-Gilles et al., 2009).

In Mediterranean environments, the seeds of many legume species must survive over dry hot summers to enable seedlings avoid potentially fatal temperatures and postpone germination until the following spring. Seeds of these species have developed a resistance mechanism to this highly stressed condition and complex machinery to perceive when environmental conditions are favourable to break dormancy.

Medicago marina (L.), known as sea medick, is a legume widely distributed from Spain and the north of Africa up to mid-Asia. In Italy, *M. marina* grows along the coastal dunes of the whole peninsula. However, despite its potential use in the renaturalization and stabilization of coastal dunes, little is known about the germination characteristics of the seed and the mechanisms it uses to break dormancy, and resist adverse conditions. We tested the hypothesis that *M. marina* seeds, like many other species of the *Medicago* genera, has developed complex mechanisms to control dormancy/germination and to tolerate the adverse environmental conditions (i.e. high temperature, drought) occurring during summer. To this aim we used a combination of physiological studies and proteomics to clarify *M. marina* seed biology and to investigate the mechanisms underlying dormancy/germination.

2. Materials and methods

2.1. Seed material and pretreatments

Dry mature *M. marina* (L.) seeds were collected in July 2008 from the Molise coastal dunes (Central-south Italy) and taken to the Molise Germplasm Bank Molise (University of Molise). The seeds were removed from pods, cleaned and subjected to one of the following treatments: freezing at −20 °C for 60 days, thawing at room temperature, mechanical scarification by sandpaper, or heating in distilled water at 100 °C for 60 min. Seeds stored at room temperature served as the control sample (untreated sample). A portion of each sample after each treatment was used for germination tests, and the remainder was frozen in liquid nitrogen and stored at −80 °C until required for ABA and proteomic analysis.

2.2. Seed moisture content and viability test

Moisture content was measured with a hygrometer (ROTRONIC model AW-DIO) on seeds collected in the field soon after arrival in the Germplasm bank. Viability was determined with the tetrazolium test (2,3,5-triphenyltetrazolium chloride) using three replicates of 30 seeds for each sample and three replicates of 20 seeds as negative control. After imbibition, carried out overnight with distilled water, intact seeds for each sample and negative control were incubated in an 0.1% aqueous solution of 2,3,5-triphenyltetrazolium chloride overnight, in the dark for 24–48 h. Imbibed seeds, used as negative control, were heated for 120 min at 100 °C and then incubated in tetrazolium solution (Bacchetta et al., 2006). Tetrazolium salts were metabolically reduced in formazans by NADH-dependent reductase (Berridge et al., 1996). Being non-diffusible, formazan stains the living tissues red thereby identifying viable cells. The viability of samples was expressed as percentage of red seeds.

2.3. Germination test

To test germination, the seeds subjected to scarification, or to freezing at −20 °C for 2 months followed by thawing at room temperature, or to boiling for 1 h were sterilized in a solution of 1% sodium hypochlorite for 10 min, rinsed in distilled water and dried with adsorbent paper. Three replicates of 30 seeds of each sample (control and treated) were placed on two sheets of Whatman filter paper moistened with distilled water in 9-cm diameter Petri dishes. All dishes were incubated in darkness at a constant temperature of 25 °C for 30 days. According to Bacchetta et al. (2006), the results are expressed as final germination percentage (*G*%) and germination speed, expressed as number of days required to reach 50% germination (T_{50}). A seed was considered germinated when the radicle emerged from the coat.

2.4. Protein extraction

For total protein extraction, 0.50 g (corresponding to around 100 mature seeds) of the control and of each treated sample were reduced to powder by mechanical breakage with liquid nitrogen and suspended in 5 ml of 10% trichloroacetic acid and 0.07% 2-β-mercaptoethanol in cold acetone at −20 °C (Rabilloud, 2000), for each sample. The mixed sample was filtered through Miracloth and precipitated overnight at −20 °C. Each mixed sample was centrifuged at 35,000 × *g* for 15 min at 4 °C, pellets were recovered, resuspended in 10 ml of 0.07% 2-β-mercaptoethanol in cold acetone at −20 °C and precipitated for 5 h at −20 °C. Pellets were recovered by centrifugation at 35,000 × *g* for 15 min at 4 °C, vacuum dried and solubilized in 300 μl of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 20 mM Tris–HCl, 1% (w/v) DTT, containing 0.2% (w/v) ampholine 3–10 and 0.15% (w/v) ampholine 5–7. The protein content was measured by the Bradford protein assay method (Bradford, 1976).

2.5. Two-dimensional electrophoresis

For isoelettrofocusing (IEF), an extract of total proteins of each sample was mixed with rehydratation buffer and loaded on 7-cm IEF strip gels in immobilized 5–8 pH gradients (IPG strip Bio-Rad). Strips were rehydrated in mineral oil for 16 h at room temperature and IEF was performed with the PROTEAN IEF Cell system (Bio-Rad) at 12 °C at the following voltages: 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 55 kVh (Rocco et al., 2006).

After focusing, the proteins were reduced by incubating the IPG strips with 1% (w/v) dithiothreitol for 15 min and alkylated with 2.5% (w/v) iodoacetamide in 10 ml of 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) sodium dodecyl sulphate and a dash of bromophenol blue for 15 min. Second dimension electrophoresis was carried out on 12% polyacrylamide gels (18 cm × 24 cm × 1 mm) with a Protean apparatus (Bio-Rad) in 25 mM Tris–HCl, pH 8.3, 1.92 M glycine and 1% (w/v) SDS, with 120 V applied for 12 h, until the dye front reached the bottom of the gel. Two-dimensional electrophoretic gels were stained with colloidal Coomassie G250. Samples were run in triplicate.

Two-dimensional electrophoretic gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Image analysis was performed using the PDQuest software (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for three different replicates of each biological sample, and Student's *t* test ($P < 0.01$) was used to identify significant changes in protein folding between samples. A two-fold change in nor-

malized spot densities was considered indicative of a differential expression.

2.6. Phosphoproteome analysis

To identify phosphorylated proteins, two-dimensional gels obtained for each sample were stained with Pro-Q Diamond according to the manufacturer's instructions (Molecular Probe). In this protocol, all gels are treated twice with 100 ml fixation solution containing 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min at room temperature under gentle agitation. Gels were washed three times for 10 min each with 100 ml ultrapure water to remove methanol and acid acetic residues. Gels were then stained with a volume of Pro-Q Diamond equal to 10 times the volume of the gels, under gentle agitation in the dark for 90 min. After staining, gels were destained in 80 ml of destaining solution (20%, v/v acetonitrile, 50 mM sodium acetate, pH 4.0) under gentle agitation in dark for 30 min at room temperature. This procedure, which was necessary to reduce the gel background due to not-specific staining, was repeated two more times. Destained gels were washed three times for 5 min per wash with ultrapure water at room temperature, and acquired using the GS-800 calibrated densitometer (Bio-Rad). To visualize total proteins, the same gels were stained with 60 ml per gel of Sypro Ruby overnight under gentle agitation at room temperature. Stained gels were then washed with 100 ml of wash solution containing 10% (v/v) methanol and 7% (v/v) acetic acid for 30 min, followed by three washes in ultrapure water for 5 min each.

2.7. Peptide identification

Protein spots were excised from the gels and digested in a 5-μl solution containing 20 μg/ml trypsin, and the mix was incubated in a solution containing 40 mM ammonium bicarbonate and 10% acetonitrile, at 37 °C overnight. Tryptic digested peptides were supplemented with a solution containing 50% acetonitrile and 0.1% trifluoroacetic acid and used for mass spectrometry analysis. The MALDI-TOF MS analyses of the tryptic digested peptides were carried out with the "Voyager-DE STR Bio Spectrometry Workstation" (Applied Biosystem). Peptides were selected in the mass range of 900–5000 Da. To identify the peptides obtained, we used the Mascot Peptide Mass Fingerprinting software (http://www.matrixscience.com; Perkins et al., 1999) to query the Mass Spectrometry Protein Sequence Data Base (MSDB). At the end of the MSDB search, we searched the literature to identify the protein functional classes.

2.8. Immunoblot analysis

Total protein of control and treated samples resolved by two-dimensional electrophoresis, as described above, were transferred (1 h at 100 V) onto a nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad). After the transfer, the nitrocellulose membrane was treated with a blocking solution (100 ml T-TBS1X, 5%, w/v powdered milk) for 1 h at room temperature, and successively incubated overnight at 4 °C with the purified primary anti-body against QP47 protein (Chiatante et al., 1995, 1997), diluted 1:500 in blocking solution. The membrane was rinsed three times in blocking solution at room temperature under gentle agitation to remove traces of unlinked primary antibody, and then incubated with alkaline phosphatase conjugated secondary antibody (anti-rabbit Ig-peroxidase, Sigma), diluted 1:2000 in blocking solution for 60 min at room temperature under gentle agitation. The membrane was rinsed three times in blocking solution, and three times in TBS 1X (0.2 M Trizma Base and 1.5 M NaCl made up to pH 7.6). Finally the membrane was developed with DAB solution (0.001% Tris pH 8.8, 0.0005% 3,3′-diaminobenzidine tetrahydrochloride, 100 μl H_2O_2 30%), at room temperature under gentle agitation until a brown reaction band appears. The stained membrane was then washed in distilled water and dried at room temperature.

2.9. Abscisic acid content determination

The level of ABA was measured in untreated, treated and germinated seeds as reported elsewhere (Chudasama and Thaker, 2007), with some variations. In detail, 500 mg of seeds were reduced to powder by mechanical breakage with liquid nitrogen, and ABA was extracted for 16 h in 5 ml extraction buffer (80%, v/v methanol and 0.1%, w/v butylated hydroxytoluene) in darkness at 4 °C under agitation. The samples were centrifuged at 35,000 × *g* for 30 min at 4 °C and the supernatant was recovered. The supernatant was air-dried for some minutes to remove traces of methanol. The dried supernatant was dissolved in 1 ml of Tris-buffered saline (25 mM Tris, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.5) and used to measure ABA content. Abscisic acid concentration was determined by competitive ELISA using the Phytodetek ABA Test Kit (Agdia Incorporated, Indiana, USA) according to the instructions included in the kit. All samples were run in triplicate and ABA standards were included in each run for construction of the standard curve. Standard and sample absorbency was read at 405 nm using a microplate reader (Labsystem, Multiscan EX) and ABA content was measured using interpolation on the standard curve.

3. Results

3.1. Seed viability

The viability of mature dry seeds was determined with the tetrazolium salts colorimetric test as reported under Section 2. The embryo and cotyledons of mature seeds were stained deep red thereby indicating viable seeds, whereas control seeds boiled for 2 h were not stained (Fig. 1, Supplementary data). The percentage of stained seeds was very low in the control (untreated, 7%) samples (Fig. 1). The percentage of red seeds increased in samples subjected to hand mechanical scarification (up to 96.5%), or to freezing at −20 °C and thawing (up to 21%), or to heating at 100 °C (96%).

3.2. Germination test

As shown in Fig. 2, the different treatments affected germination rate and percentage in different ways. In detail, control seeds had the lowest percentage of germination, namely, 5% and a T_{50} of 9.5 days. Seeds subjected to mechanical scarification had the highest percentage of germination (98%) with a T_{50} of 0.9 days (Fig. 2). The percentage of germination of seeds frozen at −20 °C for 60 days was also very high, namely, 93%, with a T_{50} of around 11 days. Only 25% of seeds subjected to heat treatment germinated with a T_{50} of 7.2 days (Fig. 2).

3.3. Abscisic acid content

To investigate the involvement of the hormone ABA acid in *M. marina* dormancy, we carried out an ELISA immunoassay on control, treated and germinated seeds. Fig. 3 shows the fold-change of ABA in treated seeds compared to control (untreated) seeds. The level of ABA was higher in all treated seeds than in control seeds. The highest concentration of ABA (an 8.7-fold increase versus controls) occurred in seeds heated for 1 h at 100 °C. The level of ABA was almost 2-fold higher in seeds subjected to freezing or to scarification than in controls.

The level of ABA was significantly lower in all samples (both treated and controls) at the beginning of germination than before germination. ABA was practically undetectable in seeds germinated

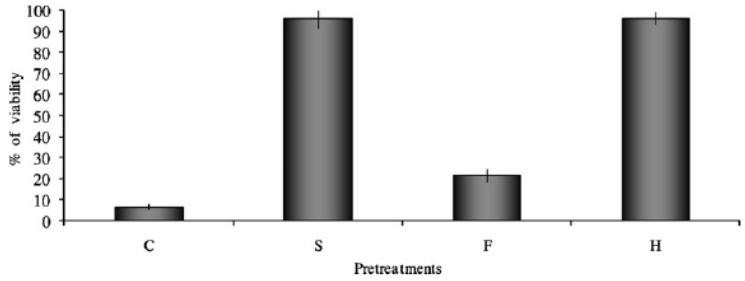


Fig. 1. Percentage viability of untreated seed (C; control) and mechanical scarification (S), freezing (F) and heating (H) seeds evaluated by the tetrazolium salt test. Vertical bars represent the standard deviation of the mean of three replicates of 30 seeds each.

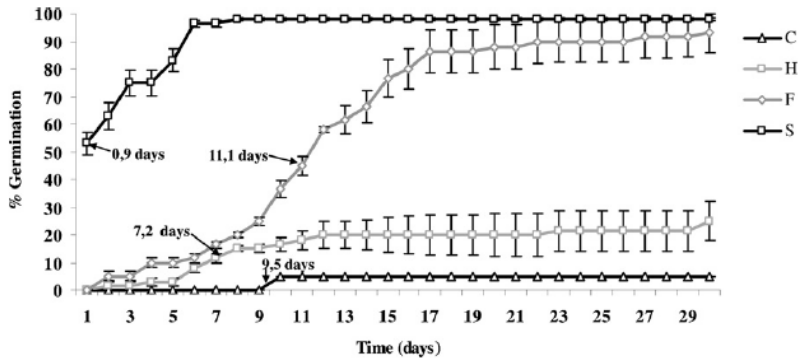


Fig. 2. Germination trend of control (C) seeds and treated seeds: mechanical scarification (S), freezing (F) and heating (H). Arrows indicate mean germination time for each treatment; vertical bars represent the standard deviation of the mean of three replicates of 30 seeds each.

from the untreated and mechanically scarified samples. The concentration of ABA was significantly lower in seeds germinated from the samples subjected to freezing or heating, whereas it was higher in heating-exposed seeds than in controls.

3.4. Proteomic analysis

We used a proteomic approach to compare the protein alterations in *M. marina* dry mature seed (control) versus treated seeds. Protein samples were separated by two-dimensional electrophoresis, which resulted in well-resolved and reproducible seed proteome maps with an average of 370 distinguishable spots (Fig. 4). We used PDQuest (Bio-Rad, version 8.0) to compare the proteome maps of untreated and treated seed. A total of 46 spots were found to be differentially expressed among treated and untreated seed (Fig. 5). These spots were excised from the gel for sequencing by MALDI TOF and liquid chromatography MS/MS, and 14 were subsequently identified by homology with other legume species, namely, *Medicago sativa, Medicago truncatula, Pisum sativum* and *Vicia faba* (Table 1). The proteins identified were classified into functional classes, and are listed in Table 1, which also shows the level of each protein spot.

Spot 5, a legumin A2 primary translation product, was expressed in control seeds, but not in the treated samples (frozen, heated and scarified). Spot 20 (legumin) was present in all samples, and its expression was lowest in frozen seeds. The expression of spot 20 was higher in heated and scarified seeds than in control seeds. Spot 6 (convicillin) was present in the control and heated samples, but not in frozen or scarified seeds.

Five spots (n. 7, 22, 23, 24 and 25) corresponded to different isoforms of RmlC-type cupin: spot n. 7 was present in control and heated seeds and absent from frozen and scarified seeds; spots 22 and 23 were detected in all samples, reached their highest level in control and scarified seeds; spots 24 and 25 were present in

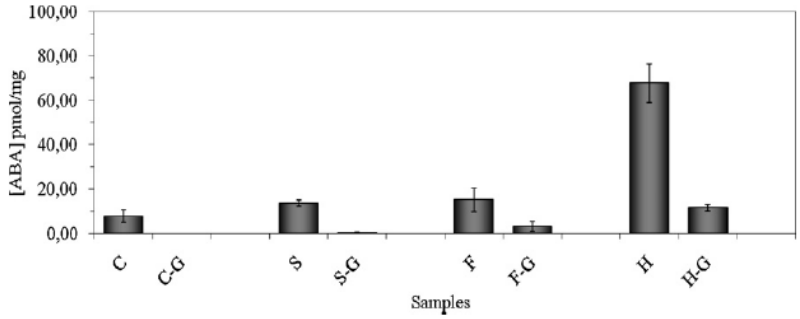
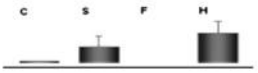
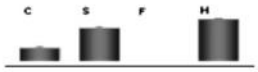
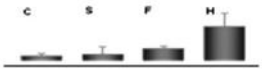
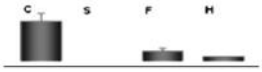
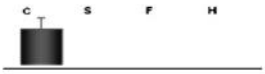
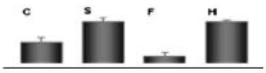
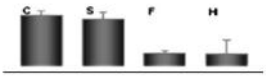
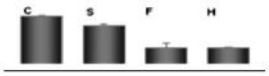
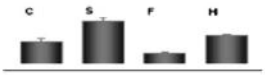
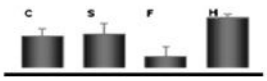


Fig. 3. Histograms of the abscisic acid content in control seeds, treated seeds, and seeds germinated after treatment. C: control seeds; S: mechanically scarified seeds F: frozen seeds; H: heated seeds; C-G: control seeds germinated; S-G: seeds germinated after mechanical scarification; F and G: seeds germinated after freezing; H-G: seeds germinated after heating. Vertical bars represent the standard deviation of the mean of three replicates obtained in three independent assays.

Table 1

Proteins identified in the 2-DE proteomic map of *Medicago marina* seeds were classified into functional classes. Spot ID number, protein name, accession number, organism, sequence coverage, theoretical and experimental pI and Mr values and peptides sequence identified, are listed, together with the expression trend in the control and different treated seed. Spot numbering refers to Fig. 5.

Spot ID	Protein name	Accession	Organism	Seq. Cov.%	Theor. Mr/pI	Exp. Mr/pI	Peptide	Expression trend
Stress e Difence								
12	Probable protein disulfide-isomerase A6 precursor (P5)	P38661	Medicago sativa	15%	40,8/5,4	45,1/5,5	K.SYELPDGQVITIGSER.F	
19	Peptidyl-prolyl cis-trans isomerase (vcCyP)	O64456	Vicia faba	32%	18,2/8,9	18,1/7,7	R.IIFELFADVTPR.T	
17	Copine like protein	Q8LBF2	Arabidopsis thaliana	19%	35,8/8,8	36,1/7,7	K.KWSSQPYLSR.R	
Transcription Factor								
9	Retrotrasposon protein	Q2QUM4	Oriza sativa	22%	35,7/8,1	28,8/6,1	K.LCHVFRLYTVVMAGSVSR.R	
Carbon Metabolism								
11	Thiamine biosynthetic enzyme	Q5G1J2	Picrorhiza kurroa	11%	37,6/5,0	37,5/5,4	M.AAMAATLTSSLTK.S	
15	Biotin carboxylase precursor	O81273	Glycine max	12%	59,3/7,2	55,2/6,9	R.HIERQVLADK.Y	
Seed Storage Protein								
5	Legumin A2 primary translation product	Q99304	Vicia faba var. minor	5%	57,0/6,1	18,8/5,8	R.DFLEDALNVNR.H	
6	Convicilin	B0BCK5	Lathyrus c. var. c.	4%	58,1/6,1	22,1/5,4	R.TLFENENGHIR.R	
7	Cupin, RmlC-type	Q2HW22	Medicago truncatula	11%	53,2/5,4	25,2/5,9	R.NSFNLER.G	
20	Legumin (minor small)	O24294	Pisum sativum	5%	65,1/5,4	44,0/6,5	K.CAGVSLR.R	
22	Cupin, RmlC-type	Q2HW22	Medicago truncatula	15%	56,4/5,9	56,5/6,2	K.CEDYHR.M	
23	Cupin, RmlC-type	Q2HW22	Medicago truncatula	22%	56,4/5,9	56,2/6,8	R.QHQHQR.E	
24	Cupin, RmlC-type	Q2HW22	Medicago truncatula	22%	56,4/5,9	56,1/6,9	K.CEDYHR.M	
25	Cupin, RmlC-type	Q2HW22	Medicago truncatula	23%	56,4/5,9	56,1/7,1	K.TCIHQCK.Q	

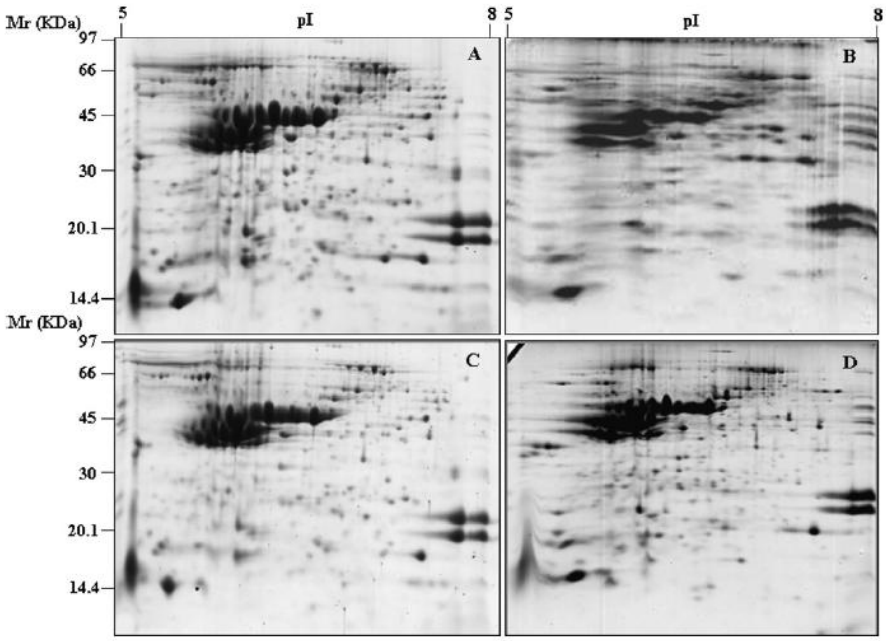


Fig. 4. Reproducible maps of control and pretreated (mechanical scarification, freezing and heating) *Medicago marina* seeds obtained by two-dimensional electrophoresis. The maps were produced in triplicate for each of two independent protein extraction experiments and the most abundant and well-separated proteins were resolved. Average proteomic maps, on a linear 5–8 pH gradient, showed 350–400 well-resolved spots, ranging in Mr from about 209–7 kDa.
A: control seeds; B: mechanically scarified seeds; C: frozen seeds; D: heated seeds.

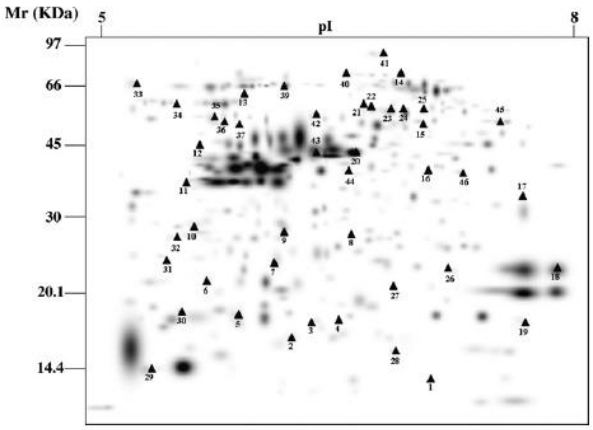


Fig. 5. Two-dimensional electrophoretic reference map of *Medicago marina* seeds. Proteins were resolved on IPGs (first dimension), 12% SDS-PAGE (second dimension) and visualized by colloidal Coomassie blue staining. Spot numbering refers to Table 1 that shows the protein identification as ascertained by MS/MS and MALDI-TOF analysis.

all samples, however spot 24 was expressed mostly in the scarified samples, and spot 25 in the heated samples. Two spots (n. 11, 15) were identified as important factors of carbon metabolism (Ferreira et al., 2006). Spot 11, corresponding to a thiamine biosynthetic enzyme, was mainly expressed in heated seeds, whereas spot 15, corresponding to biotin carboxylase, was significantly higher in control seeds than in all treated seeds. Spots 12 and 19, probably a protein disulfide-isomerase A6 precursor (P5) and a peptidyl-prolyl cis-trans isomerise respectively, were classified as chaperons. The expression of both proteins was higher in heated seed than in control seeds, neither was expressed in frozen and scarified seeds. The expression of spot 9, corresponding to a retrotransposon protein, was high in heated and scarified seeds, low in control seeds and absent from frozen seeds. Spot 17, identified as a copin-like protein, was present in control seeds, and greatly decreased in all treated samples.

3.5. Phosphoproteome analysis

The comparison of phosphoproteome maps of seeds revealed some phosphorylated proteins in untreated and treated seeds (Fig. 6). The number of phosphorylated proteins was higher in frozen and heated seeds than in control seeds, and was higher in frozen than in heated seeds (Fig. 6D). The level of phosphorylation was lower in seeds subjected to mechanical scarification than in control seeds (Fig. 6B).

A comparison of the phosphoproteome maps with the proteome maps showed that only a few of the proteins previously identified were phosphorylated. In particular, as shown in Fig. 6 and Table 1, the protein identified as legumin (spot 20) was phosphorylated in control, mechanically scarified, frozen and heated seeds. Spots 22, 23, 24 and 25, identified as RmlC-type cupins, were highly phosphorylated in heated seeds, whereas spot 12, which is probably a protein disulphide isomerase A6 precursor, was phosphorylated only in heated seeds.

3.6. Immunoblot analysis

Since 5 of the identified spots corresponded to cupin-like proteins (Fig. 5, Table 1), we verified their identification by immunoblotting analysis using a polyclonal antibody against the cupin QP47 (Chiatante et al., 1995, 1997; Castillo et al., 2005). As shown in Fig. 7, the antibody recognized four (n. 22, 23, 24, 25) of the five spots identified as cupin. However, the antibody recognized spot 21 (Figs. 5 and 7) that was not detected by mass spectrometry.

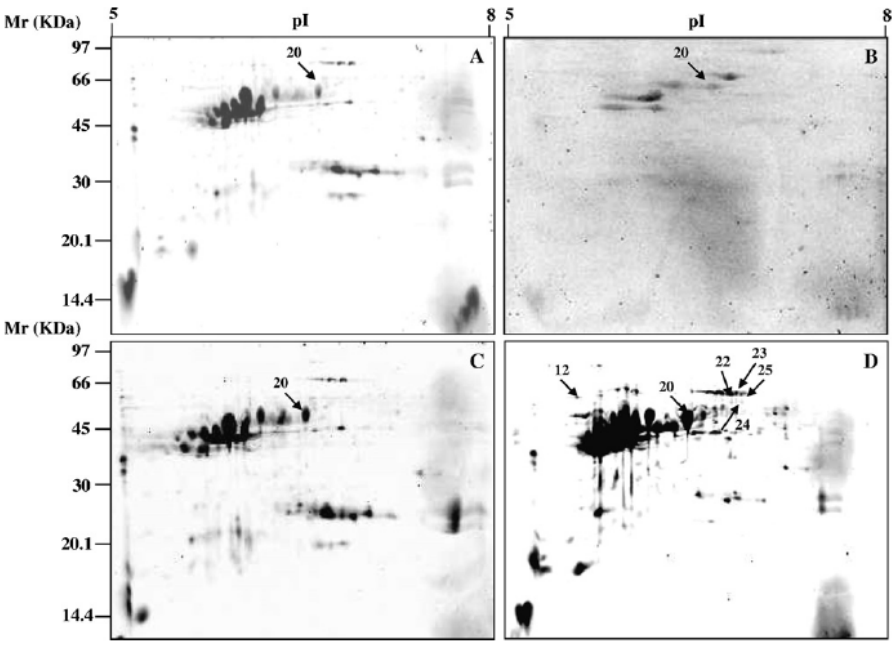


Fig. 6. Phosphoproteome maps of control, mechanically scarified, frozen and heated seeds. The arrows indicate the phosphorylated protein, previously identified in the proteomic analysis. The number of spots coincides with the numeration on the two-dimensional electrophoresis reference map in Fig. 6. (A) Control seeds; (B) mechanically scarified seeds; (C) frozen seeds; (D) heated seeds.

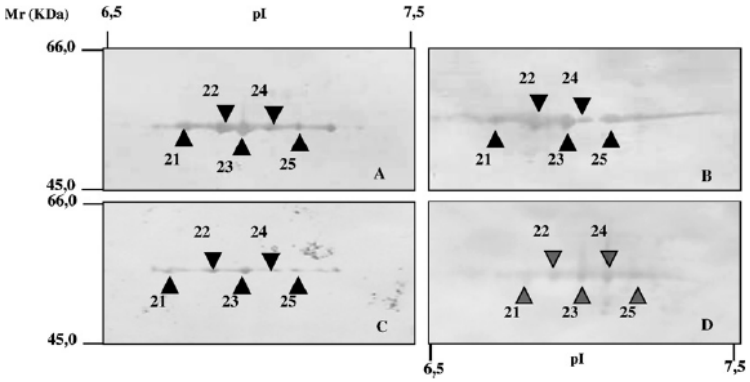


Fig. 7. Polyclonal antibody against QP47 revealed five protein isoforms probably involved in protecting the cellular structures from dehydration, during maturation of the seed. (A) Control seeds; (B) mechanically scarified seeds; (C) frozen seeds; (D) heated seeds.

4. Discussion

Only a very small fraction of *M. marina* (L.) control seeds (up to 5%) germinated, which coincides with the results of the tetrazolium salt viability test. The percentage of germination was significantly increased in seeds subjected to mechanical scarification, in seeds subjected to freezing and thawing and, albeit to a lesser extent, in seeds subjected to heating. These results indicate that, similar to other legume species (Van Assche et al., 2003), *M. marina* seeds are characterised by a coat-imposed dormancy, known as 'hardseededness', an ecological mechanism that allows seeds to germinate only when conditions are suitable for seedling growth (Argel and Paton, 1999).

Various treatments, i.e., mechanical and chemical scarification, and high and ultra-low temperatures, have been reported to be effective in reducing hardseededness and improving germination rate in several legume species (Patanè and Gresta, 2006; Ibanez and Passera, 1997; Sy and Danthu, 2001). We evaluated the capacity of three types of treatment to break seed dormancy and found that each treatment exerted distinct effects on germination percentage and rate. Mechanical scarification with abrasive paper (hand scarification), and freezing treatment efficiently removed seed dormancy, indeed they resulted in almost 100% germination. Heating at 100 °C, a scarification technique previously used to reduce hardseedness in other legumes (Cushwa et al., 1968), was substantially less effective than hand scarification and freezing in breaking dormancy.

Patanè and Gresta (2006) showed that hand scarification and freezing at an ultra-low temperature (−196 °C) considerably increased the percentage of germinated *Medicago orbicularis* seeds, whereas hot water did not significantly affect dormancy-breaking. The authors suggested that hand scarification and freezing caused small cracks in the seed coat that allowed the uptake of water thereby overcoming the coat-imposed dormancy characteristic of *Medicago* species. In our study, freezing of *M. marina* seeds at −20 °C for 60 days and thawing at room temperature resulted in a germination rate of 93%. Thus, it is reasonable to assume that the slow

thawing of seeds caused small cracks in the seed coat that allowed the uptake of water thereby interrupting dormancy. In nature, high temperatures and freezing/thawing have been reported to break coat-imposed dormancy (Baskin et al., 2000). It is conceivable that in the case of *M. marina* seeds, the alternation of low and high temperatures during winter and spring may be one of the factors involved in breaking coat-imposed dormancy.

The germination rates, measured as T_{50}, of seeds subjected to freezing, were much slower than those of mechanically scarified seeds and even slower than those of untreated seeds. The differences in germination rates, and percentages among seeds subjected to freezing or to mechanical scarification may be interpreted as a result of the different effects of mechanical scarification and freezing treatments on the hard coat of *M. marina* seed.

To monitor water uptake, we evaluated the uptake of tetrazolium salts by the embryo to obtain a measure of seed coat permeability (Debeaujon et al., 2000). The staining percentage provides a measure of water permeability, and confirms that scarification and freezing cause different cracks, in terms of size, number and morphology, in the seed coat surface thereby providing different channels for the entry of water (Patanè and Bradford, 1993).

There are no data about the anatomical structure of the *M. marina* seed coat. However, the general features of the seed coat of the model plant *M. truncatula* are well described, namely, an epidermal layer of macrosclereids (epidermis), a subepidermal layer of osteosclereids (hypodermis) and two to five rows of internal parenchyma cells, which is similar to the seed coat structure of other *Medicago* species (Small et al., 1990; Jha and Pal, 1992). An anatomical structure in the impermeable layer(s) of the seed coat functions as the "water gap" (Baskin et al., 2000); this structure in legumes is called "lens". Seed stress, for example imposed by high and low temperatures causes disruption (pulling apart) of the thin-walled cells of the lens thereby allowing water entry. The lens functions as an environmental "signal detector"; it may act as regulator of the rate of water entry into the seed, thereby affecting seed and seedling vigour. Based on these anatomical features, it is conceivable that freezing may pull the lens apart. Microscope investigations are required to verify this hypothesis. Hot water treatment did not greatly improve the percentage of germination of *M. marina* seeds, although seed viability was not affected by heat stress. Studies on the effectiveness of hot water in promoting seed germination in several *Medicago* spp. have yielded contrasting results (Patanè and Bradford, 1993; Patanè and Gresta, 2006; Uzun and Aydin, 2004). However, as proposed for other species, *M. marina* seeds may have a delaying mechanism (thermoinhibition) to prevent germination when moisture is not sufficient, during late summer and early autumn, for seedling establishment and growth (Thanos & Georghiou, 1988; Thanos et al., 1992; Abeles, 1986; Gallardo et al., 1991; Yoshioka et al., 1998).

We also investigated whether the hormone ABA, is involved in the regulation of *M. marina* seed dormancy, germination, and adaptive responses to abiotic stresses. We found that ABA was significantly higher (almost nine fold) in heated seeds than in control seeds. Moreover, it was two-fold higher in seeds subjected to scarification or freezing versus controls. The level of ABA decreased significantly in germinating seeds: it was not detectable in germinating control and scarified seeds, very low in germinating frozen seeds, and higher in germinating heat-treated seeds than in the control.

In *Arabidopsis thaliana* it has been shown that the concentration of ABA is highest during seed maturation, it decreases during seed desiccation and is relatively low in mature seeds (Karssen et al., 1983). However, the effects of ABA on regulating physiological processes in seeds depend on active hormone levels, which might be modulated by the rate of ABA precursor synthesis, hormone catabolism or its translocation from or to other sites. It is feasible that the increase of ABA in response to scarification, freezing and heating observed in *M. marina* may be due to one of the above-mentioned mechanisms. The higher level of ABA in treated seeds and in germinating seeds subjected to heating and freezing than in germinating control seeds suggests that *M. marina* might have a specific mechanism to modulate ABA content in response to stress and may use a specific ABA-signalling pathway to regulate dormancy/germination.

Proteomic analysis demonstrates altered protein patterns and post-translational modifications in seeds subjected to various types of treatment. We found that 46 spots were differentially expressed between control and treated seeds. The lack of recognition of 22/46 spots was due to the paucity of information about the *M. marina* genome. Three spots (n. 5, 6, 20) were identified as storage proteins, namely, 7S (vicilin and convicillin) and 11S (legumins) globulins, which have been reported to be functionally involved in providing the developing seedling with nitrogen, carbon and sulphur during plant germination (Shewry et al., 1995). Given their putative role in protecting seeds from environmental stress (Agizzio et al., 2003), the changes in storage proteins reported herein may be interpreted as mobilization of these proteins either in response to the abiotic stress or in preparation for the germination process.

Five spots (n. 7, 22, 23, 24, 25) corresponding to RmlC-type cupins, also classified as storage proteins, may be involved in such other relevant functions as germination and stress defence. In fact, cupins are a protein superfamily that includes enzymes, auxin-binding protein, and some nuclear or DNA-binding proteins (Dunwell et al., 2004). In our study, the identification of spots n. 22, 23, 24, 25 as cupin was confirmed by immunoblotting experiments using an antibody against a pea cupin (Chiatante et al., 1995, 1997). This antibody indicated that also spot n. 21, which was not identified by mass spectrometry, might be a cupin, whereas the antibody did not recognize spot n.7, which was identified as cupin by mass spectrometry. The antibody was produced against a pea nuclear protein, QP47 (Mr 47,000), which, have been proposed to protect chromatin against desiccation during seed maturation (Chiatante et al., 1995). Furthermore, Castillo et al. (2005) proposed that QP47 corresponds to a product of p54 processing. They also suggested that p54 is ABA-responsive and might serve as a precursor of two stress-related multifunctional seed proteins, namely p16 and p38, that are involved in nuclear processes. The mass spectrometry data and the recognition by the antibody strongly suggest that, also in *M. marina* seeds, there are several cupin isoforms that protect chromatin from abiotic stresses. Our data also show that the function of the cupins identified in *M. marina* seeds may be modulated, by post-translational alterations such as phosphorylation, which appears to be an important control mechanism in response to freezing and heating. Spot n. 7, also identified as cupin by mass spectrometry, was not recognized by the antibody, which suggests that this protein might have a role other than that of stress defence, i.e., storage. However, we found that a retrotransposon protein (spot n. 9) highly induced by heat and mechanical scarification treatments in *M. marina* seed may play also a role in chromatin protection or remodelling. Retrotransposon proteins, in fact, are induced by such abiotic stresses as mechanical wounding, drought and high temperature (Tapia et al., 2005), and being chromatin-associated may modulate the early steps of the defence gene activation pathways (Grandbastien et al., 1997).

Spot n. 11, which was up-regulated in heated seeds, corresponds to a thiamine biosynthetic enzyme that is an important carbon metabolism factor that seems to alleviate the oxidative stress response to heat shock (Ferreira et al., 2006). Other factors that seem to be involved in the response of *M. marina* to heating are two chaperones, a peptidyl prolyl isomerase (spot n. 19) and a protein disulfide isomerase A6 precursor (spot n. 12). Both chaperons are involved in protein folding and play important roles in normal

growth and heat tolerance (Kurek et al., 1999; Miernyk, 1999; Tian et al., 2009).

Spot n. 15, a biotin carboxylase precursor, level was high in control seeds, and very low in the three treated seeds. Biotin carboxylase plays an important role in lipid metabolism and in membrane stability in response to heat (Ferreira et al., 2006). Thus, the decrease of biotin carboxylase precursor in response to heating, freezing and scarification, may be due to a processing necessary to produce the active form of the enzyme required to membrane stability.

A factor that might be involved in membrane stability of the *M. marina* dry seed is a copin-like protein (spot n. 17), which was mainly expressed in control seeds. Copin-like proteins have found to be associated with lipid membranes (Creutz et al., 1998; Tomsig and Creutz, 2000), and seem to be involved in plant responses to low humidity (Jambunathan et al., 2001).

In conclusion, this study confirms that *M. marina* seeds, like the seeds of other legumes, have adopted a dormancy strategy based on a hard seed coat. However, the different scarification treatments used yielded different results on germination percentage and rate, indicating that dormancy is regulated by other mechanisms beside seed coat. Furthermore, we show that seeds perceive the three treatments used to break dormancy as environmental stress conditions that evoke an increase of ABA level and of the expression of proteins that protect important cellular component, i.e. chromatin and the cell membrane. Finally, our results demonstrate that: (a) another complex mechanism, i.e. photoinibition, besides just hardseedness is involved in *M. marina* dormancy; (b) ABA regulates *M. marina* seed dormancy/germination and seed response to environmental stress through different signalling pathways.

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Chapter II

Dormancy of *Medicago marina* (L.) seed: the role of the micropylar endosperm

1. Introduction

The endosperm is an important tissue not only as a source of nutrients for the embryo during seed development and maturation, but also as an integrator of seed growth signals. As demonstrated by Berger et al., (2006), reciprocal signalling between the embryo, the endosperm and seed integuments appears to coordinate the seed growth and development. In particular, in this work the authors highlight the role of the endosperm in mediating maternal signals that are based on memory of the transcriptional states of imprinted genes, which is decisive for harmonious seed growth (Berger et al., 2006).

When seed maturation is completed, depending on the species, the endosperm may be fully or partially lost (Linkies et al., 2010). However, in most angiosperm species which preserve an endosperm layer in their mature seeds, the endosperm can be involved in the control of germination. In mature seeds, the endosperm and in particular the one between the root embryo and micropyle, namely micropylar endosperm is able to regulate the timing of germination, by being a physical barrier for the radicle protrusion (Ni & Bradford, 1993; Bewley, 1997; Toorop et al., 2000; Leubner - Metzger, 2003; Finch-Savage & Leubner-Metzeger, 2006).

However, because the germination of mature seed can be completed, it is needed that two events occur simultaneously: 1) the radicle starts to elongate, trying to overcome the endosperm and 2) the covering layers around the embryo and the micropylar endosperm gets softer, allowing the radicle expansion (Ni & Bradford, 1993). The process making the endosperm to become soft during germination is called endosperm weakening (Ni & Bradford, 1993). This process occurs during germination, in between testa and endosperm rupture, as showed for *Lepidium sativum* seed, where it seems to be regulated trough a large number of enzymes and hydroxyl radicals (Muller et al., 2006).

Endosperm weakening requires the action of cell-wall modifying enzymes such as endo-β-mannanase, expansins (Sitrit et al., 1999; Chen & Bradford, 2000; Nonogaki et al., 2000; Chen et al., 2002, Iglesias-Fernández et al., 2011), β-1,3-glucanases (β-Glu) (Leubner-Metzger, 2003) and polygalacturonases (PGs) González-Carranza et al., (2007).

Many authors (Nonogaki & Morohashi, 1996; Chen & Bradford, 2000, Nonogaki et al., 2000) showed that endo-β-mannanase and expansins are specifically expressed in tomato endosperm

cup during seed germination. In fact, the wall of endosperm cells are rich in mannanase (galactomannans, glucomannans), hemicellulosic polysaccharides that confer a strong mechanical resistance for the radicle protrusion in germinating seeds. The endo-β-mannanase (MAN) are hydrolytic enzymes which catalyze the cleavage of β1→4 bonds in the mannan-polymer (Iglesias-Fernández et al., 2011) and their role in endosperm weakening has been widely demonstrated. Nonogaki et al., (2000) showed that in the endosperm cap of germinating tomato seeds prior radicle emergence, there is an endo-β-mannanase isoform different from the one found in the lateral endosperm after radicle emergence. Furthermore, the paper demonstrated that germinative and post-germinative mannanases are encoded by different genes and in particular, the mannanase cDNA isolated from a cDNA library prepared from imbibed seeds, termed *LeMAN2*, encodes for the endo-β-mannanase specifically expressed in the endosperm cap of tomato seeds prior to radicle emergence. In a recent work, Iglesias-Fernández et al., (2011) investigated the role of the eight *MAN* genes in the *A. thaliana* genome in different organs of this plant and in the germinating seeds. Using Real-time quantitative PCR assays and in situ hybridization analyses, the authors demonstrated that four of these genes (*AtMAN7*, *AtMAN6*, *AtMAN2* and *AtMAN5*) were expressed in germinating seeds and that their transcript accumulation was restricted to the micropylar endosperm and to the radicle, disappearing immediately after radicle protrusion.

From these results the authors pointed to a possible cooperation between embryo and endosperm MAN activities, prior to radicle emergence in *A. thaliana* seeds, suggesting the important role of *AtMAN7*, *AtMAN5* and specially *AtMAN6* genes and their products for the germination by facilitating the weakening of the micropylar endosperm through the hydrolysis of the mannan-rich cell walls.

Leubner-Metzger (2003) demonstrated that β-1,3-glucanases (βGlu), which are able to catalyse hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucanas, present in the cell wall, are expressed in some *Solanaceae* species such as tobacco and tomato. In particular β-Glu 1 has been shown to play a key role in tobacco seed germination. β-Glu 1 is induced during tobacco seed germination after testa rupture and just prior to endosperm rupture, and it is exclusively localized in the micropylar endosperm. Moreover, the hormonal interactions during endosperm weakening regulate the expression of cell wall modifying enzymes. In several works (Leubner, 2003; Kucera et al., 2005, Müller et al., 2006; Gimeno-Gilles, 2009; Linkies et al., 2009) it has been demonstrated that the endosperm weakening is promoted by gibberellins and inhibited by abscissic acid, which regulate the weakening by their antagonistic effect on the endosperm.

Endosperm rupture involves the light/gibberellins pathway. Different class of hydrolases and class I β-1-3-glucanase (βGlu I) are induced by the light/gibberellins pathway in the micropylar endosperm, allowing endosperm rupture and radicle protrusion (Leubner, 2003; Kucera et al., 2005). However it has been shown that abscissic acid is able to repress the expression of endosperm-specific βGlu I and other class of enzymes involved in cell wall loosening and expansion, such as xyloglucan endotransglucosylase, and, consequently the endosperm rupture (Leubner, 2003; Kucera et al., 2005; Gimeno-Gilles, 2009). In tobacco seeds, abscissic acid has been shown to inhibit β-Glu 1 induction, delaying endosperm rupture in a dose-dependent manner (Leubner-Metzger, 2003). Moreover, in this work is also reported that the endosperm rupture kinetic depends on a critical threshold concentration of β-Glu 1, while the kinetic of testa rupture is not affected by the β-Glu 1 accumulation, indicating the specific action of this enzyme in promoting micropylar endosperm rupture (Leubner-Metzger, 2003).

Light/gibberellins pathway can counteracts the abscissic acid effect by promoting its degradation (Leubner, 2003; Kucera et al., 2005), and also, ethylene counteracts ABA effect, antagonizing the ABA inhibition and promoting endosperm rupture (Linkies et al., 2009). In particular, ethylene action, in counteracting ABA effect, is due to specific transcription factor responsive to ethylene signal, called Ethylene Responsive Element Binding Proteins (EREBPs), that mediate hormonal regulation of cell wall modifying enzymes, such as the βGlu I (Leubner, 2003; Kucera et al., 2005).

The action of other cell wall loosening enzymes in endosperm rupture, as polygalacturonases, is reported in a work by González-Carranza et al. (2007) which analysed the endosperm cap of *Arabidopsis thaliana*. Polygalacturonases (PGs) play an important role in the process of cell separation, causing the breakdown of adhesion between neighbouring cells (Rose et al., 2003). The *A. thaliana* genome contains 69 genes that could be classified as putative genes that encode for PGs proteins and these genes can be grouped into multiple clades. From the analysis of five of these members located in two separate clades, using reporter fusion constructs and reverse transcription-PCR, the authors demonstrated that, despite these PGs exhibit high sequence similarity, they have distinct patterns of spatial and temporal expression, and sites of expression include also the aleurone and cells surrounding the micropylar endosperm in a germinating seed (González-Carranza et al., 2007). The gene *At2g43860* that encodes for a polygalacturonases expressed also in the abscission zones and pistils of tomato flowers (Hong et al., 2000), is specifically expressed in the micropylar endosperm region of *A. thaliana* seed, indicating a key role for cell wall-degrading enzymes, including PGs, in seed germination (González-Carranza et al., 2007). Sitrit et al., (1999), and subsequently Chen et al., (2002), on the basis of several studies performed on germinating tomato seeds, showed that the transcripts of expansin and gene

encoding for xiloglucan endotransglycosylase (XET), that modify xyloglucans, major components of primary cell walls in dicots, accumulate precisely in the endosperm region adjacent to the expanding radicle. These results additionally support the theory that a large number of cell wall-degrading enzymes are involved in micropylar endosperm weakening during seed germination. The expression of the weakening enzymes in endosperm cap allows cell wall loosening and cell separation that results in the endosperm rupture and, finally, in the radicle emergence.

In addition to the activities of the hydrolytic enzymes in the recent years, the role of hydroxyl radicals in cell wall loosening has been investigated. Preliminary experiments by Miller (1986) and Fry (1998) suggested that OH^-, produced in the apoplastic space of plant tissues, plays an important physiological role in cell-wall loosening process underlying cell expansion. In fact, they showed that two cell-wall polysaccharides, pectin and xyloglucan, can be broken down in vitro by OH^-. Supporting these data, Chen & Schopfer (1999), found that plant peroxidase can catalyse the production of OH^- from O_2 in the presence of a NADH, in the apoplastic space of germinating seeds. In two independent studies Fry et al. (2001), and Schopfer (2001) proposed the non-enzymatic degradation of cell wall polymers by hydroxyl radical (OH^-). The experiments reported in this work, carried out on excised embryo hypocotyl segments of different plant species, such as *Zea mays* (L.), *Cucumis sativus* (L.), *Helianthus annus* (L.), *Glycine max* (L.), *Pinus sylvestris* (L.), supported the hypothesis proposing OH^- as the wall loosening agent responsible for elongation growth, illustrating that the wall-loosening reaction appears to be equally effective in growing organs of each of these species, and also, the independence of OH^- mediated wall loosening from species-specific differences in chemical wall properties (Schopfer, 2001). Recently, Muller et al., (2009), showed that OH^- radicals play a role in endosperm weakening and rupture of *Lepidium sativum*, and that their production increase in the endosperm cap and in radicle of imbibed seeds. Furthermore, it has been also show that the increase of OH^- radicals is promoted by GA and ethylene and inhibited by ABA (Graeber et al., 2010).

Due to its involvement in regulating seed germination, endosperm can also contribute to dormancy. In fact, as shown in *Arabidopsis thaliana* and in some *Solanaceae* species (Bewley, 1997; Koornneef, 2002), such as tomato and tobacco, the micropylar endosperm can contribute to dormancy by inhibiting the protrusion of the growing radicle through the covering layers of the embryo. Bethke et al., (2007) found that the only micropylar endosperm is enough to confer coat dormancy in *A. thaliana*. As described by Windsor et al., (2000) and Debeaujon et al., (2000), the seed coats of *Arabidopsis* consist of an external layer, called testa, which consist of dead cells, and an internal single cell layer of living aleurone cells, which represent the sole endosperm tissue. As demonstrated by Bethke et al., (2007), one function of the *Arabidopsis*

aleurone layer is to maintain the dormancy of imbibed seeds, as removal of the *Arabidopsis* testa did not render seeds nondormant for up to 28 days, but removal of the aleurone layer or damage to the aleurone layer resulted in embryo growth, overcoming seed dormancy.

The studies carried out during the first phase of the PhD program and reported in the paper Scippa et al., (2011), showed that *Medicago marina* seed is characterized by a strong dormancy. Furthermore, based on the results obtained in the germination test of differently treated seed and in the hormone assay, it has been proposed that *M. marina* seed has a coat dormancy which seems to be finely regulated by complex molecular mechanisms. Since a large number of evidences point out the role of endosperm in the control of seed dormancy, the role of the endosperm in *Medicago marina* seeds dormancy has been investigated.

In particular, the weakening of endosperm was quantified trough a biomechanical approach using the puncture force test in seeds subjected to mechanical scarification and abscissic acid (ABA) treatment, along a 22 hours time course of imbibition. In addition to that, the effect of abscissic acid and gibberellic acid treatments on scarified seeds germination rate and speed, were analyzed by performing germination test, while RT-PCR analyses was used to evaluate the gene expression of key enzymes involved in abscissic acid, gibberellic acid and ethylene biosynthesis, namely NCED5 (9-cis-epoxycarotenoid dioxygenase), GA3ox (gibberellin 3-beta-hydroxylase) GA20ox (gibberellins 20 3-beta-hydroxylase) and ACO (1-aminocyclopropane-1-carboxylic acid oxidase). Then, through a proteomic approach coupled with stastical analysis, was studied the protein pattern expressed during the weakening of the endosperm and the effect of ABA on this pattern, during the time course of imbibition.

2. Materials and methods

2.1 Seed material and germination test

Dry mature *M. marina* (L.) seeds were collected in July 2010 from the Molise coastal dunes (Central-south Italy) and taken to the Molise Germplasm Bank (University of Molise). The seeds were removed from pods and cleaned. Then, seeds were sterilized in a solution of 1% sodium hypochlorite for 10 min, rinsed in distilled water, dried with adsorbent paper and subjected to mechanical scarification by sandpaper in order to promote the germination. A portion of scarified seeds were used for the germination test, while the remainder were subjected to imbibition along a 22 hours time course and used for the puncture force measurements, RT-PCR and proteomic analysis. Imbibition were performed placing scarified seeds on two sheets of Whatman filter paper moistened with 4 ml of sterilized distilled water with 0,2% (v/v) Plant Preservative Mixture (Plant Cell Technology Inc.), in 9-cm diameter Petri dishes with or without the addition of 100 μM (+/-) of Abscissic Acid (Sigma). All dishes were sealed with parafilm an incubated in darkness at a constant temperature of 25°C and, during the time course, harvested every two hours starting from 6-8 h after imbibition. After harvesting a portion of seeds were frozen in liquid nitrogen and stored at -80 °C until required for proteomic analysis and RT-PCR, while an other portion were immediately used for the puncture force measurement. Time course of imbibition was repeated in three independent replicates.

Germination was tested in presence or absence of abscissic acid (ABA) and gibberellic acid (GA_3) (Sigma) to evaluate possible differences in terms of germination rate and speed, due to hormonal treatments. At this purpose, three biological replicates of 60 scarified seeds were placed on two sheets of Whatman filter paper moistened with 4 ml of sterilized distilled water with 0,2% (v/v) Plant Preservative Mixture (Plant Cell Technology Inc.) in 9-cm diameter Petri dishes. Then, an other portion of scarified seeds were incubated in the same condition, but whit the addition of 100 μM (+/-) ABA (Sigma), 5 μM GA_3 (Sigma), 10 μM GA_3 and 100 μM (+/-) ABA plus 5 μM GA_3, individually, to test the germination. For each hormonal treatment were used three biological replicates of 60 seeds. All dishes were sealed with parafilm and incubated in darkness at a constant temperature of 25 °C for 7 days. According to Bacchetta et al. (2006), the results are expressed as final germination percentage (G%) and germination speed, expressed as number of hours required to reach 50% germination (T_{50}). A seed was considered germinated when the radicle emerged from the coat.

2.2 Puncture force measurement

Puncture force was measured on scarified seeds and scarified seeds treated with ABA, using an improved machine, showed in the figure 1, conceived and made by the Arbeitsgruppe Technik from the Albert Ludwig Universität (Freiburg, Germany), with the aim to evaluate the biomechanical changes that occur in the micropylar endosperm tissue during imbibition along a 22 hours time course, in presence or absence of ABA.

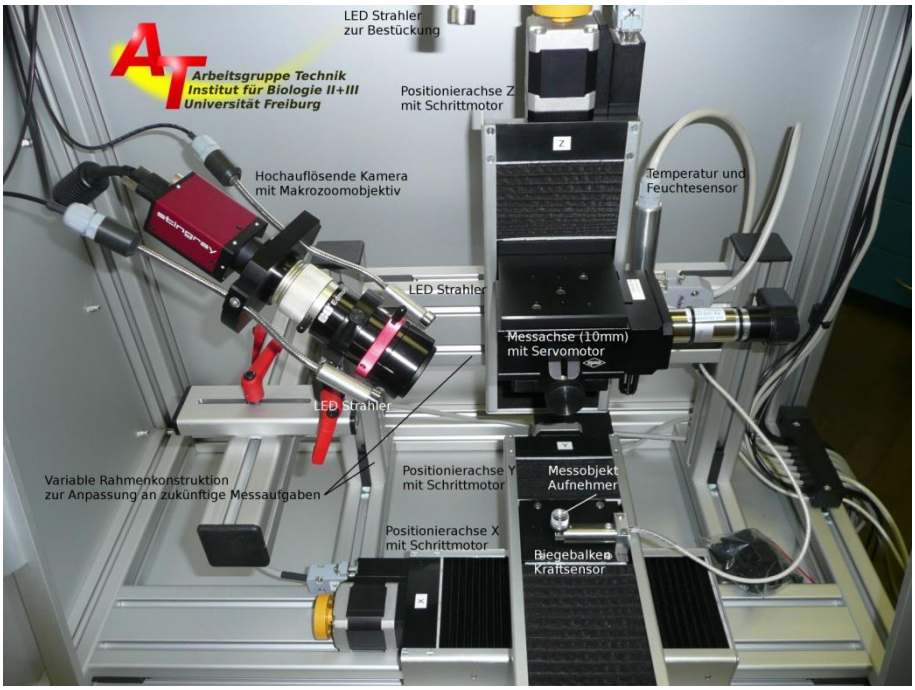

Figure 1 Puncture force machine.

Before to carry out the test, imbibed scarified seeds and ABA scarified seeds were observed during the time course, using a binocular stereoscope, to assess testa rupture, since after that, was possible remove easily the external covering layers and prepare seeds for the measurements. For scarified seeds, the measurements were performed along a 22 hours time course, while for ABA treated seeds were considered only the early germination point characterized by testa rupture and the time point just before the radicle protrusion, at 6-8 hours and 20-22 hours after imbibition, respectively. After the exportation of the outer covering layer testa, *Medicago* seeds were cut on the apex and the radicle was gently remove from the endosperm cap (Fig. 2A) and placed in a seed-shaped mould (Fig.2B).

A metal probe was slowly lowered into the individual empty endosperm cap at a constant speed of 250 nm and the puncture force was measured (Fig.3).

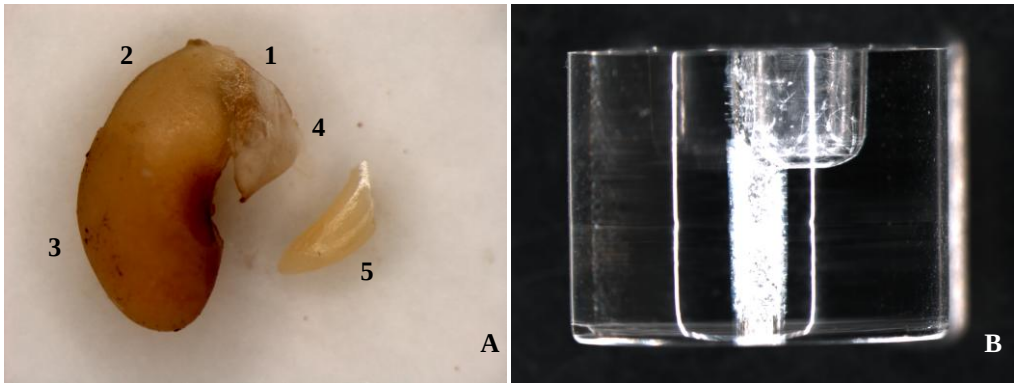

Figure 2 A: ***Medicago marina* seed prepared for the endosperm weakening measurement.** 1. Cut on the apex of seed to remove the radicle; 2. Portion of seed without the external covering layer testa; 3. External covering layer testa; 4. Empty micropylar endosperm cap; 5. Radicle removed from the micropylar endosperm. **B.** Seed shaped-mould.

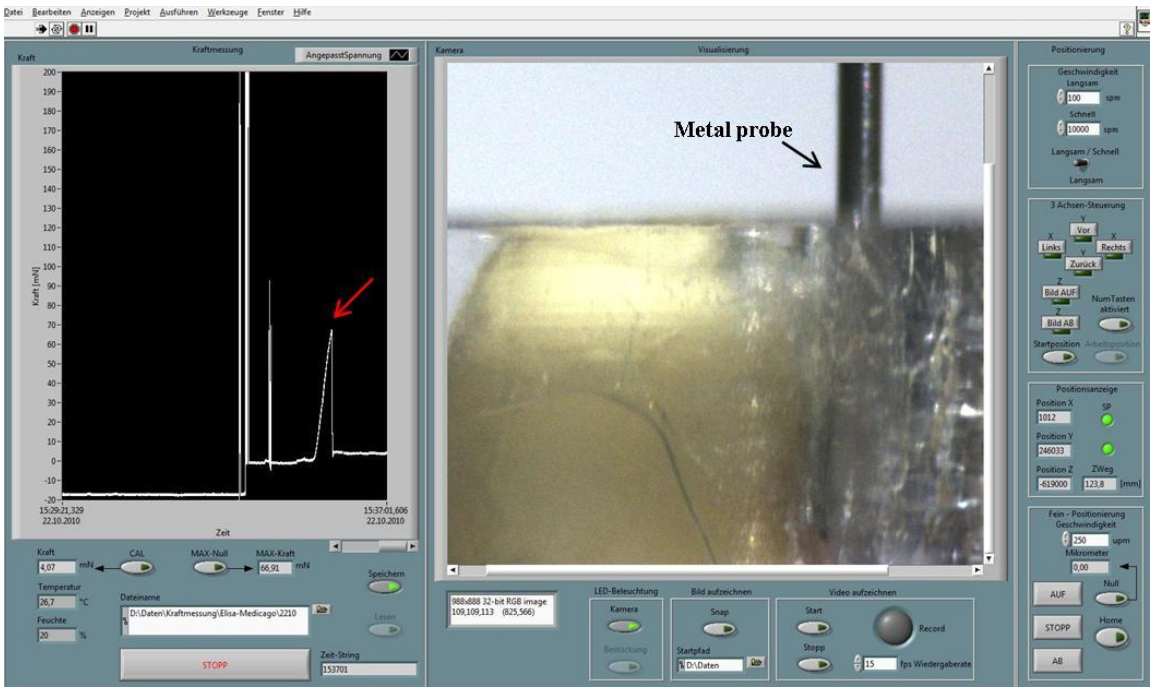

Figure 3 Quantification of endosperm weakening by direct biomechanical measurements. The radicle was removed from the mycropylar endosperm cup and the seed was placed in the seed shaped mould. A metal probe was lowered into the empty endosperm cap until it ruptures. During the measurement, the force needed to rupture was recorded in mN and plotted as a peak on the computer monitor (indicated by the red arrow in the figure).

For each time point were taken 30 measurements, using three biological replicates of 60 imbibed seeds. During the test the machine recorded the force that was needed to break the endosperm and the corresponding results were expressed in mN. These values provide an estimation of the mechanical resistance of the endosperm when the radicle starts to elongate, during germination.

2.3 Reverse transcriptase Polymerase Chain Reaction (RT-PCR) analysis

For the RT-PCR analysis were used imbibed scarified seeds not ABA (100 μM) treated and imbibed scarified seeds ABA (100 μM) treated collected at 13h after imbibition. Also, other four sample were considered, namely dry mature seeds (used as control sample), scarified seeds, scarified seeds ABA (100 μM) treated and not germinated, scarified seeds not ABA treated (100 μM) and germinated.

2.3.1 RNA extraction

RNA was extracted from approximately 0.07 g of seeds using a Qiagen RNeasy Mini kit protocol (Qiagen, Valencia, CA). Seeds were ground with a mortar and pestle to a fine powder in liquid nitrogen, lysis buffer was added to the samples and the RNA was extracted according whit the instructions included in the kit. RNA concentrations were measured at 260 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA) and RNA integrity was checked on 1.5% (w/v) agarose Tris-acetate EDTA ethidium bromide gels.

2.3.2 cDNA synthesis and RT-PCR

cDNA was synthesized using 1.0 μg of total RNA, poly(A) oligonucleotide primer and Superscript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). Standard PCR conditions and gene specific primers were used.

In particular the following primers were used: for *GA3ox,* primers GA3ox Forw (ACCCAAATGCTTCAAATTAATAG) and GA3ox Rev (TCACAGTGTTTGGCATAATCT) (Di Giacomo et al., 2008); for *GA20ox,* primers GA20ox Forw (GAGGGGAACATTGTGGTTATGC) and GA20ox Rev (GTCTTGTTGTTGTTCACCACTGCC) (Aubert et al., 2006); for *NCED5,* primers NCED5 Forw (GGACGGTTTTATGCATGATCCT) and NCED5 Rev (TGGCAAAGTCATGCATCATGAT) (Planchet et al., 2011).

The following gene-specific primers pair, used for *ACO*, was designed: primers ACO Forw (AGAGCACTATAGGAAATGCA) and ACO Rev (TTGACAACGATGGAGTGG).

18S rRNA cDNA fragment was used as a loading control using the same RT reaction and the following primers: 18S Forw (CCAGGTCCAGACATAGTAAG) and 18S Rev (GTACAAAGGGCAGGGACGTA) (Pislariu & Dickstein, 2007).

All PCR reactions were performed in 25 µl volumes using Taq recombinant polymerase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol and cDNA obtained previously was used as template in all reactions.

Reaction conditions for thermal cycling were 94 °C for 4 min, 30 cycles of 94 °C for 45 s, 50°C for 45 s, 72 °C for 50 s and 72 °C for 4 min for GA3ox, GA20ox, NCED5 and ACO. For 18S, reaction condition for thermal cycling were 94 °C for 4 min, 28 cycles of 94 °C for 45 s, 52°C for 45 s, 72 °C for 50 s and 72 °C for 4 min. Two independent biological replicates were run for each sample, each with two technical replications. Results were analyzed using Image J 1.41o software (Wayne Rasbanb National Institute of Health, USA; http//rsb.info.nih.gov/ij). To account for small differences in RNA loadings data was normalized to 18S gene expression.

2.4 Proteomic analysis

For the proteomic analysis were used imbibed scarified seeds not ABA (100 µM) treated and imbibed scarified seeds ABA (100 µM) treated collected at 6h, 8h, 13h, 18h, 20h, and 22h after imbibition. Also, other four sample were considered, namely dry mature seeds (used as control sample), scarified seeds, scarified seeds ABA (100 µM) treated and not germinated, scarified seeds not ABA treated (100 µM) and germinated.

2.4.1 Protein extraction

For total protein extraction, 0.50 g (corresponding to around 100 mature seeds) of each seeds samples were reduced to powder by mechanical breakage with liquid nitrogen and suspended in 5 ml of 10% trichloroacetic acid and 0.07% 2-β-mercaptoethanol in cold acetone at -20°C (Rabilloud, 2000), individually. The mixed sample was filtered through Miracloth and precipitated overnight at -20°C. Each mixed sample was centrifuged at 35,000xg for 15 min at 4°C, pellets were recovered, resuspended in 10 ml of 0.07% 2-β-mercaptoethanol in cold acetone at -20°C and precipitated for 5 h at -20°C. Pellets were recovered by centrifugation at 35,000×g for 15 min at 4°C, vacuum dried and solubilised in 300 ml of 7M urea, 2M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 20mMTris–HCl, 1% (w/v) DTT, containing 0.2% (w/v)

ampholine 3-10 and 0.15% (w/v) ampholine 5-7. The protein content was measured by the Bradford protein assay method (Bradford, 1976), using the BioRad (Bio-Rad, Hercules, CA, USA) protein assay and BSA as standard.

2.4.2 Two-dimensional electrophoresis

For isoelettrofocusing (IEF), an extract of total proteins of each sample was mixed with rehydratation buffer and loaded on 7-cm IEF strip gels in immobilized 5–8 pH gradients (IPG strip Bio-Rad). Strips were rehydrated in mineral oil for 16 hours, at room temperature and IEF was performed in PROTEAN IEF Cell system (Bio-Rad) at 250 V for 1 h, 500 V for 1 h, 4000 V for 10.000 "volthours" and 500 V for 99:00 h. After focusing, the proteins were reduced by incubating the IPG strips with 1% (w/v) dithiothreitol for 20 min and alkylated with 2.5% (w/v) iodoacetamide in 10 ml of 50mM Tris-HCl pH 8.8, 6M urea, 30% (w/v) glycerol, 2% (w/v) sodium dodecyl sulphate for 20 min. Second dimension electrophoresis was carried out on 12% polyacrylamide gels with a Mini-Protean apparatus (Bio-Rad) in 25mM Tris-HCl, pH 8.3, 1.92M glycine and 1% (w/v) SDS, applying a voltage of 100 V until the dye front reached the bottom of the gel. Two-dimensional electrophoretic gels were stained with colloidal Coomassie G250 o/n and then washed several times in distilled water to remove the excess dye.

2.4.3 PDQuest analysis

Two-dimensional electrophoretic gels were acquired using a GS-800 calibrated densitometer (Bio-Rad), and gels analysis was performed using PDQuest 2-D Analysis Software (Bio-Rad), to identify differentially expressed proteins. The intensities of the differential expressed protein were obtained from three independent experiment of each biological sample, followed by comparison automatically executed by the software (and then, manually verified), which make a quantitative measurement to obtain statistical information on variations in the protein levels. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for the three different replicates of each biological sample and statistical Student's t test analysis at significance level $p < 0.01$ was chosen to find out significant changes in protein folding between samples. A two-fold change in normalized spot densities was considered indicative of a differential expression.

2.5 Statistical analysis and identification of specific markers

On the basis of the results obtained from PD-Quest analysis, protein that showed a differentially expressed pattern, were also analyzed trough uni - and multivariate analysis to identify the proteins able to discriminate significantly the different samples. The protein variation among and within the different samples was obtained through the analysis of variance (ANOVA). ANOVA was computed at several different levels: first it was computed between the control seeds (dry mature seeds) and scarified seeds, second it was computed between control seeds and germinated seeds not ABA (100 μM) treated, third it was computed between scarified seeds and scarified germinated seeds not ABA (100 μM) treated, fourth it was computed between scarified germinated seeds not ABA (100 μM) treated and not germinated scarified seeds ABA (100 μM) treated, to identify spots that are specific markers of a physiological state (germination - dormancy) and/or of a treatment (ABA - scarification). Moreover, ANOVA was computed between the scarified imbibed seeds samples along the 22 hours time course, treated and not treated whit ABA (100 μM), to highlight the proteins with significant expression pattern among these samples, during the weakening of the micropylar endosperm and in relation to the ABA treatment. Subsequently, the cluster analysis, using the Ward's method as linkage, was performed among proteins resulted discriminating by ANOVA ($P \leq 0.05$) to group all significant protein in relation to their quantitative relationship.

These variable protein were manual excised from the gels and analyzed by mass spectrometry technologies.

3. Results

3.1 Puncture force measurement

To define when testa rupture, which characterizes the early germination event, takes place, and to verify the involvement of ABA, imbibed scarified seeds (control) and imbibed scarified seeds treated with ABA (100 µM) were kept at the constant temperature of 25°C and observed during a time course, using a binocular stereoscope. The observation taken during a 24 h time course showed that both ABA treated and untreated seeds undergo to testa rupture after 6-8 hours of imbibition (Fig. 4B). However, the micropylar endosperm rupture, assessed trough the radicle emergence, occurred between 22 and 24 hours after imbibition only in ABA untreated scarified seeds.

For this reason the time point at 22 hours after imbibition was took as the final time point just before the completion of germination and consequently, it was considered as the last imbibition time useful for the puncture force measurement. After this time point, because of the radicle emergence, it was not possible measure the micropylar endosperm resistance.

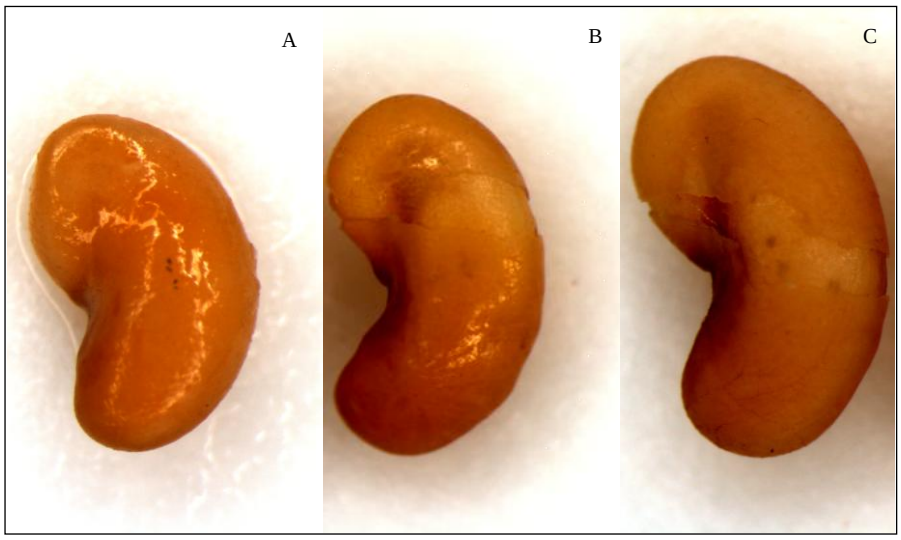

Figure 4 Testa rupture of imbibed scarified seeds. **A**: scarified seed before testa rupture; **B** and **C:** testa rupture of scarified not ABA treated seeds; **B**: testa rupture after 6- 8h hours of imbibition; **C**: testa rupture after 20-22 hours of imbibition.

The mechanical resistance of the micropylar endosperm was used as a measure of endosperm weakening and quantified by puncture-force method (Müller et al., 2006). Puncture force measurements, performed on scarified seeds during imbibition, showed a constant decrease of micropylar endosperm resistance. As shown in figure 5 the highest endosperm cap puncture force was measured after 6-8 h of imbibition, when testa rupture, characterizing the early germination event, occurred (Fig. 4B).

Puncture force values decrease quickly after 6-8 h of imbibition (97,55 ± 8,47 mN), and then slowly between 10 h and 18 h of imbibition, where the puncture force resulted equal to 90,48 ± 7,27 mN and 71,94 ± 7,27 mN, respectively. A new significant decrease of the puncture force that reached the value of 52,31 ± 5,01 mN, was visible only after 20 h of imbibition. The lowest puncture force (42,67 ± 4,55 mN) was necessary to puncture the endosperm at 20-22 hours of imbibition (Fig. 5).

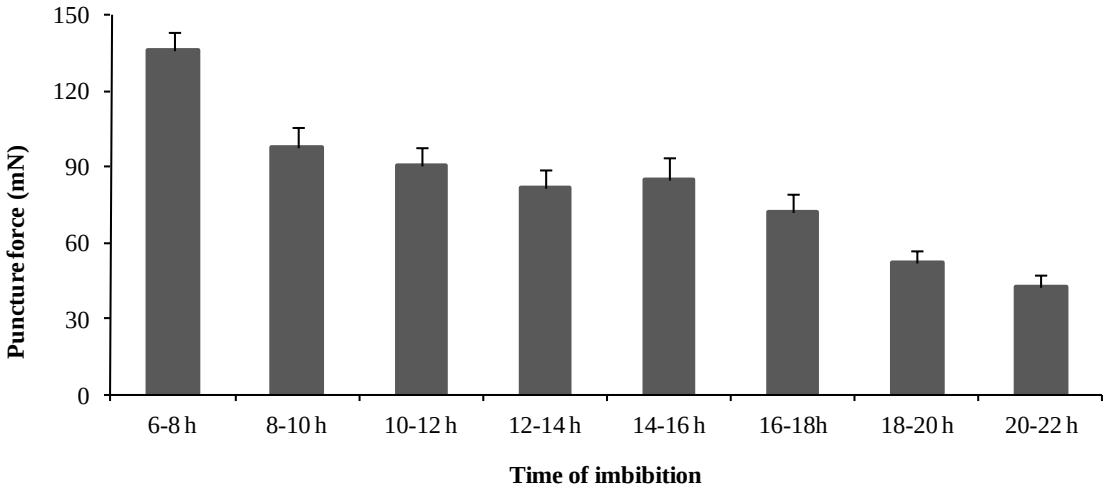

Figure 5 Modification of the micropylar endosperm resistance during imbibition of scarified seeds measured by the puncture force method. For each time point, the histograms represent the mean values of at least 30 individual endosperm cap measurements and the vertical bars represent the standard error.

Puncture force was measured also on scarified seeds subjected to ABA (100 µM) treatment, but only after 8 hours and 22 hours of imbibition. These two time points represented, as previously mentioned and shown in figure 4, the early germination event characterized by testa rupture, and the time point just before the radicle protrusion, respectively. The endosperm cap rupture for ABA treated seeds measured at 8 and 22 h after imbibition are compared with untreated seeds (Fig.6). After 6-8 h of imbibition the endosperm cap puncture force, measured in ABA treated seeds, was of 76,17 mN ± 9,49. This value is significantly lower to the one obtained for scarified seeds not treated with ABA, where the puncture force resulted in an average value of 135,89 ± 7,19 mN (Fig. 6). However at 20-22 h after imbibition, this value remained almost unchanged in the ABA treated seeds, resulting higher compared to the control (Fig.6).

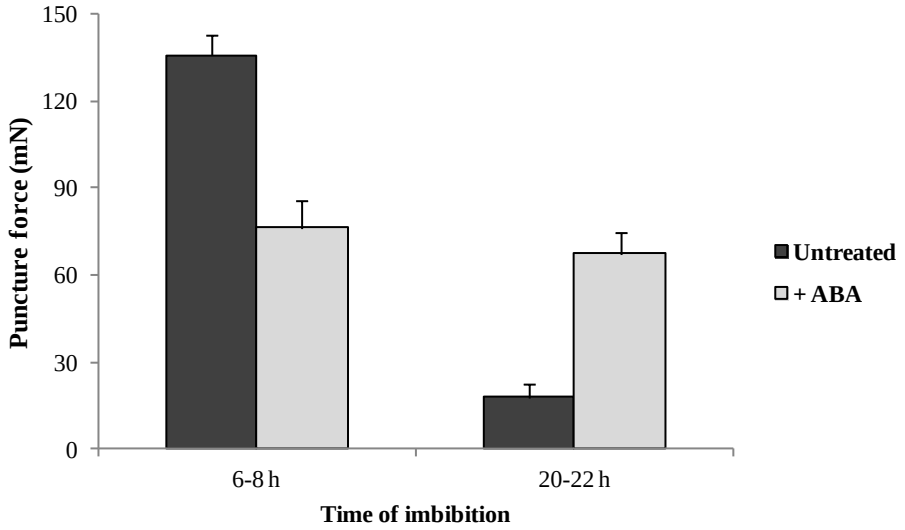

Figure 6 Micropylar endosperm rupture after 6-8 h and 20-22 h after imbibition in scarified seeds and 100 µM ABA treated scarified seed. For each time point, the histograms represent the mean values of at least 30 individual endosperm cup measurements and the vertical bars represent the standard error.

3.2 Germination test

The effects of hormones as ABA and GAs in dormancy breaking and germination regulation, was also evaluated. At this purpose, germination tests were performed on scarified seeds subjected or not to ABA and GA_3 treatments. Three biological replicates of 60 scarified seeds each, used as control sample (S), were kept for germination without hormones addition, while for hormonal treatments, three biological replicates of 60 scarified seeds each, and for each treatment, were subjected to 5 µM GA3, 10 µM GA_3, 100 µM ABA, 100 µM ABA plus 5 µM GA_3 respectively (as described in the materials and methods section).

As shown in figure 7, the ABA treatment significantly affected the germination capacity of seeds. In fact, while scarified untreated seeds achieved the 97,8% of germination whit a T_{50} equal to 27,8 h, scarified ABA treated seeds did not germinate even when the test was extended for more than a week.

Different results came from the germination trend of scarified seeds subjected to GA_3 treatment. In fact, scarified seeds treated with 5 µM GA_3 achieved a germination percentage of 86,7 with a T_{50} of 16,6 h, while only 3,3% of seeds treated whit 10 µM GA germinated whit a T_{50} of 56,2 hours (Fig.7). However, seeds subjected to 5 µM GA_3 plus 100 µM ABA treatment did not germinate, in a similar manner to that obtained for the seeds treated with 100 µM ABA alone (Fig.7).

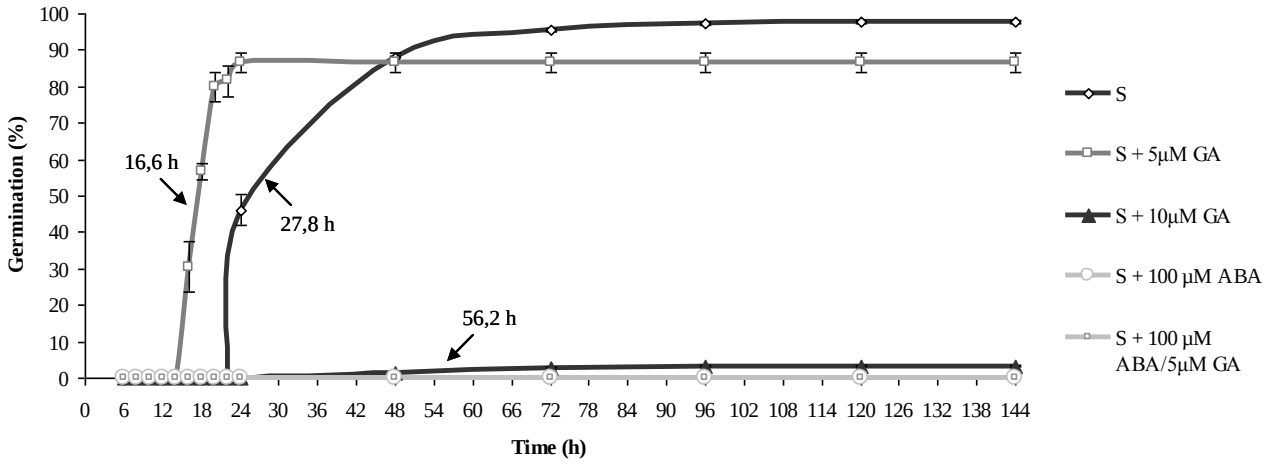

Figure 7 Germination trend of scarified seeds (S) and scarified, GA_3 (5 µM), GA_3 (10 µM), ABA (100 µM) and GA_3 (5 µM) plus ABA (100 µM) treated seeds during time. Vertical bars represent the standard error of the mean of three biological replicates of 60 seeds each. The arrows indicate the T_{50} of each treatment.

3.3 Reverse transcriptase Polymerase Chain Reaction (RT-PCR) analysis

RT-PCR analysis was used to evaluate the gene expression of key enzymes involved in abscissic acid (NCED5), gibberellic acid (GA3ox, GA20ox) and ethylene (ACO) biosynthesis to investigate the role of these hormones in dormancy breaking and germination. In particular, the expression pattern of *GA3ox*, *GA20ox*, *NCED5* and *ACO* genes in dry mature seeds (C), scarified seeds (S), scarified seeds subjected or not to ABA (100 µM) treatment collected at 13 h after imbibition (13h + ABA; 13h - ABA), scarified germinated seeds not ABA treated (G - ABA), scarified not germinated seeds ABA treated (NG + ABA), were analysed by reverse transcriptase-PCR using gene specific primers and optimised PCR conditions, as described in materials and methods section.

GA3ox and *ACO* genes showed a similar expression pattern as both appeared to be expressed in scarified seeds not treated with ABA collected at 13 h after imbibition and in scarified germinated seeds, while they were undetectable in all the other samples (Fig. 8). However, transcripts of *GA3ox* gene seemed to increase during germination showing almost a 0,8 fold induction in germinated seeds, while *ACO* transcripts remained constant in both imbibed (13 h - ABA) and germinated seeds (Fig.8).

GA20ox gene was preferentially expressed in germinated seeds, resulting undetectable in the remaining samples; *NCED5* resulted to be induced by the scarification and up-regulated in scarified seeds collected at 13 h after imbibition not subjected to ABA treatment. In fact, as reported in figure 8, *NCED5* showed approximately 1,4 fold induction for both, compared with control seeds and germinated seeds not ABA treated. Furthermore, it resulted that *NCED5* transcript was not detected in ABA treated samples (scarified seeds ABA treated collected after 13 h of imbibition and scarified not germinated seeds ABA treated).

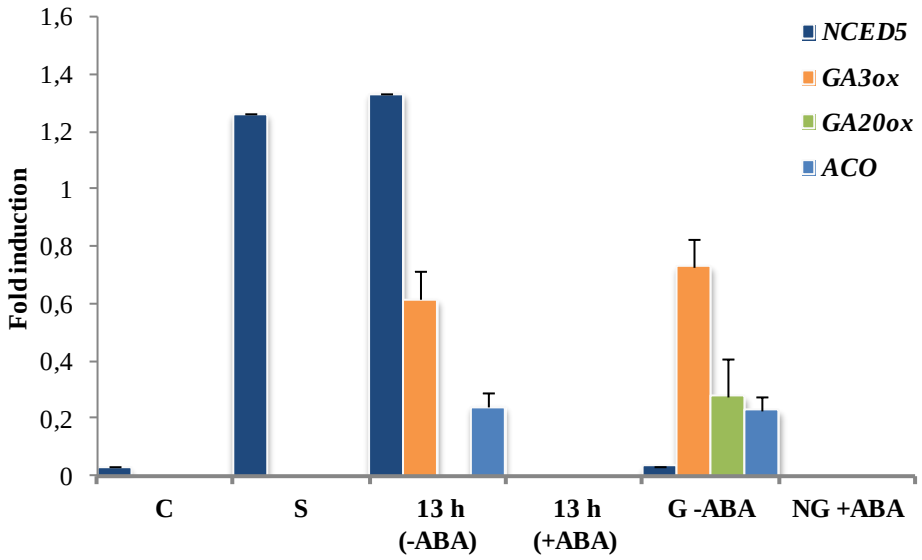

Figure 8 ***NCED5, GA3ox, GA20ox*** **and** ***ACO*** **genes transcriptional profiling**. Histograms represent the quantification of specific PCR products normalised with the control 18S rRNA. Error bars indicate the standard deviation for three biological replicates.

3.4 Proteomic analysis

For proteomic analysis, proteome maps obtained from dry mature seeds (used as control sample), scarified seeds, scarified germinated seeds not ABA (100 µM) treated, scarified not germinated seeds ABA (100 µM) treated and scarified seeds ABA (100 µM) treated and not ABA treated, collected along a 22 h time course, were compared to analyse the protein pattern in *M. marina* dry mature seed during the endosperm weakening in preparation to germination, after the dormancy release, and to identify proteins regulated by ABA.

Total protein extracts from each sample were resolved by 2-DE, as described in materials and methods section, and proteins detected by blue comassie.

As reported in figure 9, proteomic maps, resolved on a 5-8 pH gradient, showed a high level of reproducibility with an average of 450-500 distinguishable spots that reached Mr values between about 104 and 15 Da.

Software assisted proteome analysis was carried out by matching all the replicate gels with the dry mature seed (control) used as reference.

The comparison of relative spot densities between the different proteomic maps revealed a total of 351 spots differentially expressed among the dry mature seeds, scarified seeds,

germinated seeds, not germinated seeds and among scarified ABA treated and not treated seeds along the 22 hours time course, while the remaining spots showed a constant expression.

The 351 spots resulted differentially expressed by the Student‘s t test ($P \leq 0.01$), were subjected to Analysis of Variance (ANOVA) and further grouped according to their similar expression profile by cluster analysis.

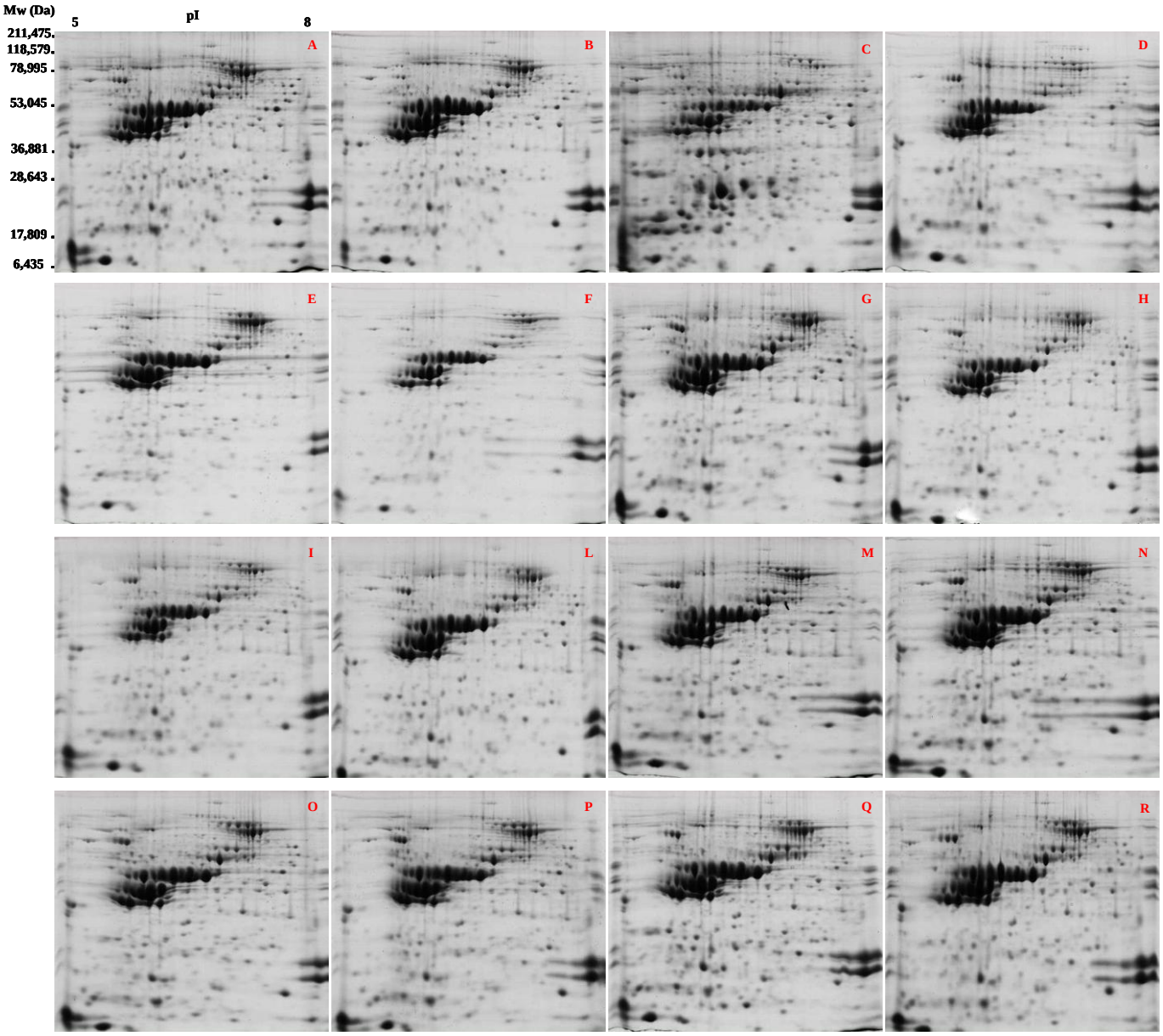

Figure 9 Reproducible maps of control (dry mature seeds), scarified seeds, scarified and germinated seeds not ABA treated (100 µM), scarified ABA (100 µM) treated seeds and not germinated, scarified seeds collected after 6, 8, 13, 18, 20, 22 hours of imbibition, ABA (100 µM) treated (+ABA) and not ABA treated (-ABA), obtained by two-dimensional electrophoresis. The maps were produced in triplicate for each of three independent protein extraction experiments and the most abundant and well-separated proteins were resolved. Proteomic maps, on a linear 5-8 pH gradient, showed an average of 450-550 well-resolved spots, with a Mr from between 211,5 and 6,5 Da.

A: control seeds (dry mature seeds); **B**: scarified seeds; **C**: scarified and germinated seeds not ABA (100 µM) treated; **D**: scarified ABA (100 µM) treated and not germinated; **E**: scarified seeds 6 h (-ABA); **F**: scarified seeds 6 h (+ABA); **G**: scarified seeds 8 h (-ABA); **H**: scarified seeds 8 h (+ABA); **I**: scarified seeds 13 h (-ABA); **L**: scarified seeds 13 h (+ABA); **M**: scarified seeds 18 h (-ABA); **N**: scarified seeds 18 h (+ABA); **O**: scarified seeds 20 h (-ABA); **P**: scarified seeds 20 h (+ABA); **Q**: scarified seeds 22 h (-ABA); **R**: scarified seeds 22 h (+ABA).

3.5 Identification of specific markers

Results of ANOVA ($P \leq 0.05$) revealed that, among the 351 differentially expressed spots obtained from the PDQuest analysis ($P \leq 0.01$), 215 spots were highly discriminating all the different samples.

To identify spots that are specific markers of a physiological state (germination - dormancy) and/or of a treatment (ABA - scarification) the 215 differentially expressed protein spots were further analysed on the base of their presence or absence within the following groups: dry mature seeds and scarified seeds (group I); dry mature seeds and scarified germinated seeds not ABA treated (group II); scarified seeds and scarified germinated seeds not ABA treated (group III); scarified germinated seeds not ABA treated and scarified not germinated seeds ABA treated (group IV).

A total of 95 protein spots, showed in the master gel (Fig.10), were selected among the 215 for their presence/absence within the different groups.

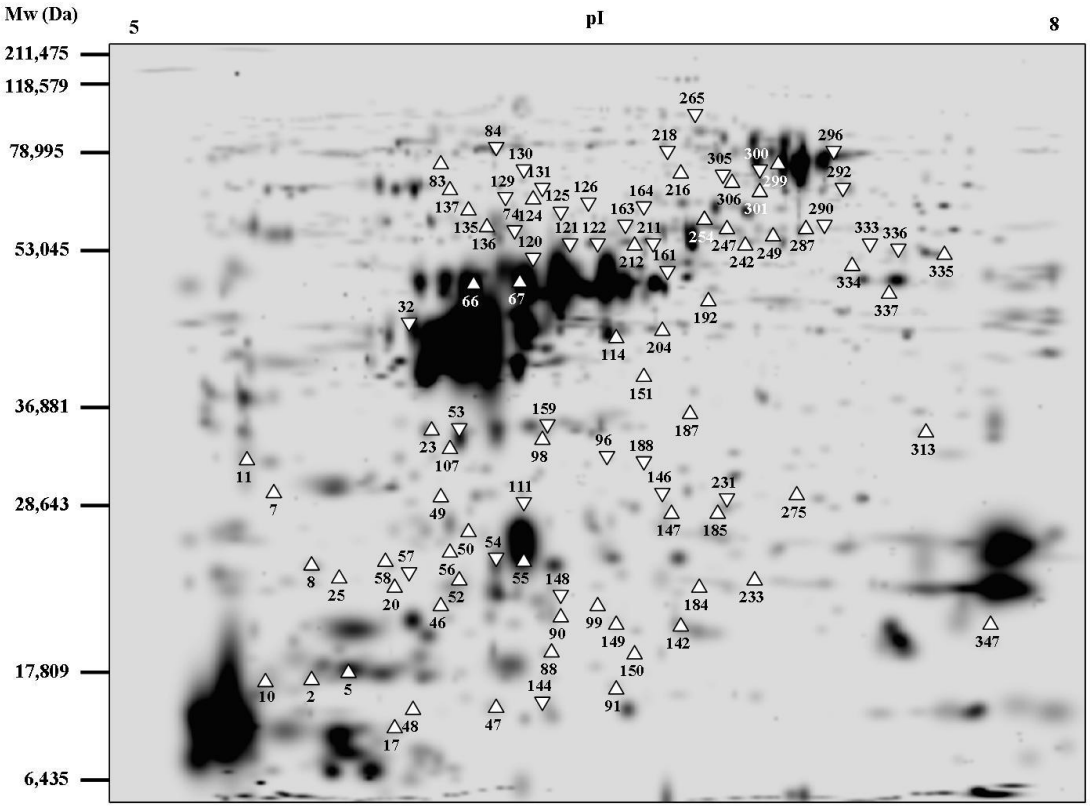

Figure 10 Master gel of *Medicago marina* seed. Two dimensional electrophoresis reference map created on the basis of the spot detection in each gel. On the map are indicated the 95 significant and differentially expressed spots resulted by the multiple comparison analyses between the groups. The numbers are correlated with the dendrograms obtained trough the cluster analyses.

However, while no protein spots were found to discriminate dry mature from scarified seeds (group I), within the other groups the following results were obtained:
65 spots discriminated samples within group II; 66 spots discriminated samples with the group III while 64 samples within group IV.

Furthermore, the comparison of the 95 spots expression between different groups generated the data represented in the Venn diagram (Fig.11). In particular, while five and six spots are specifically expressed in group II and group III respectively, 60 were present in both (Fig. 11A); twelve and seventeen spots characterize group II-III and IV respectively, wile remaining 35 were commonly expressed in all the three groups (Fig. 11B).

A complete list of these specific spot-markers is reported in table 1 in the supplementary material section.

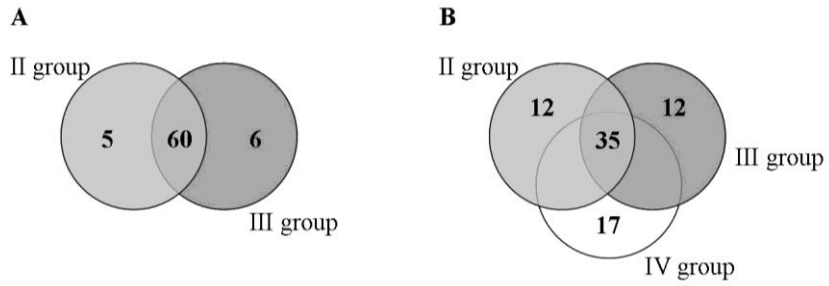

Figure 11 Venn diagrams of the expressed proteins between and within the four groups. **A**: sixty proteins were present in both the second and third group, while a few proteins discriminated in only one of these groups. **B**: thirty-five proteins were expressed in the second, third and fourth group, twelve discriminated only the second and the third group, while seventeen were expressed only in the fourth group. II group: dry mature seed and germinated seed; III group: scarified seed and scarified germinated seed not ABA treated; IV group: scarified germinated seed not ABA treated and scarified not germinated seed ABA treated. The first group (scarified seed and dry mature seed) is not represented in graph because no proteins discriminated within the group.

3.6 Markers expression pattern

The expression profile of the 95 spots in control (C), scarified (S), germinated (G - ABA) and not germinated plus ABA (NG + ABA) was organized by cluster analysis.

Results reported in figure 12 shown that the three samples: C, S and NG + ABA are very similar, and all significantly differ from the germinated sample (G - ABA).

In fact, protein spots discriminated, for their qualitative or quantitative different expression, the samples as following:

- sixty-eight spots discriminated, for their high expression, germinated seeds (G - ABA) from control (C), scarified (S) and not germinated seeds (NG + ABA);
- eleven spots discriminated control (C) and scarified seeds (S) from germinated seeds (G - ABA) and not germinated seeds (NG + ABA), for their high expression in C and S and their low expression in the other two samples;
- three spots discriminated control (C), scarified (S) and germinated seeds (G - ABA) from not germinated seeds for their low expression in not germinated seeds (NG + ABA);
- two spots discriminated for their high expression, not germinated samples (NG + ABA) from the other three samples (C, S, NG + ABA);
- two spots discriminated, for their high expression samples C, S and NG + ABA from sample G - ABA.

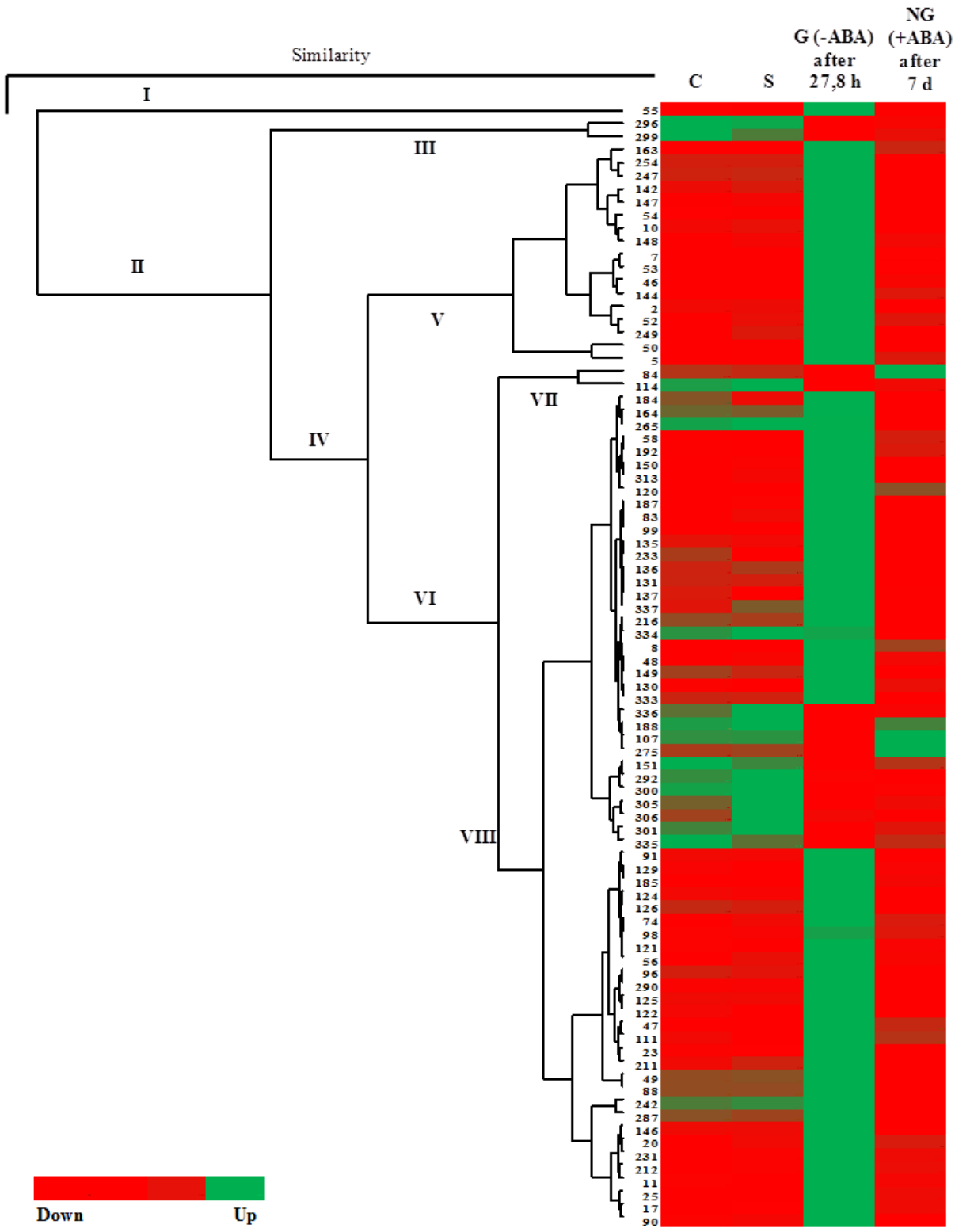

Figure 12 Cluster analysis. The protein spots resulted differentially expressed from the ANOVA analysis between and within the dry mature seeds (C), scarified seeds (S), scarified and germinated seeds not ABA treated (G - ABA after 27,8 h) and ABA treated scarified and not germinated seeds (NG + ABA after 7 days), were grouped by cluster analysis on the basis of their low or high quantity. Two different clusters (I and II) were generated, in turn, divided into several sub-clusters (III - VIII). The down regulated proteins are indicated by the red colour, while the up regulated proteins are indicated by the green colour.

To further investigate the effect of ABA treatment on these 95 spots, their expression was evaluated in all the samples (dry mature seeds, scarified seeds, germinated and not germinated seeds, scarified imbibed seeds along the 22 hours time course in presence or absence of ABA), respect to the ABA treatment. Twenty-two spots were found to be altered by the ABA treatment along the entire time course (spot n. 32, 57, 66, 67, 114, 126, 131, 149, 150, 151, 159, 161, 164, 188, 204, 218, 231, 247, 313, 333, 336). In particular, according with their profile during time, these spots can be grouped in spots (n. 32, 57, 66, 67, 151, 188, 218) up regulated after the ABA treatment (Fig. 13); spots (n. 114, 126, 131, 149, 150, 159, 161, 164,) down regulated by the ABA treatment (Fig. 14), and spots (n. 204, 231, 247, 313, 333, 336, 347) showing an heterogeneous expression profile during the time course (Fig.15).

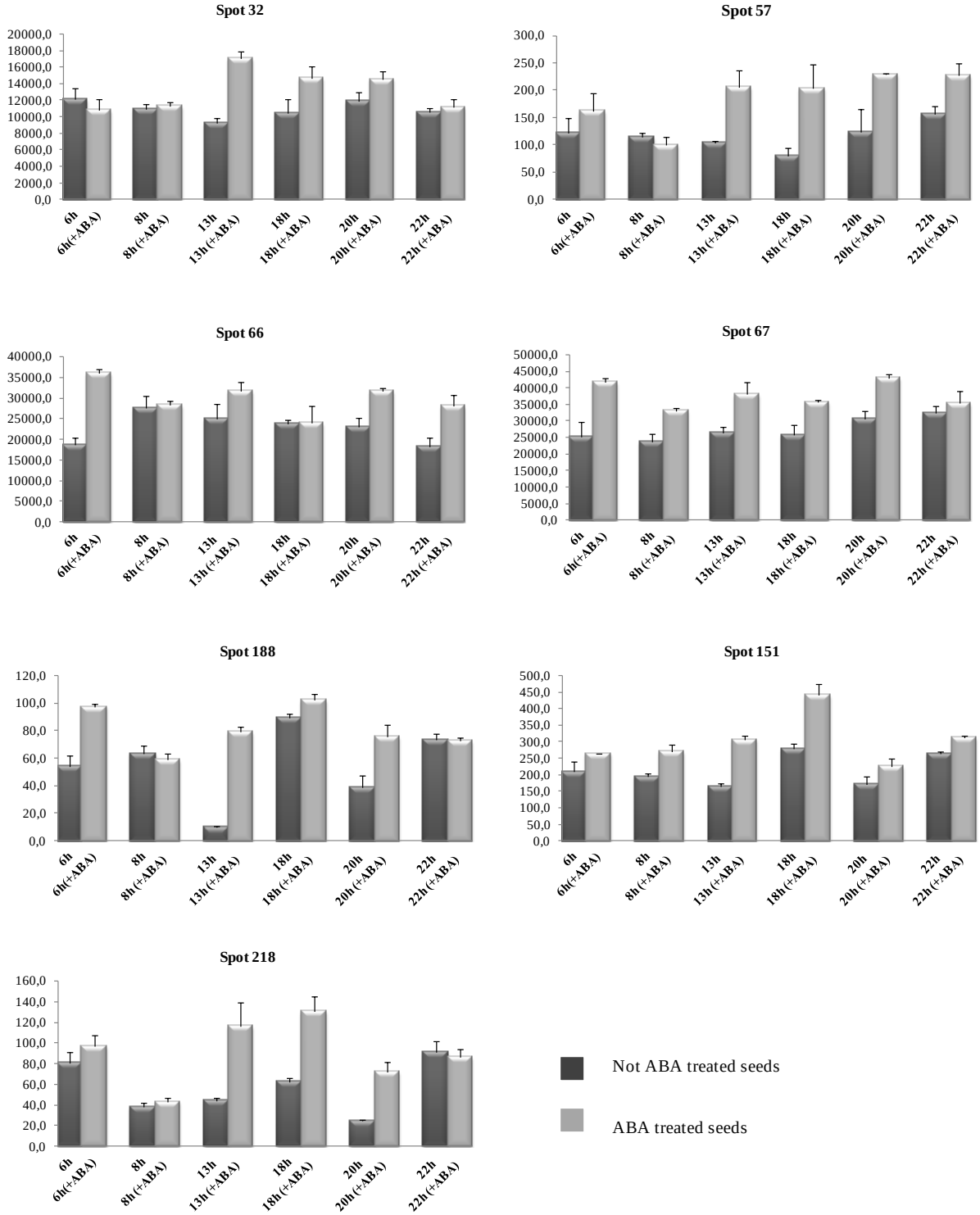

Figure 13 Spot expression pattern of ABA treated seeds and not ABA treated seeds along a 22 h time course. Histograms represent the expression profiling of spots resulted up-regulated along the 22 hours time course following the ABA treatment, while the vertical bars represent the standard error.

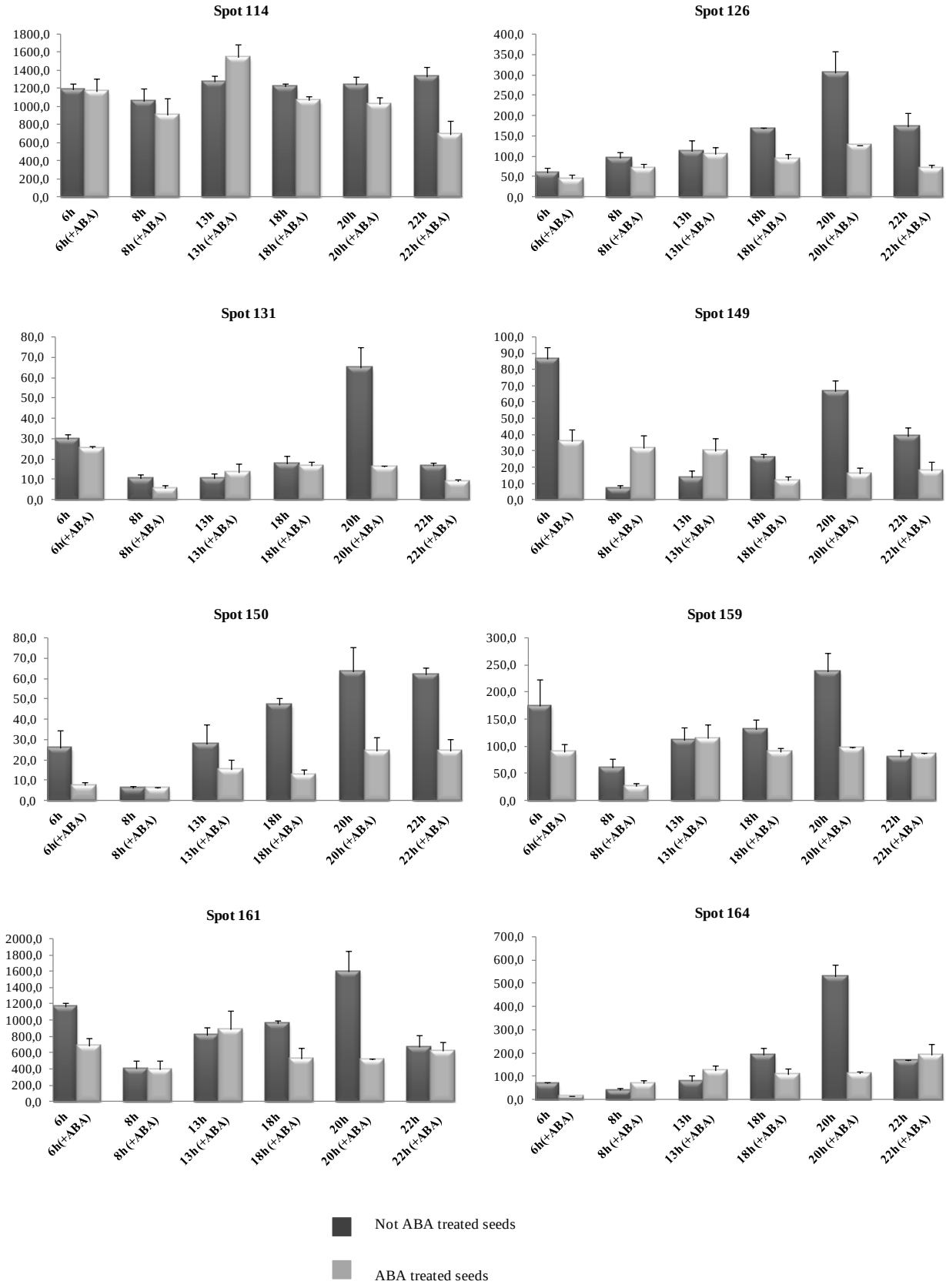

Figure 14 Spot expression pattern of ABA treated seeds and not ABA treated seeds along a 22 h time course. Histograms represent the expression profiling of spots which resulted generally down regulated along the 22 hours time course following the ABA treatment, while the vertical bars represent the standard error.

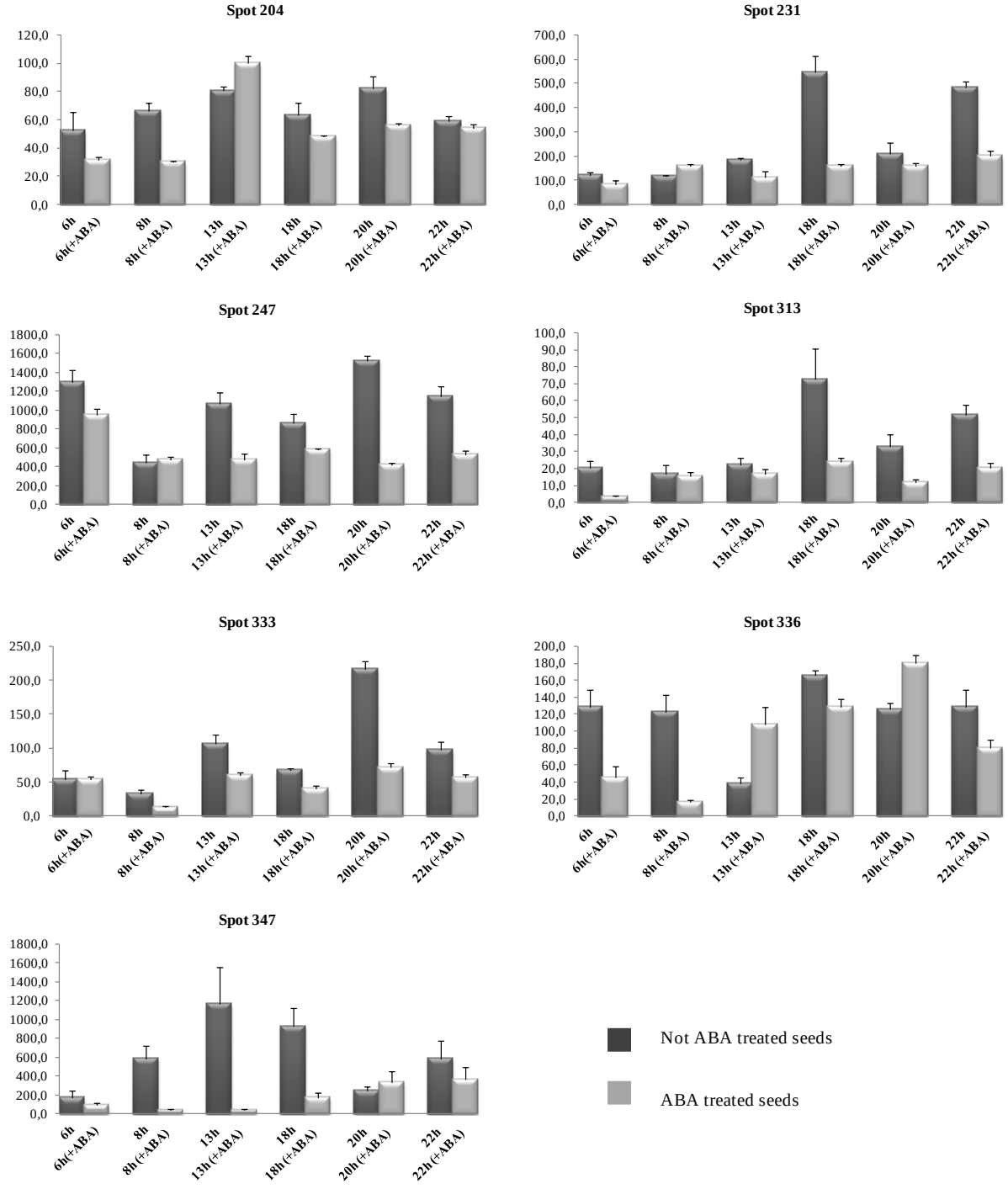

Figure 15 Spot expression pattern of ABA treated seeds and not ABA treated seeds along a 22 h time course. Histograms represent the expression profiling of spots showing a heterogeneous expression profile during the 22 hours time course following the ABA treatment, while the vertical bars represent the standard error.

Lastly, these 95 protein spots were manual excised from the gel for sequencing by mass spectrometry.

4. Discussion and conclusions

Results obtained in the first phase of the PhD program and published in the paper Scippa et al., (2011), showed that *Medicago marina* seeds, like in other legumes, have adopted a dormancy strategy based on a hard seed coat which may be also regulated by other complex mechanisms.

As widely reported, the micropylar endosperm contributes to coat dormancy. In fact, micropylar endosperm can act as a barrier for radicle protrusion and is able, in that way, to regulate the timing of germination (Ni & Bradford, 1993; Bewley, 1997; Toorop et al., 2000; Leubner-Metzger, 2003; Finch-Savage & Leubner-Metzeger, 2006). Several works, conducted on different seeds species, such as lettuce (Ikuma & Thiamann, 1963), tomato (Chen & Bradford, 2000; Wu et al., 2001, Leubner-Metzeger, 2003) tobacco (Leubner-Metzeger, 2003) and coffee (Da Silva et al., 2004) report that the weakening of the micropylar endosperm is a prerequisite for the completion of germination, which is visible when the radicle protrudes the external covering layers surrounding the embryo. Furthermore, it has been reported that the micropylar endosperm weakening requires the involvement of many hydrolytic enzymes and is finely regulated by plant hormones (Leubner-Metzger, 2003; Finch-Savage & Leubner-Metzeger, 2006). Since the micropylar endosperm can represents an obstacle for the radicle protrusion, it is possible to quantify directly the weakening of the micropylar endosperm by measuring puncture force (Müller et al., 2006).

In this work, puncture force measurements were performed on scarified imbibed seeds collected along a 22 hours time course to verify the involvement of micropylar endosperm in *M. marina* dormancy. Results obtained showed a constant decrease of micropylar endosperm resistance, starting from 6-8 h after imbibition (testa rupture), when the highest endosperm cap puncture force (135,89 ± 7,19 mN) was obtained, until the time point at 20-22 h after imbibition (time point just before the radicle protrusion), when only an average force of 42,67 ± 4,55 mN was enough to puncture the endosperm.

In between these two time points, with the exception for 10 h (97,55 ± 8,47 mN) and 20 h after imbibition (52,31 ± 5,01 mN), in which the corresponded puncture force values quickly decreased, the micropylar endosperm resistance decreased slowly in a linear manner in all points included between 12 and 18 hours.

Thus, the micropylar endosperm puncture force seems to decrease in two significant drop phases: the first occurred after 10 and the second occurred after 20-22 hours from the beginning of imbibition, (Fig.5). This second reduction coincided whit the radicle protrusion. In fact, 22 h after imbibition seed radicle was able to generate enough force to protrude the endosperm and

complete the germination within the next 2 hours. Indeed, scarified seeds completed the germination in 24 h with a germination rate of almost 98% (Fig. 7).

As stated before, a large number of evidences demonstrated that the endosperm weakening is a common phenomena of many plant species, that precedes the radicle protrusion, and occurs when, during seed imbibition, the water uptake starts (Müller et al., 2006; Linkies et al., 2009, Graber et al., 2010, Morris et al., 2011).

The plant species *Lepidium sativum* has been widely used as a model species to investigate the role of endosperm weakening (Müller et al., 2006; Linkies et al., 2009, Graber et al., 2010, Morris et al., 2011). The completion of germination in *Lepidium* seed, measured trough the radicle emergence, is regulated by two opposing forces, the growth potential of the radicle, and the resistance to this growth from the seed covering layers (testa and endosperm cap) (Müller et al., 2006; Morris et al., 2011). After testa rupture, the strength of the endosperm progressively decreases toward germination completion. Furthermore, in *Lepidium sativum* seeds it has been observed that the endosperm weakening occurs after 8 h of imbibition on medium or in water without hormonal addition and its progression results in the occurrence of endosperm rupture and germination completion after 18 h of imbibition (Müller et al., 2006; Linkies et al., 2009, Graber et al., 2010, Morris et al., 2011).

By the puncture force measurements, two related work Müller et al., (2006) and Linkies et al., (2009), demonstrated that in *Lepidium* seeds, the mechanical resistance of the micropylar endosperm decreases during imbibition, starting from the testa rupture that occurring 8 hours after imbibition. Then, this mechanical resistance continued to decrease until 18 h after imbibition, when the radicle protruded the endosperm within the next 2-4 h.

The weakening of the endosperm was quantified also in tomato seeds (Chen & Bradford, 2000; Wu et al., 2001), in *Coffea Arabica* (Da Silva et al., 2004) and *Solanum lycocarpum* (Pinto et al., 2007). In two different works, Chen & Bradford (2000) and Wu et al., (2001), demonstrated that the physical weakening of tomato cap tissue occurs during seeds imbibition in water, starting within the 12 h, and reaches the minimum value after 36 h of imbibition, when seeds complete the germination. Additionally, in water-imbibed *Coffea arabica* seeds (Da Silva et al., 2004), the puncture force required to break the micropylar endosperm, strongly decrease just before the completion of germination, that in these seeds occurs after 9 days of imbibition. A similar behavior was demonstrated by Pinto et al., (2007) for *Solanum lycocarpum* seed imbibed in water. Data emerged in the present work for *M. marina* seeds, in which the micropylar endosperm mechanical resistance decreases along a 22 h of imbibition time course allowing the radicle to protrude and complete the germination process, provide additional evidence of the role of endosperm in seed dormancy. However, the two step drops of endosperm resistance may

indicate the involvement of different mechanisms in the control of dormancy/germination during time, and data supporting this suggestion seem to derive from the analysis of the effect of ABA on endosperm weakening.

Several studies show that hormones like ABA and GAs are involved in the control of endosperm weakening in germinating seeds (Leubner-Metzger, 2003; Kucera et al., 2005; Finch-Savage & Leubner-Metzeger, 2006).

In the present paper, the effect of 100µM ABA treatment on micropylar endosperm weakening of scarified imbibed seeds was evaluated for the time point characterized by the testa rupture, (6-8 hours of imbibition), and the time point just before the radicle protrusion, (22 hours of imbibition). The endosperm cap puncture force measured at 6-8 h after imbibition resulted in an average force of 76,17 mN ± 9,49, 1,8-fold lower if compared whit the average value registered in control seeds (135,89 ± 7,19 mN) at the same time. Instead, after 22 h of imbibition while only an average force of 42,67 ± 4,55 mN was enough to protrude the endosperm of scarified imbibed seeds, a force of 67,36 ± 7,69 mN was needed to puncture the endosperm of scarified imbibed seeds treated with ABA. Thus a 1,5-fold increase of the puncture force value was evident in micropylar endosperm following the ABA addition. Based on these values, it seems that ABA may have different effects on endosperm during time. In fact although at the beginning of imbibitions (6-8 h), the ABA seems to increase the endosperm weakening, later it seems to counteract the further rupture which is the prerequisite for germination. Indeed, the higher value of puncture force compared to the control, measured at this time point seems to be related to the germination trend of scarified seeds treated with ABA. The treatment with 100µM of ABA determined a significant delay of germination, as demonstrated from the inability of these seeds to germinate, even when the test was extended for more than a week (Fig. 7). The increase of force required to puncture the endosperm cap after 22 h of imbibition and the germination trend shown by the ABA treated seeds, may suggest that, while ABA treatment had no visible effect on testa rupture, it was able to delay the endosperm rupture through an increase of the micropylar endosperm resistance that, consequently, delayed or completely inhibited the germination. In a recent work, Gimeno-Gilles et al., (2009) showed that the germination pattern and radicle elongation of scarified *Medicago truncatula* seeds, were significantly affected by the addition of ABA (100 µM). In this work is reported that ABA treatment inhibited the germination rate of scarified seeds by about 50% and blocked the radicle elongation. More recently, Bolingue et al., (2010) reported that non dormant *Medicago truncatula* seeds imbibed in a solution containing ABA underwent to testa breaking but not to endosperm rupture, indicating the involvement of ABA in the endosperm weakening but not in testa rupture.

Additional evidences are reported in the literature on the effect of the ABA in delaying endosperm weakening and germination. As for example the works carried out by Muller et al., (2006) in *Lepidium sativum* and *Arabidopsis thaliana* seed, and by Graber et al., (2010) in two different accessions of *Lepidium sativum* seeds. In these works, seeds germinated in medium containing ABA showed a delay in endosperm weakening and consequently in germination in a dose dependent manner, if compared with seed geminated in medium without ABA. Morris et al., (2011) demonstrated again, that the addition of ABA delayed completion of endosperm weakening in *Lepidium sativum* seed, shifting its onset to more than 30 h of imbibition and its completion to 96-120 h; while, without ABA addition, the endosperm weakening of *L. sativum* seed occurred within 18 hours, indicating that ABA strongly affects the timing of this process. Also, the effect of the ABA in delaying micropylar endosperm rupture and germination was demonstrated in tomato (Chen & Bradford, 2000; Wu et al., 2001), and in coffee (Da Silva et al., 2004).

As for other plants, in *M. marina* seed the decrease of the micropylar endosperm resistance leads seeds toward the germination, indicating that the weakening of the micropylar endosperm is required to allow the germination to be completed. Thus, it is conceivable that also in *M. marina* seed the physiological phenomena of endosperm weakening takes place when, during imbibition, the mechanical opposition of the micropylar endosperm is progressively lost, contextually to the radicle elongation in preparation to germination (Ni & Bradford, 1993). Like other species as *L. sativum*, *A. thaliana* and *M. truncatula*, endosperm weakening was affected by ABA treatment that again, delayed or inhibited the germination, trough the increase of the micropylar endosperm resistance occurring, in *M. marina* seeds, after 22 h of imbibition just before the radicle emergence.

To better assess the role of hormones in *M. marina* dormancy, scarified seeds were subjected to different GA_3 and GA_3 plus ABA treatments. In literature, is well documented that mechanical or chemical scarification improve the germination of species affected by "hardseededness", like other species of *Medicago* genus (Patanè & Bradford, 1993; Patanè, & Gresta, 2006; Bolingue et al., 2010), but little is known about the combined action of GA application and mechanical scarification on dormant legume seeds. In the present work emerged that the GA_3 treatment can act synergically with the mechanical scarification enhancing the speed of germination, but in a dose-dependent manner. In fact, scarified seeds treated whit 5 μM of GA_3 showed a decrease in T_{50} value of about 11 hours, respect to scarified seeds not subjected to GA treatment used as control.

Different works documented the action of GA_3 in enhancing germination on different plant species as soybean (Wang et al., 1996, Zhang et al., 1997) and corn (Wang et al., 1996).

Additionally, in a similar manner to that obtained for *Medicago marina* seeds, Commander et al., (2009) demonstrated that not only the germination, but also the speed of germination of *Anthocercis littorea*, *Diplolaena grandiflora*, *Solanum orbiculatum* are promoted by the addition of GA_3.

On the other hand, the application of 10 µM of GA_3 on scarified seeds, reduced the germination percentage (only the 3,3% of seeds germinated) and increase the time of 50% germination of about 28 hours more in comparison with control seeds, establishing a slowing down germination pattern. This latter result suggests that the application of gibberellic acid in combination whit mechanical scarification, could act in a dose dependent-manner on germination speed and rate, and that the concentration of 10 µM, not only inhibited the germination but also significantly delayed the germination speed.

Moreover, the simultaneous application of GA_3 and ABA on scarified seeds had no effects in restoring germination, as these seeds, like 100 µM ABA treated seeds, failed to germinate.

The literature reports many evidences indicating that the key of seed germination and dormancy regulation is the antagonistic action of the three hormones abscisic acid, gibberellins and ethylene (Hedden & Phillips, 2000; Wang et al., 2002; Kucera et al., 2005; Seo et al., 2006; Sun, 2008; Cheng et al., 2009; Seo et al., 2009). Several studies have demonstrated that ABA catabolism and GAs biosynthesis are required for dormancy breaking and for the completion of germination (Ogawa et al., 2003; Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006). Because of this, we can assume that seeds receiving concomitant ABA and GA_3 treatment, failed to germinate for the antagonistic effects of these two hormones in the molecular pathway underling the germination process. With the aim to elucidate the action of these hormones in *M. marina* seed dormancy and germination, RT-PCR analysis on genes encoding the key enzymes involved in the final step of ABA, GA_3 and ethylene biosynthesis was carried out in dry mature seeds, scarified seeds, scarified seeds subjected or not to 100 µM ABA treatment collected at 13 h after imbibition, scarified germinated seeds ABA untreated and scarified not germinated seeds ABA treated.

RT-PCR analysis revealed that the expression pattern of *GA3ox*, *GA20ox*, *NCED5* and *ACO* encoding respectively gibberellin 3-beta-hydroxylase, gibberellins 20 3-beta-hydroxylase, 9-cis-epoxycarotenoid dioxygenase and 1-aminocyclopropane-1-carboxylic acid oxidase enzymes were all influenced by ABA treatment, showing very interesting results.

The pattern of expression of all analysed genes was very similar in dry mature seed (control), in seed treated with ABA after 13 h of imbibition and in non-germinate ABA treated seeds (NG+ABA). In fact, in all the three samples the level of the analysed gene transcripts was too low to be detected.

Scarified seeds showed a very high level of *NCED5* transcript, which was 1,2 fold higher compared to the control, while all the other gene transcripts remained undetectable.

In seed imbibed for 13 h, in absence of ABA, a very high level of *NCED5* together with *GA3ox* and *ACO* transcripts were detected; while in germinated seed not treated with ABA the level of *NCED5* transcript was very low, the level of *GA3ox*, *GA20ox* and *ACO* was rather high compared to the other samples.

A variety of studies demonstrated that *NCED* genes family are strongly up regulated during seed development and dormancy (Tan et al., 2003; Lefebvre et al., 2006; Millar et al., 2006) and in the pathways controlling stress induced ABA biosynthesis, like stress related to drought (Qin & Zeevart, 1999, 2002; Iuchi et al, 2000; Tan et al, 2003, Wan & Li, 2006) and high temperature (Toh et al., 2008), or biotic stress (Fan et al., 2009).

As described in the general introduction of this thesis, 9-cis-violaxanthin and 9-cis-neoxanthin are oxidized by 9-cis epoxycarotenoid dioxygenase (NCED) to generate the intermediate product xanthoxin and this step is considered to be the rate-limiting step in ABA biosynthesis (Xiong & Zhu, 2003). Thus, *NCED* not only is an important gene involved in the biosynthesis, but it also regulates the level of ABA. In *Arabidopsis,* Lefebvre et al., (2006) described nine *NCED* genes, five of which (*AtNCED2, 3, 5, 6*, and *9*) are thought to play a role in ABA production. Using RT-PCR, they showed that in *Arabidopsis*, *AtNCED5,* together with *AtNCED6* and *AtNCED9* were detected only in seed tissue and were required for ABA biosynthesis during seed development. While *AtNCED6* and *AtNCED9* transcripts were detected at early and mid-maturation stages, *AtNCED5* expression was observed only at later stages. Results obtained in this thesis show that while *NCED5* is expressed at very low level in dry mature *M. marina* seeds, it is significantly induced in seeds subjected to scarification and in seed after 13 h of imbibition in absence of ABA treatment. Many works documented the expression of *NCED* gene family members also in response to abiotic stress (Qin & Zeevart, 1999, 2002; Iuchi et al, 2000, 2001; Tan et al, 2003, Ruggiero et al., 2004, Wan & Li, 2006) but just few work describe the specific expression of *NCED5* in response to abiotic stress like high temperature exposure (Toh et al., 2008) or drought stress (Frey et al., 2011). In my previous work it has been suggested the scarification pretreatment, used to remove seed dormancy, can also be perceived as a stress condition from *M. marina* seeds (Scippa et al., 2011). Despite the fact that there is no available information in the recent literature about the induction of *NCED5* in seed subjected to mechanical or chemical scarification, we can assume that, like drought or high temperature exposure, mechanical scarification could induce the expression of *NCED5* in response to this specific stress condition. This result is also confirmed by the level of ABA measured in seeds subjected to scarification (Scippa et al., 2011).

Using qRT-PCR, Millar et al., (2006) showed that in non dormant seeds of *Arabidopsis* and barley the expression of *NCED5* together with *NCED9* increased during the first 6 h of imbibition in water but declined after this time, in preparation to the germination. These authors demonstrated that the modulation of *NCED* gene during seed imbibitions is an important factor able to determine if seed will germinate or will remain dormant. In fact, in *Arabidopsis*, *NCED* genes were induced soon after imbibition, during the first 3-6 h in non dormant seeds, remaining constant within the first 12 h from the onset of imbibition. After this time the expression of *NCED* gene decreased, leading to a rapid decline of ABA level into the seed, culminating in the completion of germination after 48-72 h. Similar results were obtained for barley seeds (Millar et al., 2006). The involvement of *NCED5* gene in seed dormancy establishment has been further proved by Frey et al., (2011) in *Arabidopsis*, trough the phenotypic analysis of triple mutant seeds. The increase of *NCED5* here obtained in *M. marina* seeds after 13h of imbibition may be, as reported in *Arabidopsis* and other species, linked to the role of ABA in determining if seed will germinate or will remain dormant.

Scarified seeds imbibed for 13 h in absence of ABA, are also characterized by an elevated amount of *GA3ox* and *ACO*, while *GA20ox* is not detectable. Instead, germinated ABA untreated seeds showed low level of *NCED5* and high *GA3ox*, together with *GA20ox* and *ACO* transcripts levels. Liu et al., (2010) reported that during the imbibition in water of *Arabidopsis* seeds, the expression of three *GA20ox* and two *GA3ox* genes was significantly induced in the initial hours of imbibition. While transcription levels of *GA20ox* genes increased rapidly during the first 6 h, and then decreasing to a lower level, *GA3ox* genes displayed a delayed initiation, reaching their maximum at 12 h of imbibition with a peak at 24 h of imbibition. In *Pisum sativum*, Ayele et al., (2006) showed that following seed imbibition in water *GA20ox* transcripts levels decreased, while *GA3ox* levels and consequently the amount of bioactive GA_1 increased. Based on these results it has been suggested that the GA_{20} serves as substrate for *in situ* 3β-hydroxylation into bioactive GA_1 required, for the storage mobilization during the germination (Ayele et al., 2006). Since similar results were obtained for *M. marina* seeds, we can assume that, like *pisum*, during the imbibition GA_{20} was used for de novo synthesis of bioactive GA needed for the storage mobilization during the germination. This situation may result in the depletion of *GA20ox* transcripts and in a constant expression of *GA3ox* gene during the imbibition until the completion of germination. The increase of *GA20ox* gene measured further in germinated *M. marina* seed may be explained with its involvement in radicle protrusion and subsequent growth of seedling. In fact, as observed in germinated *Pisum sativum* (Ayele et al., 2006) and *Arabidopsis thaliana* seeds (Ogawa et al., 2003), transcripts abundance of *GA20ox* increased markedly in embryonic-axis when the initial radicle protrusion was visibile, remaining high also

when germination was completed. These authors suggested that the large increase in amount of *GA20ox* transcripts serves to synthesize and maintain growth-active GA in the embryo axis for the rapid expansion of radicle. Moreover, after germination, the high levels of *GA20ox* gene serve for the growth of young seedlings.

Like *GA3ox* gene, *ACO* gene resulted expressed during imbibition remaining constant up to the radicle protrusion. Petruzzelli et al., (1995) showed that the action of ethylene was needed only during the early phases of the germination process in *Pisum sativum*, assuming that the germination in *Pisum* was dependent on ethylene. Subsequent studies on germinating *Pisum sativum* seeds (Petruzzelli et al., 1999; 2000) have shown that *ACO* gene induction and ethylene biosynthesis and responsiveness were confined to the embryonic axis, demonstrating that ACO enzyme activity is mainly localized to the radicle during seed germination. In addition to that, these authors demonstrated that maximal *ACO* gene induction was achieved after radicle emergence and was correlated with maximal ethylene amount (Petruzzelli et al., 1999; 2000). Similar findings were obtained for *Cicer arietinum* seeds (Gómez-Jiménez et al., 1998; Gómez-Jiménez et al., 2001). Thus, on the basis of the expression pattern obtained for *ACO* gene in *M. marina* seeds, it can be proposed that like in *Pisum*, ethylene, acting together with gibberellins, plays a pivotal role in allowing the completion of germination process.

Results reported in this thesis showed that none of these genes resulted expressed when seed were subjected to the ABA treatment.

It is well known that seed germination is regulated by dormancy and different environmental factors such as light, oxygen, temperature. Moreover, it is also thought that the key to this regulation is the balance of the negative and positive effects due to abscisic acid, gibberellins (Hedden & Phillips, 2000; Seo et al., 2006; Sun, 2008; Seo et al., 2009). Studies on numerous mutants have demonstrated that ABA catabolism and GA biosynthesis are required for seed germination (Ogawa et al., 2003; Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006). Moreover, Seo et al., (2006) indicated that endogenous ABA suppressed GA biosynthesis in developing *Arabidopsis thaliana* seeds, through the suppression of *GA20ox* and *GA3ox* genes.

Recently, Toh et al. (2008) showed that high temperature can stimulate ABA biosynthesis, which in turn repress GA biosynthesis and signaling during *Arabidopsis* seeds germination. In addition to the action of endogenous ABA, the application of exogenous ABA on germinating seeds may regulate the expression of gibberellins biosynthetic genes (Zentella et al., 2007; Liu et al., 2010).

Zentella et al., (2007) showed that the transcript levels of *GA20ox* in *Arabidopsis* were significantly reduced by application of exogenous ABA, and in a more recent study, Liu et al., (2010) reported that also the expression of *GA3ox* gene was significantly inhibited by exogenous

application of 0,5 µM of ABA in *Arabidopsis* seeds. Similar results were obtained here for *M. marina* in which the application of 100 µM of ABA completely inhibited the expression of GA biosynthetic genes in all the seeds subjected to ABA treatment. This finding suggests that exogenous ABA was able to inhibit the biosynthesis of bioactive GA, trough the suppression of *GA20ox* and *GA3ox* genes. Also *NCED5* gene transcripts were not detectable in presence of ABA treatment. Because the *NCED* gene product has been suggested to catalyze the rate-limiting step in the ABA biosynthesis pathway (Tan et al., 1997; Qin & Zeevaart, 1999; Taylor et al., 2000; Thompson et al., 2000b, Xiong & Zhu, 2003), whether or not the expression of this gene is regulated also by the application of exogenous ABA is a very important information regarding the capacity of ABA to auto-regulate its own biosynthesis. Results obtained for *M. marina* seeds were in agreement with those found for tomato seeds and cowpea. In tomato, it was found that the *NCED* gene was not induced by the application of exogenous ABA (Thompson et al., 2000a), and similarly, in cowpea, ABA was unable to activate *NCED* family genes (Iuchi et al., 2000). These observations would support the hypothesis that exogenous ABA may regulate the biosynthesis of endogenous ABA trough the suppression of *NCED* genes.

Finally, the application of exogenous ABA on *M. marina* seeds suppressed also the expression of *ACO* gene. Several studies indicate a role for ethylene in promoting seed germination, mainly through the negative regulation of ABA action (Ghassemian et al., 2000; Cheng et al., 2009). However, some evidence suggests ethylene influences germination by the accumulation of *ACO* transcripts in seeds of numerous species (Petruzzelli et al., 2000; Chiwocha et al., 2005; Matilla et al., 2005; Hermann et al., 2007; Iglesias-Fernandez & Matilla, 2009). Linkies et al., (2009) showed that *ACO* transcripts accumulate in the embryo and endosperm of germinated *Arabidopsis thaliana* and *Lepidium sativum* wild-type seeds. For both species, this accumulation was inhibited following the application of 10µM of ABA on wild-type seeds, in a similar manner as occurred for *M. marina* seeds treated with 100 µM of ABA.

Taken together, these findings generally demonstrated the presence of an antagonistic interaction between ABA, GAs and ethylene and a synergy between GAs and ethylene in controlling seed germination. *Medicago marina* seed germination was inhibited by the application of exogenous ABA which is able to act on the repression of GAs and ethylene biosynthetic genes, avoiding, in that way, the accumulation of these two hormones into the seeds. As stated before, GAs and ethylene regulate the expression of cell wall modifying enzymes, counteracting the ABA inhibitory effect and promoting endosperm weakening (Leubner-Metzger, 2003; Kucera et al., 2005, Müller et al., 2006; Gimeno-Gilles, 2009; Linkies et al., 2009). Exogenous ABA, used for hormonal treatments in this work, trough the repression

of *GA3ox*, *GA20ox* and *ACO* genes, may prevent the accumulation of cell wall loosening enzymes regulated by GAs and ethylene, leading to a final block of germination.

In conclusion, results emerged from this work suggest that the micropylar endosperm can contribute to the establishment of *M. marina* physical dormancy, that once again, it can be removed by mechanical scarification. Moreover, abscisic acid seems to be strongly involved in regulation of germination timing, and its action may be on micropylar endosperm weakening and on the expression of GA and ethylene biosynthetic genes.

In this work, the preliminary results of a proteomic analysis, focused on the identification of molecular factors controlling dormancy, germination and ABA response are also presented. In particular using of 2D analysis coupled whit the multivariate statistical analysis were identified 95 spots that are markers of specific physiological state (dormancy-germination) or of a specific treatment (ABA-scarification). From these analyses emerged that a large number of spots (sixty-eight) were strongly up regulated during germination and strongly down regulated in control, scarified seeds and not germinated seeds treated with ABA. Thus, the three samples control, scarified seeds and not germinated seeds subjected to ABA treatment, showed a very similar proteins expression profile, indicating the involvement of ABA in the molecular pathways allowing dormancy induction and repression of germination. Indeed, in non germinated seeds ABA treated, it appears that exogenous ABA influences all those spots resulting strongly up regulated in germinated seeds, making their expression similar to that found in dormant mature seeds. On the basis of these information, it can be assumed that these proteins represent specific markers of different physiological states and might be expressed in seeds when dormancy is overcome in order to complete germination.

The sequencing by mass spectrometry and the identification of these spots is actually in progress to understand the role of the specific protein markers of dry mature seeds (dormant seeds), scarified seeds, germinated and not germinated seeds and ABA treatment, and to unravel their role in dormancy breaking and during the weakening of the micropylar endosperm.

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Supplementary material

Spot ID			
Control (C)	**Scarified seeds (S)**	**Germinated seeds (G-ABA)**	**Not germinated seeds ABA treated (NG+ABA)**
2	2	2	2
5	5	5	5
7	7	7	7
8	8	8	10
10	10	10	11
11	11	11	17
17	17	17	20
20	20	20	23
23	23	23	25
25	25	25	31
31	31	46	36
46	46	47	46
47	47	48	47
48	48	49	48
49	49	50	49
50	50	52	50
52	52	53	52
53	53	54	53
54	54	55	54
55	55	56	55
56	56	58	56
58	58	74	58
74	74	83	74
83	83	84	83
84	84	88	84
88	88	90	88
90	90	91	90
91	91	96	91
96	96	98	96
98	98	99	98
99	99	107	99
107	107	111	107
111	111	114	111
114	114	120	114
120	120	121	120
121	121	122	121
122	122	124	122
124	124	125	124
125	125	126	125
126	126	129	126
129	129	130	129
130	130	131	130
131	131	135	131
135	135	136	135
136	136	137	136
137	137	142	137
142	142	144	142
144	144	146	144
146	146	147	146
147	147	148	147
148	148	149	148
149	149	150	149
150	150	151	150

Spot ID			
Control (C)	**Scarified seeds (S)**	**Germinated seeds (G-ABA)**	**Not germinated seeds ABA treated (NG+ABA)**
163	163	185	163
164	164	164	185
184	184	184	164
185	185	187	181
187	187	188	184
188	188	192	187
192	192	211	188
211	211	212	192
212	212	216	204
216	216	231	211
231	231	233	212
233	233	242	216
242	242	247	231
244	244	249	233
247	247	254	242
249	249	265	244
254	254	275	247
265	265	287	249
275	275	290	254
287	287	292	265
290	290	296	275
292	292	299	287
296	296	300	290
299	299	301	292
300	300	305	296
301	301	306	299
305	305	313	300
306	306	333	301
313	313	334	302
333	333	335	305
334	334	336	306
335	335	337	313
336	336		326
337	337		333
			334
			335
			336
			337
			343

Table 1 In the table are listed spots found in control seeds (C), scarified seeds (S), germinated seeds not ABA treated (G-ABA) and in not germinated seeds ABA treated (NG+ABA).

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