The structure and function of maize scutellum during early stages of germination

Hédia Tnani

Universidad de Barcelona
Facultad de Farmacia
Departamento de Bioquímica y Biología Molecular

Centro de Investigación en Agrigenómica
Consorcio CSIC-IRTA-UAB-UB
Laboratorio de Genética Molecular Vegetal

The structure and function of maize scutellum during early stages of germination

Hédia Tnani

Tesis doctoral

Barcelona, Mayo 2012

UNIVERSIDAD DE BARCELONA
FACULTAD DE FARMACIA
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR
PROGRAMA DE DOCTORADO EN BIOTECNOLOGÍA
BIENIO 2006-2008

The structure and function of maize scutellum during early stages of germination

Memoria presentada por Hédia Tnani para optar al título de Doctor por la Universidad de Barcelona

Trabajo realizado en el Departamento de Genética Molecular del Centro de Investigación en Agrigenómica (CRAG)

Dr. Ignacio López Ribera
(Director de la tesis)

Dr. Carlos M. Vicient Sánchez
(Director de la tesis)

Dr. Albert Ferrer Prats
(Tutor de la tesis)

Hédia Tnani
(Autora de la tesis)

Barcelona, Mayo 2012

À mes chers parents,
À ma chère sœur Fati,
À mon cher oncle Férid,

To all my beloved family,

Agradecimientos

Esta es sin duda la parte más complicada en escribir por todos los sentimientos y las emociones que conlleva. Este trabajo no hubiera sido posible sin la variada y generosa contribución de muchas personas. Esta tesis es tan mía como vuestra. Para ello, me gustaría agradecer a todos aquellos que con su dirección, sus consejos, su apoyo, su ayuda, sus críticas, sus sugerencias, su estímulo, me han ayudado a desarrollar este trabajo:

- A mis directores de Tesis: los Drs. Carlos M. Vicient e Ignacio López Ribera por su dirección, su asesoramiento científico, su predisposición permanente e incondicional en aclarar mis dudas, su paciencia, su apoyo y consejos y por haberme introducido y enseñado todo del fascinante y tan interesante mundo de la investigación y de la biología molecular. Me faltan palabras para expresarles todos mis agradecimientos y toda mi gratitud solo puedo decir ¡¡ Muchas gracias por todo!!

- Al Prof. Pere Puigdomenech por darme la oportunidad de realizar esta tesis en el CRAG.

- A mi tutor de tesis el Dr. Albert Ferrer Prats por aceptar la tutela de esta tesis y por sus consejos en la redacción de la misma. Gracias por aclararme muchas de las dudas que tenía.

- Je remercie également le Professeur Thierry Jouenne, responsable scientifique de la plate-forme protéomique IBiSA PISSARO, pour m'avoir accepté dans son laboratoire et pour sa précieuse collaboration au niveau de la partie protéomique.

- J'aimerai également remercier mon oncle, le Professeur Férid Limam, pour tous les conseils et les encouragements qu'il m'a prodigué tout au long de cette thèse.

Mis agradecimientos van también:

- Al Dr. Irar, por toda su ayuda en un tema tan novedoso para mí como el de la proteómica. ¡Gracias Sami!

- A la Dra. Nora Garcia, por su inestimable ayuda y sus buenos consejos y por estar siempre dispuesta a echar una mano.

- A Núria, mi gran compañera y amiga por tus consejos nacidos de tu gran experiencia científica que me sirvieron y me ayudaron mucho. He aprendido tanto estando a tu lado. Gracias por aguantarme tanto tiempo y por hacer de los momentos de convivencia dentro del laboratorio una experiencia muy rica tanto en lo profesional como personal.

- A mis compis del lab verde: muchos de vosotros ya seguís en el mismo lab, otros os habéis cambiado de lab y otros ya no están: A Cristian, Chus, José et Mercé muchas gracias por haber aguantado a la más caótica y desordenada. Gracias por vuestros consejos y vuestra ayuda y sobretodo por vuestra sonrisa y vuestro buen humor durante todos estos años.

- A Bea, Céline y Catalina muchas gracias por la gran amistad que me habéis ofrecido, por vuestro constante apoyo y por animarme siempre que haga falta. Gracias por escucharme y brindarme ayuda cuando ha sido necesario y por haber hecho de mi estancia en el CRAG una maravillosa aventura y una continúa caja de sorpresas. ¡¡¡ Sois las mejores!!!

- A Imen A. mi compañera de desventuras y sobre todo amiga. Gracias por tus consejos, ayuda y apoyo. Cuantas veces acudí a ti cuando las cosas salían mal. ¡¡No me cabe ninguna duda de que serás una gran investigadora!!!

- A Ángel por la ayuda prestada en la toma y trato de imágenes y a Montse por toda su ayuda y sus consejos en la realización de los experimentos del confocal y en la toma de imágenes para tener la imagen "más chula".

- A los invernaderos: Pilar, Eva, Jordi y Alejandro por la enorme labor de cuidarme mis plantas de Arabidopsis con gran profesionalidad.

- A todos los colores y a todos los compañeros y personal del departamento de Genética Molecular del CRAG, no puedo nombrarlos a todos pero cada uno de vosotros ha contribuido de manera especial a la realización de este trabajo. Habéis llenado mi vida de sonrisas y alegrías que hizo de mi vida una eterna y maravillosa aventura y una gran experiencia. Gracias por todos vuestros consejos, ayudas y por animarme siempre que haga falta y sobre todo gracias por todos estos momentos inolvidables que hemos compartido dentro y fuera del CRAG.

- A todos los amigos que tuve fuera del CRAG. A toda esta gente ajena o no al mundo de la ciencia que siempre me ha apoyado y animado durante los peores momentos. A ellos también les debo mucho.

- A la GRAN FAMILIA TUNECINA, a los TUNECINOS DE ADOPCIÓN: A Cris T., Ali, Imen C., Jaco, Zohra, Emna, Olfa, Sami, Chris M., Adem, Aymen, Bader, Emine, Skander …etc Porque a pesar de todo seguís allí a mi lado al pie del cañón brindándome ayuda siempre que haga falta. Porque ser extranjero no siempre es fácil, gracias por ser mi pequeña Túnez en España.

- A los IO: a Antonieta, Xavi, Laura, Judith y Paula que hubiese sido de mi vida sin vosotros. Gracias por esta gran experiencia y por todos los valores que me habéis enseñado. Espero ser un día "una consumidora muy responsable". Cleo gracias por tu amistad y por ser tal como eres UNA PERSONA MUY ESPECIAL. Hemos compartido muchas risas, cines, fiestas y viajes pero también momentos difíciles. Tuviste que aguantar a la más desordenada y caótica futura Dra.

- A mis compis de piso, los de antes y los de ahora: a la "Peris Peris", Jaime, Lorenzo y Eric por ser los mejores.

- A Hanaa, Tabet y Diana gracias por haber hecho de este año muy difícil de redacción y muchas veces de desesperación: un año tan divertido de aprendizaje de una cultura tan fascinante como la PALESTINA.

- Y sobre todo A ESTA MARAVILLOSA FAMILIA que tengo que a pesar de la distancia ha estado apoyándome SIEMPRE en todas las decisiones que he tomado y sobre todo durante todos estos largos años de doctorado. A ellos les dedico esta tesis.

Y ahora en francés: Aucun remerciement ne serait à la hauteur du soutien de mes parents tout au long de cette thèse. Ils ont su me transmettre l'appétit du savoir et la persévérance face aux problèmes.

“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Curie

Abbreviations

ABA	abscisic acid
ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1-carboxylic acid
Ala	Alanine
AOA	aminooxyacetic acid
AOBs	artificial Oil Bodies
AP	acid phosphatase
BAP	6-benzylaminopurine
dai	Days after imbibition
ER	endoplasmic reticulum
FAs	fatty acids
GA	gibberellin
IAA	indole acetic acid
IBA	indole-3-butyric acid
ICL	isocitrate lyase
MLS	malate synthase
NAA	naphtalen acetic acid
NBD	nucleotide binding domains
NBF	nucleotide-binding folds
OBs	oil bodies
OPT	oligopeptide transporter
ORFs	open reading frames
PAC	paclobutrazol
PEPC	phosphoenol pyruvate
PSV	protein storage vacuoles
PTR	peptide transporter
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SDS	sodium dodecyl sulfate
SPS	sucrose-phosphate synthase
STS	silver thiosulfate
TAGs	triacylglycerides
TIBA	2,3,5- triiodobenzoic acid
TMD	transmembrane domains
YFP	yellow fluorescent protein
YSL	yellow stripe-like

INDEX

1. INTRODUCTION

1 INTRODUCTION

1.1 Structure of cereal grain

Grass plants produce one-seeded dry fruits known as caryopsis (popularly known as grain). This type of fruits are moncarpelate and indehiscent, and the ovary wall is tightly bound to the seed coat. In maize, the caryopsis is also known as kernel.

Cereal grains are divided into three main parts: embryo, endosperm and carpel wall (seed coat and pericarp) (Fig. 1.1). Embryo and endosperm are products of the double fertilization (Evers and Millar, 2002).

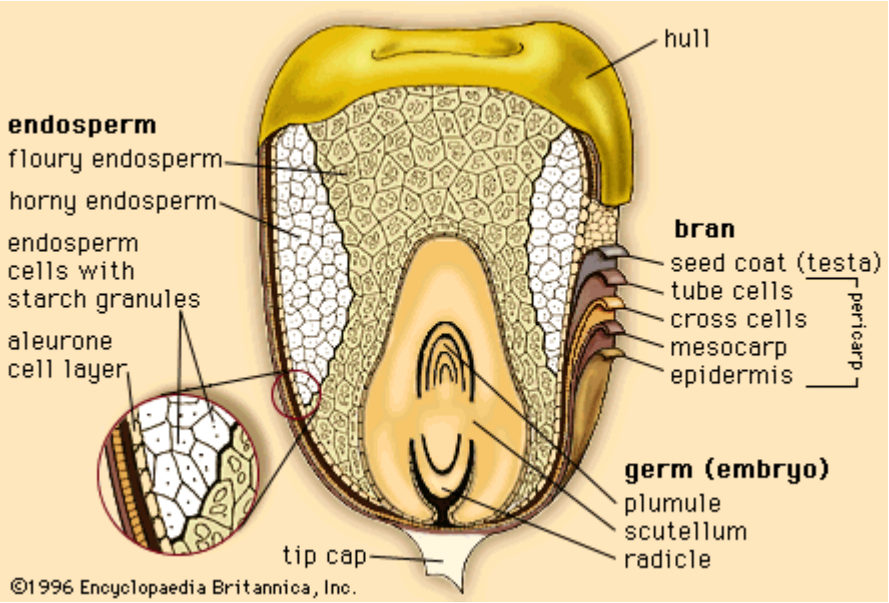


Figure 1.1. The outer layers and internal structures of a kernel of corn.
From Encyclopædia Britannica, Inc

1.1.1 Endosperm

The endosperm is the product of the second fertilization event in double fertilization (Lopes and Larkins, 1993). Endosperm is formed when the two sperm nuclei inside a pollen grain reach the interior of the embryo sac (female gametophyte). One sperm nucleus fertilizes the egg, forming a zygote, while the other sperm nucleus fuses with the two polar nuclei at the centre of the embryo sac, forming a primary endosperm cell, which develops into the endosperm. The endosperm is composed of three major cell types: the starchy endosperm, the basal transfer layer and the aleurone layer (Olsen, 2001).

1.1.1.1 Transfer cell layer

The transfer cell layer is located at the basal part of the grain and functions in nutrient uptake from the mother plant during seed development. There is no vascular connection between the maternal plant and developing caryopses and all nutrients entering the seed pass through a specialized group of cells called transfer or basal endosperm cells by symplastic or apoplastic methods (Hueros *et al.*, 1995, 1999).

Moreover, endosperm transfer cells are characterized by the presence of cell wall ingrowths, which increase the surface of the cellular membrane up to 22-fold and make endosperm transfer cells very efficient in the uptake of nutrients from adjacent maternal vascular tissue to the endosperm. Sucrose synthase and the cytoskeleton probably play a primary role in the wall ingrowth formation (Wang *et al.*, 1994).

Sugar, especially glucose, is found to modulate the promoter activity of ZmMRP-1, a determinant of transfer cell-specific expression. The ZmMRP-1- encoded protein can transactivate the promoters of transfer cell-specific genes. The major solutes transferred by endosperm are amino acids, sucrose, and monosaccharides, which is consistent with the expression of their transporters and transport-associated genes (Thompson *et al.*, 2001; Zheng and Wang, 2010).

1.1.1.2 Starchy endosperm

The starchy endosperm comprises the major part of the endosperm and is formed by relatively uniform cells in shape and content. Mature starchy endosperm if formed by dead cells filled of starch granules and protein bodies. These reserves, which account for most of the mass of dead starchy endosperm cells, are not broken down until germination has been triggered and are basic for fuel the growing seedling until autotrophy. The nutrients are made available as a result of hydrolysis by enzymes produced in the embryo and in the aleurone layer (Saito *et al.*, 2010).

The major storage proteins in maize (*Zea mays*) kernels are the alcohol-soluble prolamins, a type of storage proteins present only in grasses. Maize prolamins (zeins) are divided into different types (α-, β-, γ-, and δ-zeins) that differ in their amino acid composition and structural properties (Herman and Larkins, 1999; Shewry and Halford, 2002).

Zeins are synthesized in the endoplasmic reticulum (ER) where they form accretions called protein bodies. Zeins and other prolamins do not contain a canonical ER retention signal, and the mechanism responsible for their aggregation involves specific protein–protein interactions among each other, the action of the chaperone binding protein (BiP), and the formation of disulfide bridges (Vitale and Ceriotti, 2004; Randall *et al.*, 2005; Pompa and Vitale, 2006; Kumamaru *et al.*, 2007). Maize seeds also contain smaller quantities of legumin-1 and α-globulin (Woo *et al.*, 2001; Yamagata *et al.*, 2003). These storage proteins accumulate in vacuoles during the early stages of endosperm development and are then retained in ER protein bodies at later stages (Müntz, 1998; Müntz *et al.*, 2001; Arcalis *et al.*, 2010).

Although oil is not the main reserve accumulated in the starchy endosperm, triacylglyceride deposits are present in small quantities throughout the starchy endosperm (Hargin *et al.*, 1980; Barthole *et al.*, 2012). Oil bodies in the starchy endosperm have diffuse boundaries and are fused with each other and with protein vacuoles during grain development, forming a continuous oil matrix between the protein and starch components (Heneen *et al.*, 2008).

Starchy endosperm cells undergo cell death during seed maturation. Unlike programmed cell death of most other plant cells, including aleurone cells, the death of starchy endosperm cells is not followed by a rapid destruction of the corpse (Young *et al.*, 1997; Young and Gallie, 1999, 2000b).

1.1.1.3 Aleurone layer

The aleurone layer consists of a single or few cell layers that surround the starchy endosperm and, in a modified form, the embryo (Olsen, 2001). In most cereals, including typical maize lines, the aleurone is a single cell layer, although in barley (*Hordeum vulgare*) it comprises about three cell layers and is variable in rice (*Oryza sativa*). Aleurone cells are distinguished from starchy endosperm cells by their morphology, biochemical composition, gene expression profiles and because they remain alive in dry seeds. During seed maturation, abscisic acid (ABA) induces aleurone cells to acquire desiccation tolerance and survive seed drying (Young *et al.*, 1997; Young and Gallie, 2000a).

Aleurone cells have a regular cuboidal shape with thick walls and prominent large nuclei. Aleurone cells have relatively high concentrations of protein, lipid, vitamins and minerals. The cytoplasm of the mature aleurone cell is filled with organelles, the most prominent of which are the protein storage vacuoles (PSV). They also contain oleosomes embedded in their limiting membranes (Bethke *et al.*, 1998).
PSV store a small amount of non-starch carbohydrate (Jacobsen *et al.*, 1971) and abundant mineral reserves that are chelated into phytin (Ca, Mg, and K salt of inositol hexaphosphate) (Stewart *et al.*, 1988). Mitochondria, ER, glyoxysomes and Golgi are also prominent in aleurone cells (Jones, 1969; Lonsdale *et al.*, 1999).

While performing some storage function, the primary role of the aleurone is digestive. Upon imbibition, the embryo produces gibberellin (GA), which induces cells in the aleurone layer to secrete amylases and proteases that break down the stored starch and proteins in the dead starchy endosperm, making free sugars and amino acids available to the growing seedling (Fath *et al.*, 2000). The aleurone is also a major site of mineral storage (Stewart *et al*., 1988) and serves to protect the nutrient-rich endosperm by expressing an array of stress- and pathogen- protective proteins such as PR-4 (Jerkovic *et al.*, 2010).

1.1.2 Embryo

The embryo in grasses, at grain maturity, comprises the embryonic axis and the scutellum (Fig.1.2). The scutellum is supposed to be the single cotyledon in the monocotyledoneus embryos and is attached to the embryo axis in the scutelar node. The embryo has the highest concentration of lipid and lipid soluble vitamins in cereal grains (Barthole *et al.*, 2012).

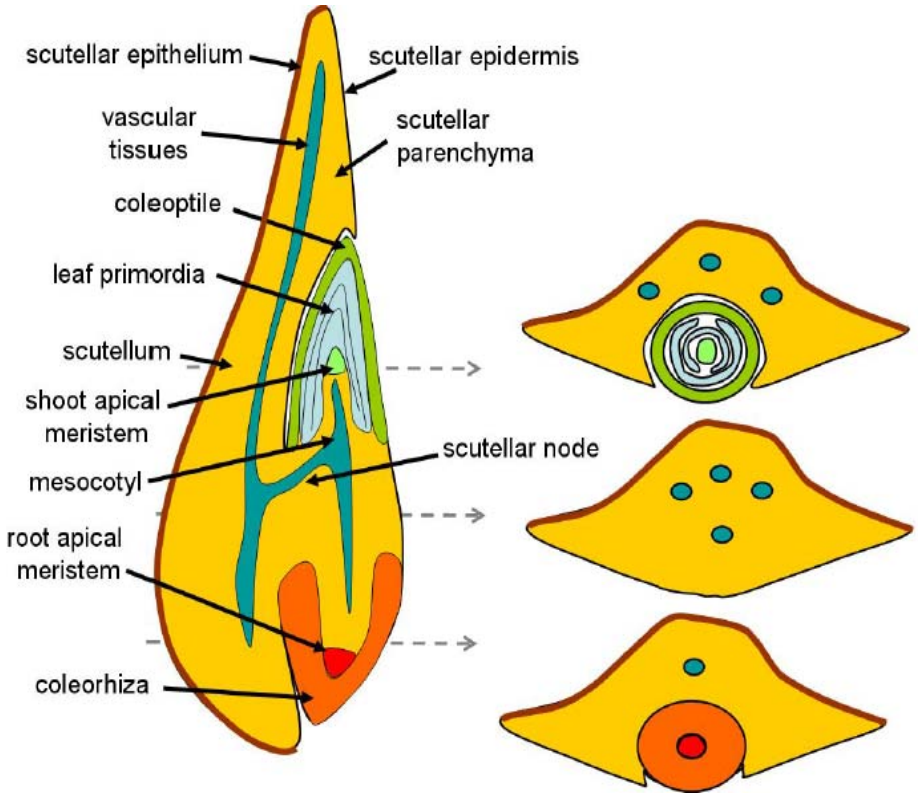


Figure 1.2. Schematic representation of a mature maize embryo.

1.1.2.1 Embryonic axis

The embryonic axis originates the root, leaves and stem of the new plant. In the mature seed, the embryo axis is formed by the primary root, protected by the coleorhiza, and the stem tip with five or six short internodes and leaf primordia which, as a whole, form the plumule that is surrounded by the coleoptile (Figure 1.2). In grasses, the embryo axis can be divided in:

- Epicotyl: the apical end of embryo axis above the coleoptile insertion site.
- Mesocotyl: the embryonic axis between the scutellar node and the coleoptile insertion site. It growth quickly during germination and serves to elevate the coleoptile out of the soil.
- Hypocotyl: the embryonic axis between the the scutellar node and the radicle.

1.1.2.2 Scutellum

The name scutellum (small shield, in latin) derives from its shield-like shape and it lies between the embryonic axis and endosperm (Figure 1.2). Dissected scutellum constitutes 11% or the kernel mass, and about 90% of the embryo. The scutellum consists in four tissues (Swift and O'Brien, 1970):

- **Epithelium**: a single cell layer bordering the starchy endosperm. It is constituted by cylindrical, densely cytoplasmic cells attached to each other only near their bases. During germination, they secrete hydrolytic enzymes which diffuse into the endosperm where they digest starch and proteins. The resulting sugars, peptides and amino acids are then translocated through the scutellum into the embryo axis. In many places, the epithelium folds inward in the scutellum, forming "glands" up to 1 μm in length, thus providing additional surface for enzyme secretion. The walls of the secretory cells are different from other cell walls in being comprised only of one type of hemicellulose and very little cellulose.
- **Parenchyma**: Comprises the major part of the scutellum. Parenchyma tissue is composed by cells that contain a nucleus, dense cytoplasm, some starch granules, and clear bodies that contain liquid oil (oleosomes). Parenchymal cells are short cylindrical with thick walls bearing numerous pits and intracellular spaces that facilitate movement of materials among cells.

One constituent of the scutellum cell walls is similar to the hemicellulose in pericarp walls. It is a polymer made up of a xylose backbone with short side branches of xylose, arabinose, galactose, and glucuronic acid.

- Epidermis: a single layer of cuticulized cells bordering the embryonic cavity.

- Provascular tissue: The vascular system of the scutellum in the mature embryo is undifferentiated but only a few hours after germination (3 to 30 hours) it differentiates into phloem and xylem. In maize, there is a main bundle connecting the embryonal axis with the scutellum that travels from mesocotyl almost to the scutellar tip where many of their branches turn downward like a weeping willow. Additional bundles supply the posterior scutellar appendices. The main bundle is collateral, with xylem in the adaxial side and phloem in the abaxial. However, the branches are usually amphivasal, with the phloem surrounded by xylem. In the smaller ones, which approach the epithelium, the xylem ring around the phloem is either incomplete or completely missing (Negbi, 1984). The temporal and spatial differentiation of the scutellar vascular system indicates that it plays an important role in secretive and absorptive functions of the scutellum (Swift and O'Brien, 1970).

The scutellum functions as a storage organ but it also plays an important role in endosperm digestion during germination. As a storage organ, the maize scutellum accumulates 90% of the lipids in the kernel, 19% of the proteins, 10% of the sugars and 10% of the minerals. Deposition of lipids begins in the scutellum at about 22 days after pollination and continues until maturity. As the kernel approaches maturity, the scutellum cell walls thicken appreciably and develop large pits. Lipid deposition continues in the scutellum after starch and protein synthesis has stopped in the endosperm. Most of the triacylglycerides in maize scutellum are contained in oil bodies, which have similar properties to oil bodies in other plant tissues and species (Huang, 1992). The oil appears to be synthesized on the ER and the whole oil body assembled almost simultaneously (Napier *et al.*, 1996). Protein bodies and phytate are also present in the epithelium and parenchyma scutellar cells (Herman and Larkins, 1999).

The scutellum also plays a role in the digestion of the endosperm reserves during the early growth of the seedling. On one hand, in addition to the aleurone layer, the scutellum secretes enzymes to dissolve the endosperm starch (Nomura *et al.*, 1969).

On the other hand, since the scutellum lies between the reserves stored in the starchy endosperm and the embryonic roots and shoot, the scutellum must provide the pathway along which these food reserves move to the growing axis during germination. Moreover, the products of the mobilization of the scutellum own reserves (fat, proteins, and minerals) must also be transported towards the embryo axis. The vascular tissues of the scutellum must play a key role in nutrient transport. Scutellar vascular tissues are connected to the embryo axis vasculature in the scutelar node and then extend to the scutellum parenchyma. They remain in a non-completely differentiated form in the mature seeds but they rapidly differentiate after imbibition, allowing a quick nutrient transport (Swift, 1972).

1.2 Oil bodies

Eukaryotic cells store lipids in specialized cytoplasmic organelles called oil bodies, oleosomes, lipid bodies or lipid droplets (Fujimoto and Parton, 2011). These organelles protect lipid reserves against oxidation and hydrolysis. Oil bodies (OBs) were first observed in plants, in cotton seeds, and were described as spherical bodies of about 1 μm in diameter and highly refractive to light (Huang, 1992; Tzen and Huang, 1992).

In plants, oil bodies (OBs) are especially abundant in the embryo and, in some species, they represent the main nutrient reservoir of the seed. For example, lipid content in rapeseed (*Brassica napus*) represents about 40% seed weight, 20% in soybean (*Glycine max*), and 46–54% in some subtropical species like sesame (*Sesamum indicum*) (Hiremath *et al.*, 2007). Even in non-oilseed species such as pea (*Pisum sativum*) lipids represent 2% of their seed weight. The average content of oil in corn seed is 4% but oil content can be as high as 20% in specially selected lines (Moose *et al.*, 2004).

In most species, seed lipids accumulate mainly in the embryo (maize, sunflower, rapeseed), but there are species in which the highest lipid accumulation is observed in the endosperm (carrot, cilantro) or in both organs (tobacco) (Zweytick *et al.*, 2000). Lipid accumulation has also been observed in pollen and tapetum and in fruit carpels (olive, coconut). Pollen and tapetum OBs are similar in shape and size as the observed in seeds, but fruit OBs are larger and irregularly shaped compared to embryo ones (Murphy, 1993). Other organs in which the presence of OBs has been described, although at lower quantities, are the young root meristem (Hernandez-Pinzon *et al.*, 2001), apical meristem (Rinne *et al.*, 2001), root nodules (Gurusamy *et al.*, 2000), phloem (Madey *et al.*, 2002) and leaf epidermal cells (Wahlroos *et al.*, 2003).

In corn, seed oil accumulates mainly in the embryo, especially in the scutellum and in the aleurone layer. The composition of the lipid reserves may vary between species and varieties but always the most abundant compounds are triacylglycerides (TAGs). Other minor components are free fatty acids, phospholipids, sterols, tocopherols, waxes and carotenoids. TAG synthesis takes place in the endoplasmic reticulum (ER) (Tzen *et al.*, 1990; Murphy, 2011; van der Schoot *et al.*, 2011).

1.2.1 Structure of oil bodies

The OBs are remarkably stable even when extracted out of the cells. This stability is due to their structure. The OBs are spherical structures with diameters ranging 0.6 to 2.0 μm (Tzen *et al.*, 1993). They have an internal matrix comprised mainly of triacylglycerides which is bounded by a phospholipid monolayer embedded with unique proteins (Fig.1.3) (Lee *et al.*, 1995). The set of proteins depend on the species and organs (Huang, 1992). In plants, OB associated proteins are principally oleosins, caleosins and steroleosins (Fig.1.3) (Purkrtova *et al.*, 2008). OBs in maize scutellum have a relative simple set of proteins and structure (Lee *et al.*, 1995).

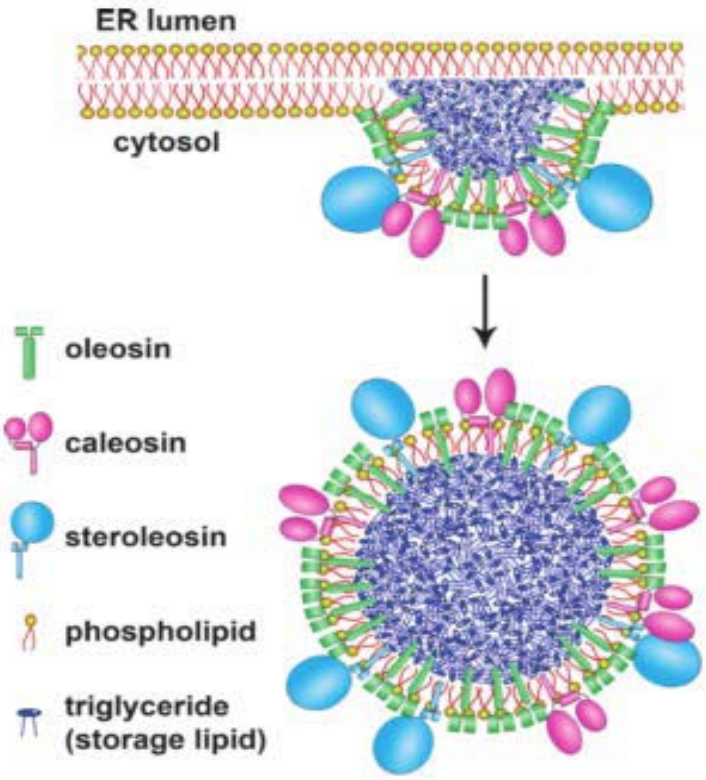


Figure 1.3. A Schematic Model of Forming Oil Body (Shimada and Hara-Nishimura, 2010).

1.2.1.1 Oil body associated proteins

OB associated proteins are a class of proteins localized on the surface of these organelles. They exist in both Bacteria and Eukaryotes. The set of Oil Body Associated proteins present in a cell depends in the species, the variety, the cell-type and the physiological and developmental conditions (Murphy, 2005). The most important role of these proteins is to maintain the integrity of the OBs and to prevent their fusion. Indirectly, they also control the size of the OBs. The most abundant proteins in plant OBs are oleosins. These proteins are plant-specific and abundant in seeds (Shimada and Hara-Nishimura, 2010). Some other proteins have been described as caleosins and steroleosins. They may be involved in specific roles as, for example, the interactions with other organelles (Purkrtova *et al.*, 2008; Pu *et al.*, 2011). These proteins may constitute important targets for seed improvement both in term of oil seed yield and optimization of technological processes for extraction of oil and storage proteins (Murphy and Hernendez-Pinzon, 2000).

1.2.1.1.1 Oleosins

Oleosins are the most abundant proteins in plant OBs. They are relatively small proteins comprising three domains: a hydrophilic N-terminal domain, a hydrophobic central domain, and a hydrophilic C-terminal domain. The central domain is highly conserved and forms a hairpin like structure that is composed of two anti-parallel β– strands. This structure is inserted into the triacylglyceride matrix of the OB and enables oleosins to target into the growing OBs already attached to the endoplasmic reticulum (Fig. 1.3) (Huang, 1992, 1996; Capuano *et al.*, 2007).

Numerous data indicate that oleosins stabilize the OBs preventing merging with each other. The high negative charge of the hydrophilic parts contributes to repel the oleosomes from other OBs. Thus oleosins would be responsible for maintaining the small size of oleosomes. The average size of OBs affects the germination rate of seeds. During germination lipid reserves must be mobilized quickly. This mobilization begins with the attack of lipases, which takes place on the surface of the OB. If the OBs are large the surface/mass ratio is small and, therefore, lipases can only mobilize the triacylglycerides with a low rate. In consequence, germination rates are positively associated with oleosin content (Lin *et al.*, 1983; Wang and Huang, 1987; Barros *et al.*, 2010).

Oleosin-deficient mutants show an inverse proportion between OBs sizes and total oleosin content. The Arabidopsis double mutant ole1/ole2 has irregularly-enlarged OBs throughout the seed cells, and hardly germinates (Shimada *et al.*, 2008).

Concerning the total oil content, seeds of oleosin deficient mutants have lower oil contents than wild type, suggesting that over expression of oleosins increases oil contents because oleosins stabilize oil bodies (Shimada and Hara-Nishimura, 2010). Accordingly, *Brassica napus* lines with high oil contents accumulate more oleosins than those with low oil contents (Katavic *et al.*, 2006).

On the other hand, Arabidopsis oleosin mutants are more sensitive to freezing during germination. The freezing treatment accelerates the fusion of OBs and generates eccentric nuclei in ole1 mutant seeds, which caused seed mortality. On the other hand, overexpression of oleosins increases freezing tolerance of seeds, especially freezing-sensitive seeds (Shimada and Nishimura, 2010).

Plant oleosin genes constitute small families. Two transcripts (Ole-1 and Ole-2) encoding two oleosin isoforms have been isolated from developing barley embryos and

aleurone layers where OBs are highly abundant organelles. For each of the isoforms the aleurone and embryo transcripts are identical, indicating that the same genes are expressed in both tissues. The temporal accumulation of the two transcripts during seed development is similar (Aalen, 1995).

Oleosins are also expressed in floral anther. Anther-type oleosins with molecular masses of 10 to 50 kDa have glycine-rich domains which do not exist in seed-type oleosins. Anther-type oleosins function in stabilizing pollen-oil bodies, and are important for the formation of pollen and pollen coat (Tzen *et al.*, 1998; Liu *et al.*, 2009; Bonsegna *et al.*, 2011).

The structure and topological orientation of oleosins make them interesting for biotechnological purposes such as emulsifying agents or carriers of recombinant proteins. Artificial oil bodies (AOBs) have been successfully constituted and used for purification, refolding and immobilization of recombinant proteins, as well as for encapsulation of probiotics (Chen *et al.*, 2004; Liu *et al.*, 2009; Bhatla *et al.*, 2010).

1.2.1.1.2 Caleosins

Caleosins constitute a second group of integral OB proteins found in a wide range of plants (Purkrtova *et al.*, 2007). Caleosins are bigger than oleosins and have molecular masses about 30 kDa, but share with oleosins a similar three-domain structure composed of a hydrophilic N-terminal domain, a conserved hydrophobic central domain and a hydrophilic C-terminal domain (Naested *et al.*, 2000).

However, unlike oleosin, caleosin has a calcium-binding site, an EF-hand motif in an N-terminal domain and has an enzyme activity as peroxygenase. Both EF-hand motif and calcium are necessary for peroxygenase activity of caleosins (Chen *et al.*, 1999; Frandsen *et al.*, 2001; Jiang *et al.*, 2008; Jiang and Tzen, 2010).

Caleosins are able to stabilize oleosomes and it has been reported that during germination caleosin plays a role in the degradation of storage lipids in OBs (Poxleitner *et al.*, 2006). Its role involves both the normal modification of storage vacuole membrane and the interaction of OBs with vacuoles. Some caleosin homologues might be involved in stress responses because their expression seems to be up-regulated by both biotic and abiotic stress factors (Takahashi *et al.*, 2000; Partridge and Murphy, 2009; Aubert *et al.*, 2010; Kim *et al.*, 2011).

Caleosins may also be involved in other processes as in signal transduction via calcium binding or phosphorylation/dephosphorylation processes during membrane expansion, lipid trafficking and OB biogenesis and mobilization (Frandsen *et al.*, 2001; Poxleitner *et al.*, 2006). It has been hypothesized that the peroxygenase activity of the caleosin may be involved in changes in the fatty acid components of OBs (Hanano *et al.*, 2006). Caleosin genes are also expressed in tissues other than embryos in which OBs are also accumulated, like in anthers (Zienkiewicz *et al.*, 2011).

1.2.1.1.3 Steroleosins

Steroleosins are proteins found in animals and plants, some of them associated to oleosomes, that have homology to a sterol-binding dehydrogenase/reductase domain involved in signal transduction in diverse organisms (Lin *et al.*, 2002). Steroleosins are present in plant genomes as small families (for example, eight genes in Arabidopsis) and at least some of them are associated to oil bodies in oil accumulating organs through a hydrophobic anchoring segment preceding the sterol-binding dehydrogenases/reductases domain (Frandsen *et al.*, 2001; Purkrtova *et al.*, 2008). Little is known about the role of steroleosins. It has been suggested that they might be involved in the regulation of the formation or the degradation of the oleosomes mediated by sterols during seed maturation or germination (Lin *et al.*, 2002; Lin and Tzen, 2004; Lin *et al.*, 2005).

1.2.1.1.4 Other proteins

Different proteomic studies in plant seeds have identified, in addition to the well-known OB-associated proteins, some less abundant proteins, including enzymes (lipase and beta-glucanase), proteins involved in membrane traffic and proteins of unknown function (Jolivet *et al.*, 2004, 2009; Katavic *et al.*, 2006). Some of these enzymes are associated with OBs only during germination. It has been proposed that lipases attach the OBs through their interaction with oleosins or other OB-associated proteins. The maize OB membrane has been shown to contain a prolipase that is activated on germination (Barros *et al.*, 2010).

1.3 Germination

Germination is the stage of the plant life cycle at which the resting embryo begins to produce functional roots and shoot. Germination is initiated when a viable grain absorbs enough water. Reserves stored in the endosperm are solubilised by hydrolytic enzymes to support the embryo's early growth. Germination is completed with the elongation of the embryonic axis giving rise to the radicle breaks through the covers surrounding the seed.

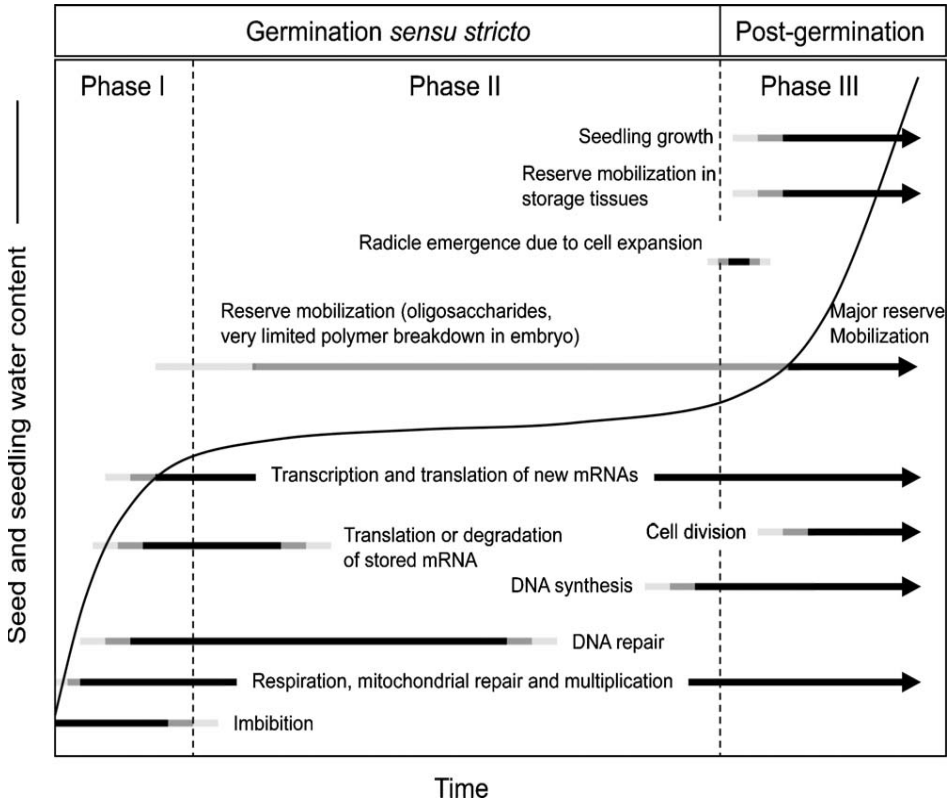


Figure 1.4. Time course of physical and metabolic events occurring during germination (Phases I and II) and early seedling growth (Phase III). The time taken for these events to occur varies between species and is influenced by germination conditions. The curve shows a stylized time course of water uptake. (Nonogaki *et al.*, 2010).

A useful depiction of the progress of germination has evolved around the time course of water uptake by a germinating seed (Fig.1.4). Initially there is rapid imbibition of water by the dry seed (Phase I) until all of the matrices and cell contents are fully hydrated. This is followed by a period of limited water uptake (Phase II), which remains unchanged in seeds that do not complete germination, such as in dormant or dead seeds. The increase in water uptake associated with Phase III is initially, and briefly, related to completion of germination.

The slight increase in water content is followed by a much larger uptake as the cells of the growing radicle, and subsequently the rest of the seedling, increase due to mitotic divisions and cell expansion. While the use of these Phases is convenient to illustrate the events taking place in germinating seeds, in some species they are not so well defined. For example, in larger seeds water uptake into the reserve-laden cotyledons still may be proceeding even following emergence of the radicle. Also, the Phases do not temporally define the metabolic events occurring within the germinating seed, for these are confluent. The cellular contents of a dry seed are laid down during development, which allows for the resumption of metabolic events (Nonogaki *et al.*, 2010).

During water uptake phases I and II, large metabolic changes take place in seeds which set the course for subsequent radicle protrusion. Metabolism is reactivated with enzymes that were stored in the seed during maturation. This has been shown in proteomic approaches in Arabidopsis, where a large number of enzymes involved in the major metabolic pathways were found in dry seeds and remained stable or even accumulated further during early germination (www.seed-proteome.com; Gallardo *et al*., 2001; Rajjou *et al*., 2004; Fu *et al*., 2005). Upon imbibition, dramatic changes in the transcriptome can be observed after as little as 1–3 h that is in phase I of water uptake (Howell *et al*., 2009; Preston *et al*., 2009). Some of these changes have been shown to be tissue specific (Okamoto *et al*., 2010).

1.3.1 Morphological changes in scutellum during germination

Different morphological changes occur in the *Zea* scutellum during imbibition and germination, In general, the scutellum enlarges during germination and the fresh weight increases during the early days of germination. On the other hand, vascular bundles quickly differentiate a few hours after imbibition.

The cells of the scutellar epithelium undergo a series of morphological changes once the seed imbibition starts. In the mature seeds, epithelial cells are slightly elongated towards the outer layer and are densely packed without inter-cellular spaces. During imbibition the epithelial cells lose their adhesion and elongate perpendicularly to the scutellar surface and form finger-like extensions that project into the starchy endosperm (Bewley, 1997). This process contributes to increase the contact area between the endosperm and scutellum, thus providing the exposure of a greater surface for secretion and absorption.

During the first 72 hours of imbibition a number of morphological changes mark the switch from a dormant to an active state of the epithelium. Rough endoplasmic reticulum appears in all cells, and the mitochondria increase in number. These changes are more pronounced in the epithelium than in the parenchyma cells. The number of amyloplasts increases in the epithelium, but they appear to be smaller and less frequent than in parenchyma cells. Later, starch begins to disappear from the epithelial cells. The amyloplasts are surrounded by a double membrane and contain a granular ground substance, internal membranes, growing starch granules, and occasionally, plastoglobuli. Often amyloplasts are in close contact with rough endoplasmic reticulum. Many well defined Golgi apparatuses are observed in the epithelial cells at this stage (probably necessary for the formation of scutellar enzymes). Following 162 hours, the epithelial cells contain a large number of Golgi apparatuses, each consisting of 4-6 Golgi *cisternae*. The Golgi *cisternae* appear to increase in thickness from the forming face to the maturing face. Often Golgi apparatuses are found in groups, which may indicate previous duplications, and rough endoplasmic reticulum is observed in the immediate vicinity of the Golgi apparatus. All together indicate an increase in the metabolism of the scutellar epithelium. Small bodies, lomasomes, consisting of membranes devoid of ribosomes are frequently observed near the cell wall in epithelial cells in seeds 72 hours, or more, after imbibition. Some of the lomasomes are confluent with the plasmalemma and appear to release their content into the cell wall. Often the lomasomes are in close contact with oleosomes. At the same time, large aggregates of membranes (membrane bodies) are observed in epithelial cells. Membrane bodies are always found inside or in direct contact with the vacuoles originated from protein bodies, and often membrane bodies enclose oleosomes (Daussant *et al.*, 1982).

The analysis of serially sectioned epithelial cells 72 hours after the start of malting showed that membrane bodies appear to pinch off small bodies, which often fuse with the plasmalemma. The pinched-off bodies are indistinguishable in structure from the lomasomes. After two weeks of germination the epithelial cells were completely occupied by big vacuoles, while the cytoplasm was restricted to a narrow layer in contact to the cell wall (Daussant *et al.*, 1982).

1.3.2 Expression profile during seed germination

The messenger RNAs stored during embryo maturation are mobilized during germination. Dynamic proteomic analyses during Arabidopsis seed germination have demonstrated that translational activity is low during the first 8 h of imbibition, reflecting the use of stored proteins in this early phase. Translational activity then strongly increases (by more than ten-fold) to reach a maximum during 8 to 24 h. A significant proportion of the gene expression and metabolic signatures of seed desiccation resemble those characterizing seed germination, implying that the preparation of the seeds for germination begins during seed desiccation (Jiménez-López *et al.*, 2011; Rajjou *et al.*, 2011). All components of the transcriptional machinery are stored in dry seeds and are quickly activated upon imbibition, as has been demonstrated by the fact that the addition of the translation inhibitor cycloheximide does not alter early transcript up-regulation in Arabidopsis (Kimura and Nambara, 2010).

Wilson *et al*. (2005) reported that while some transcripts are made *de novo* upon germination, the majority of the transcripts required for germination accumulated in the embryo before germination. In barley, more than 12,000 different transcripts are stored in the embryo of dry grains, many of which are presumably translated during germination (Sreenivasulu *et al.*, 2008). In Arabidopsis, over 10,000 transcripts were differentially expressed during cold stratification, with subequal numbers up-regulated as down-regulated, revealing an active period in preparing seeds for germination, where transcription and RNA degradation both play important roles in regulating the molecular sequence of events (Narsai *et al.*, 2011).

Potokina *et al.* (2002) observed that, in mature barley grain, the endosperm contained substantial mRNA produced during seed development, which was degraded during early stages of germination. They observed that genes specifically expressed in the embryo and the scutellum during germination belong to the functional classes of protein translation, carbohydrate metabolism, nucleotide metabolism, cell cycle and transporters.

Penfield *et al.* (2006) reported microarray-based expression analysis in the endosperm of germinating Arabidopsis seeds at 24 h after stratification. They found that genes that are expressed abundantly in the endosperm are shown to be enriched for those related to seed reserve mobilization, cell wall modifications, carbon metabolism and the transport of metabolites and metals.

Other studies have observed the same results. Notable increases in abundance during the first three days of germination included proteins involved in protein degradation (proteosome subunits, ubiquitin), protein folding (protein disulfide isomerase), cytoskeletal activities (tubulin chains, ascorbate peroxidase), and enzymes of energy metabolism. Marked decreases in abundance were noted for β-amylase, protease inhibitors, heat shock proteins, alcohol dehydrogenase, peroxidases, and ADP-glucose pyrophosphorylase (Mak *et al.*, 2009).

1.3.3 Role of scutellum during germination

Seed imbibition initiates several biochemical events necessary for seedling development. For example, enzymes secreted from the aleurone layer, break down starch in the endosperm converting it to simple sugars which nourish the embryo. The scutellum provides a quick source of energy for the developmental process, whereas the endosperm provides energy for later stages of development. Germination is considered complete when the primary root breaks the coleorhiza and emerges from the seed (Bewley, 1997).

During germination, the epithelium is a secretory cell layer and the source of the initial complement of hydrolytic enzymes causing the breakdown of the endosperm (Briggs, 1972) and the source of the initial gibberellins stimulus (Stoddart *et al.*, 1973). The scutellum also serves to absorb nutrients from the endosperm. Glucose derived from starch in the endosperm is converted to sucrose in the scutellum which is then transported to the embryo axis (Nomura *et al.*, 1969).

Most of the cells in the embryo are potentially metabolically active upon hydration during germination. This also activated hydrolytic and synthetic enzymes and growth hormones to mobilize nutrients and synthesize all ingredients for growth (Lin *et al.*, 1983).

1.3.3.1 Breakdown of scutellum nutrients

In addition to the endosperm reserves, nutrients accumulated in the scutellum also contribute to the flow of energy and materials needed for growing of the embryo axis. In old maize seeds the endosperm loses its ability to provide nutrients to the embryo axis and the scutellum appears to provide all nutrients to the respiration and growth of the axis (Cruz Pérez *et al.*, 2003).

In the scutellum of maize kernel after imbibition, lipase activity increased rapidly, concomitant with the decrease in storage triacylglycerols. The enzyme activity peaked at day 6, but remained at the same level from day 6-10 when most of the triacylglycerols had been depleted. Lipase is *de novo* synthesized in postgermination. Lipase mRNA was already present 2 days after imbibition. At day 6 when lipolysis was most active, more than 60% of the lipase activity was recovered in the lipid body fraction and specifically associated with the organelle membrane. From day 6-10, the lipase activity gradually shifted from the lipid body fraction to other subcellular fractions. Lipase in these subcellular fractions was attributed to represent the enzyme associated with membrane ghosts of the lipid bodies which were fusing with the fragile cell vacuoles; such fusions were observed *in situ* by electron microscopy (Wang and Huang, 1987).

Sucrose-phosphate synthase (SPS), an enzyme that plays a central role in the conversion of fatty acids into sucrose, is expressed in the scutellum of germinating seedlings (Chávez-Bárcenas *et al.*, 2000). The expression in scutellum is independent of both light and plastid development.

Phytine (Mg2+, Ca2+ and K+ salt of myoinositol hexakisphosphate) accumulates in a subcellular compartment of the embryo scutellar cells. Acidification of the phytate containing compartment in the seed embryo takes place, reaching a minimum at about pH 4 after three days of germination (Barba *et al*., 1997). This acidification could be important in allowing phytate solubilisation for export to growing parts of the maize seedling. An accumulation of phytase mRNA was observed at the early steps of germination in the maize scutellum (Maugenest *et al.*, 1999).

The barley scutellum contains high activities of the 'acid carboxypeptidases' and 'alkaline peptidase'. Carboxypeptidase I from germinated barley grain is expressed in the scutellum (Doan and Fincher, 1988). A cathepsin B gene of wheat is expressed in the scutellum and the aleurone layer of germinating grains where its expression is regulated by gibberellin (Cejudo *et al.*, 1992).

In the scutellum the expression was restricted to the parenchyma, suggesting that may have a role other than for mobilization of the endosperm. In rice, the mRNAs for type I and type III-like carboxypeptidases were abundant in germinated embryos composed of leaf, root, and scutellum (Washio and Ishikawa, 1994).

The peptidase activities in the scutellum suggest that they are involved in the hydrolysis of the scutellum protein reserves, but also in the hydrolysis of peptides absorbed from the starchy endosperm, which are hydrolysed to amino acids in the scutellum before transport to the growing seedling tissues (Enari and Mikola, 2008).

Enzymatic activity of beta-N-acetyhexosaminidase was analyzed in maize seeds during germination and activity increased before germination (48 h) but exclusively in the embryo, and mostly in the scutellum (Oikawa *et al.*, 2003).

Three different groups of acid phosphatase isozymes (AP-1, AP-2, and AP-3) have been identified in scutellum extracts of different inbred maize lines (El-Metainy and Omar, 1981). Acid phosphatases may be involved in phytate processing (Senna *et al.*, 2006).

In germinating wheat grains phosphoenolpyruvate carboxylase (PEPC) mRNA accumulated transiently in the scutellum and aleurone layer, showing a sharp maximum 24 h after imbibition. A clear increase in PEPC was observed in the scutellar epithelium of grains 24 h after imbibition. The data suggest that the transiently formed PEPC mRNA in the scutellar epithelium encodes the 108-kD PEPC subunit (González *et al.*, 1998).

Soluble and mitochondrial malate dehydrogenases are synthesized in the scutella during germination and early growth of young maize seedlings (Yang and Scandalios, 1975).

1.3.3.2 Digestion of the endosperm nutrients

The break-down of the endosperm reserves during germination is mediated by the aleurone layer and by the scutellum. Scutellum is activated first, while the aleurone becomes activated later (Smritimala and Swati, 1993).

The scutellum secretes hydrolytic enzymes and gibberellins, which trigger the formation of enzymes in the aleurone cells and their secretion into the starchy endosperm after a lag period. It is noteworthy that ABA, an inhibitor of gibberellin-induced synthesis of hydrolases in the aleurone, does no inhibit the production of α-amylases and cell wall hydrolases by the scutellum of barley (Gibbons, 1981; Woodger *et al.*, 2010).

After imbibition, the synthesis and secretion of α-amylases is first initiated in the scutellum followed by the aleurone layer (Miyata *et al.*, 1981; Subbarao *et al.*, 1998).

α-amylase gene *RAmy3D* have a high-level of expression in the scutellum of isolated rice embryos that could be inhibited by a variety of sugars as well as endosperm extracts from germinated rice grains (Karrer and Rodriguez, 1992).

Barley α-amylase expression in the embryo is localized to the scutellar epithelium (Perata *et al.*, 1997). On the contrary, the maize β-amylase is synthesized *de novo* in the aleurone cells but not in the scutellum during seed germination (Wang *et al.*, 1997).

The barley cysteine proteinase B (EPB) is the main protease responsible for the degradation of endosperm storage proteins providing nitrogenized nutrients to support the growth of young seedlings. The expression of this enzyme is induced in the germinating seeds by gibberellin, and suppressed by ABA (Mikkonen *et al.*, 1996). EPB is expressed in the scutellar epithelium within 24 h of seed germination, but the aleurone tissue surrounding the starchy endosperm eventually becomes later the main tissue expressing this enzyme.

1.3.3.3 Transport of nutrients towards the embryo axis

Plant morphogenesis and development depend on a constant, fine regulation of transporter production and activity, which allows for coordinated exchanges between the different cells, tissues and organs. During germination, the nutrients accumulated in the seeds must be transformed into soluble molecules and transported quickly to the embryonic axis to nourish its growth until it is able to perform the photosynthetic function. Enzymic hydrolysis of starch and storage proteins in the endosperm during germination forms a reservoir of sugars, small peptides and amino acids which are translocated across the scutellum to supply the growing embryo (Higgins and Payne, 1977b, 1978a, 1978b, 1981).

Transport of peptides and amino acids has been reported in scutella isolated from barley, wheat, rice and maize (Salmenkallio and Sopanen, 1989).

In barley, peptide transport is strongly pH dependent with a pH optimum of 3.8–5.0, which is similar to the pH of the endosperm (Higgins and Payne, 1977a). Transport is proton-coupled and is effectively inhibited by reagents that disrupt the transmembrane proton gradient (Walker-Smith and Payne, 1984). The peptide carrier is stereospecific, and di- and tripeptides are transported preferentially, with some evidence for transport of tetra- and pentapeptides (Hardy and Payne, 1992).

Competitive inhibition of uptake between di- and oligopeptides suggests that transport is mediated by a common carrier, although the presence of multiple carriers with overlapping substrate specificity cannot be eliminated (Sopanen and Väisänen, 1985).

Peptide transport activity in the scutella of germinating barley grains starts to develop after 6 h imbibition, increasing rapidly to a maximal rate at 21–24 h imbibition (Sopanen, 1979).
The development of peptide transport activity is inhibited by cycloheximide and cordycepin, inhibitors of protein and mRNA synthesis, respectively, consistent with *de novo* synthesis the protein and transcript during germination (Sopanen, 1979; Walker-Smith and Payne, 1985).

Moreover, during the initial maize germination phases, a net flow of sucrose takes place from the scutellum towards the embryo axis and regions that undergo elongation. During radicle extension, sucrose and hexose transporters, as well as H+-ATPase, become the fundamental proteins that orchestrate the transport of nutrients required for successful germination. Sucrose and hexose transporters were active in the embryo tissues, together with the plasma membrane H+-ATPase, which was localized in all embryo regions involved in both nutrient transport and active cell elongation to support radicle extension (Sanchez-Linares *et al.*, 2012).

1.3.4 Peptide transport proteins in plants

Plants have evolved multiple transport systems for nitrogen (N) to facilitate the uptake from soil and internal reallocation to support development, growth, and reproduction (Glass *et al.*, 2002; Rentsch *et al.*, 2007). Completion of the Arabidopsis genome has revealed that this plant has ten times more predicted peptide transporters than other sequenced organisms. Database comparisons indicate that other plant species contain multiple peptide transporters. Thus, peptides probably play an important and unexplored role in plant growth and development (Stacey *et al.*, 2002).

Plant root can take organic N from soil in the form of amino acids and small peptides, or even proteins (Paungfoo-Lonhienne *et al.*, 2008). Protein reserves in the cereal endosperm are sequentially degraded to small peptides and amino acids during germination and these are translocated across the scutellum to support growth of the embryo (Higgins and Payne, 1981).
Peptide transport in the germinating grain is mediated by specific carriers localized to the plasma membrane of the scutellar epithelium in an energy-dependent manner

(Waterworth *et al.*, 2005). Recently, many peptide transporters have been reported to be localized in the tonoplast (Weichert *et al.*, 2012; Komarova *et al.*, 2012).

Internalized peptides are rapidly hydrolysed by peptidases and the resulting amino acids are transported to the embryo axis and used for protein synthesis or as alternative sources of nitrogen and carbon. Peptide transport also occurs in mature leaves (Jamai *et al*., 1994), in the developing ovules, germinating pollen, etc (Hammes *et al.*, 2010).

Peptide transport systems have been extensively studied in bacteria, fungi and animals, but less in plants. Peptide transport systems in Eukaryotes and Prokaryotes display common but distinct functional features. For example, similar to Prokaryotes, eukaryotic peptide transport is carrier mediated energy dependent and usually restricted to small peptides (two to six amino acids) (Stacey *et al.*, 2002).

Plant peptide transporters generally belong to three different gene families, each recognizing peptides of specific length (Rentsch *et al*., 2007):

1) Peptide Transporter/Nitrate Transporter 1 (PTR/NRT1): di-and tripeptides.

2) Oligopeptide transporters (OPT): tetra- and pentapeptides, glutathione.

3) ATP Binding cassette (ABC) transporters: large peptides.

1.3.4.1 Peptide transporter/Nitrate transporter 1

Peptide transporter/Nitrate transporter 1 (PTR/NTR1) proteins are membrane proteins involved in the transport of di- and tri-peptides across the membrane. They are present in all Eukaryotes, but whereas in bacteria, animals and fungi there are few genes, in plants there are a very much higher number (Tegeder *et al.*, 2011). Functional di-/tripeptide transport has been shown for members from Arabidopsis (AtPTR1, AtPTR2, AtPTR3, and AtPTR5; (Frommer *et al.*, 1994; Song *et al.*, 1996; Rentsch *et al.*, 1995; Chiang *et al*., 2004; Dietrich *et al.*, 2004; Rentsch *et al.*, 2007; Komarova *et al.*, 2008), faba bean (VfPTR1; Miranda *et al.*, 2003), barley (HvPTR1;West *et al.*, 1998; Waterworth *et al.*, 2000, 2005) and *Hakea actites* (HaPTR4; (Paungfoo-Lonhienne *et al.*, 2009). Besides, some plant PTRs function as nitrate transporters and are termed as Nitrate Transporter NRT1 (Rentsch *et al.*, 2007).

The first member of the peptide transporter family characterized in plants was the CHL1 from Arabidopsis, which when mutated confers resistance to the herbicide chlorate and a decrease in nitrate uptake. This transporter was found to encode a protein with 12 putative membrane spanning segments. CHL1 gene encodes an electrogenic nitrate

transporter predominantly found in roots and displays nitrate and pH-dependent regulation (Tsay *et al.*, 1993).

One year after the identification of the CHL1, several peptide transporter genes were independently isolated by functional cloning. These peptide transporters were found to share sequence similarity with CHL1, and were classified together with CHL1 to form the new transporter family, PTR, this name being preferred because the majority of members are peptide transporters. CHL1 and these peptide transporters then served as prototypes to search for homologs in other organisms or tissues (Zhao *et al.*, 2010). A phylogenetic analysis of peptide transporter sequences of Arabidopsis and rice showed that these proteins comprise a distinct, separate group of proteins (Fig.1.5) (Tsay *et al.*, 2007).

Some peptide transporters have been functionally characterized in Arabidopsis, as, for example:

- AtPTR1: it recognizes a broad spectrum of di- and tripeptides and substrates lacking a peptide bound, but not amino acids, amino fatty acids or peptides with more than three amino acid residues. AtPTR1 has a role in long-distance transport of di- and tripeptides (Dietrich *et al*., 2004; Bernard *et al*., 2008; Hammes *et al.*, 2010). The protein is localized in the plasma membrane (Weichert *et al.*, 2012). AtPTR1 plays a role in uptake of peptides by roots indicated by reduced nitrogen (N) levels and reduced growth of atptr1 mutants on medium with dipeptides as the sole N source (Komarova *et al.*, 2008).

- AtPTR2-A: it is able to transport a wide spectrum of di- and tripeptides but not nitrate (Chiang *et al.,* 2004). It is expressed in the roots of Arabidopsis seedlings (Song *et al.*, 1996). It is localized at the tonoplast (Weichert *et al.*, 2012).

- AtPTR2-B: it is constitutively expressed in all plant organs and may play a general role in plant nutrition (Song *et al.*, 1997).

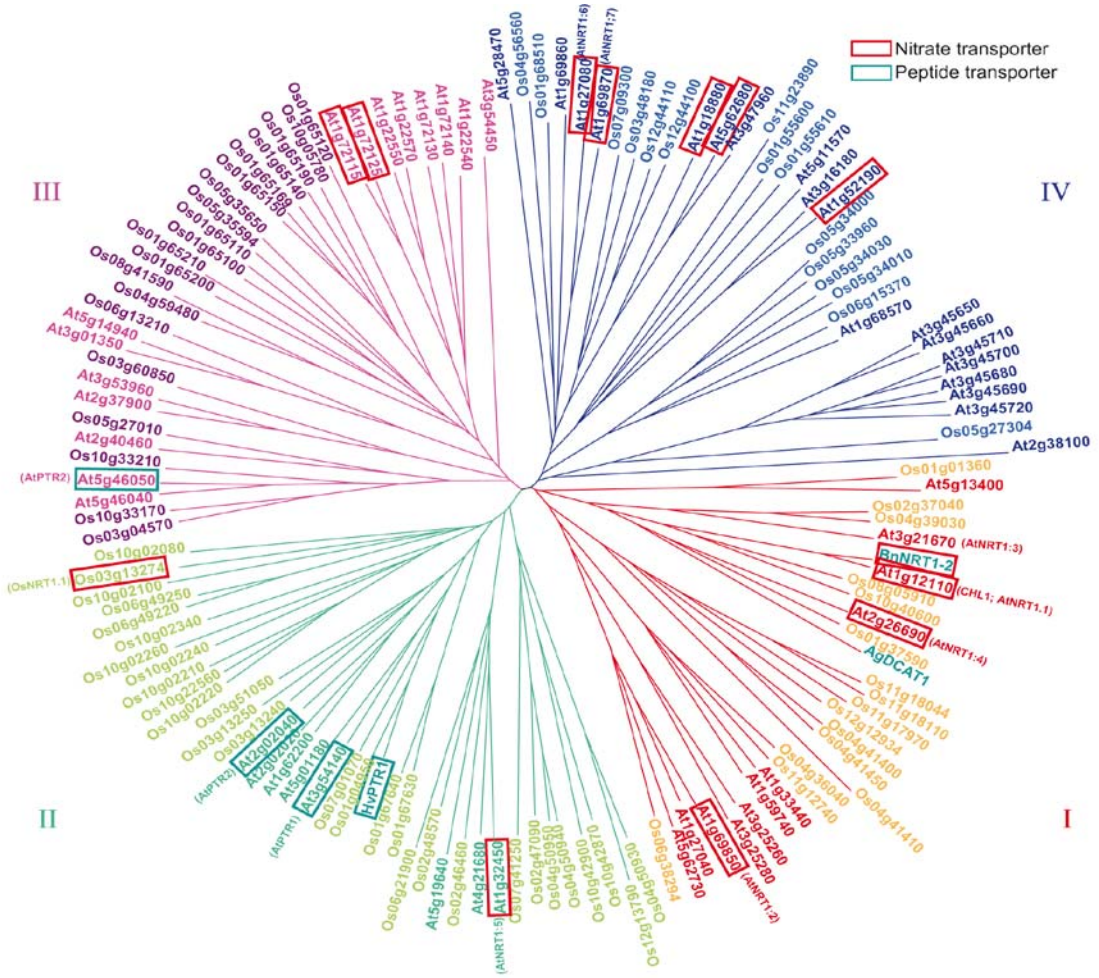


Figure 1.5. Phylogenetic tree of the 53 Arabidopsis PTR/NRT1 family transporters, 80 rice PTR/NTR1 transporters and Hordeum HvPTR1 (Tsay *et al.*, 2007).

- AtPTR3: It transports di-and tripeptides. Its expression is induced by wounding, salt and pathogens (Karim *et al.*, 2005). The germination frequency of the mutant line was reduced on salt- containing media, suggesting that the AtPTR3 protein is involved in stress tolerance in seeds during germination (Karim *et al.*, 2007).

- **AtPTR4**: It is expressed in the vasculature of the plants and the protein is localized at the tonoplast (Weichert *et al.*, 2012).

- **AtPTR5**: It transports dipeptides and the protein is localized in the plasmamembrane (Komarova *et al.*, 2009; Hammes *et al*., 2010; Weichert *et al*., 2012).

atptr5 mutants and AtPTR5-overexpressing lines showed that AtPTR5 facilitates peptide transport into germinating pollen and possibly into maturating pollen, ovules, and seeds *(*Komarova *et al.*, 2008). Overexpression of AtPTR5 resulted in enhanced shoot growth and increased N content.

- AtPTR6: it is highly expressed in pollen and during senescence. The protein is localized at the tonoplast (Weichert *et al.*, 2012).

Plasma membrane localization of Arabidopsis PTR1 (Dietrich *et al.*, 2004), PTR5 (Komarova *et al.*, 2008), and barley PTR1 (Waterworth *et al.*, 2000) as well as tonoplast localization of Arabidopsis PTR2, PTR4 and PTR6 (Chiang *et al.*, 2004; Shimaoka *et al.*, 2004; Weichert *et al.*, 2012; Komarova *et al.*, 2012) suggests involvement in inter- and intracellular transport, respectively.

Rice genome contains more than 80 PTRs coding genes (Tsay *et al.*, 2007). However, the exact physiological function for most of these genes is unknown. In germinating seeds, seven of the eight OsPTRs studied were expressed at various levels, but *OsPTR5* was not expressed. In grain-filling seeds, expression of *OsPTR4*, *OsPTR7* and *OsPTR8* increased from early to late stages of seed development and that of *OsPTR2*, *OsPTR3* and *OsPTR6* decreased. Expression of *OsPTR1*, *OsPTR2*, *OsPTR5*, *OsPTR7* and *OsPTR8* were up regulated by drought and salt, and *OsPTR4* was down regulated. However, only *OsPTR1* displayed a slight upregulation by cold. In a complementation assay with the yeast ptr2 mutant, only OsPTR6 was able to substitute ptr2. This indicates that OsPTR6 transports di-/tripeptides (Zhao *et al.*, 2010; Ouyang *et al.*, 2010). However, functional assays demonstrated that it cannot transport any di-/tripeptide and has substrate selectivity (Tsay *et al.*, 2007).

In barley (*Hordeum vulgare)* one peptide transport have been isolated and characterized, HvPTR1, which transports the peptide Ala-Phe and is expressed in the scutellar epithelium (West et al., 1998). HvPTR1 protein is localized to the plasma membrane of scutellar epithelial cells during germination (Waterworth *et al.*, 2000; Waterworth and Bray, 2006) and its activity is inhibited at the post-translational level by phosphorylation in response to rising levels of amino acids emanating from the endosperm as a result of storage protein breakdown and mobilization (Waterworth *et al.*, 2005).

Two peptide transporter (PTR) homologs have been isolated from developing seeds of faba bean (*Vicia faba*) (Miranda *et al.*, 2003). VfPTR1 was shown to be a functional peptide transporter through complementation of a yeast mutant. In developing seeds, the highest levels of *VfPTR1* transcripts were reached during cotyledon development.

During early germination, *VfPTR1* mRNA appeared first in cotyledons and later, during seedling growth, also in axes and roots. Expression of *VfPTR2* was delayed compared with *VfPTR1*, and was restricted to the nascent organs of the seedlings. Localization of *VfPTR1* transcripts showed that this PTR is temporally and spatially regulated during cotyledon development. In germinating seeds, *VfPTR1* mRNA was localized in root hairs and root epidermal cells, suggesting a role in nutrient uptake from the soil. In seedling roots, *VfPTR1* was repressed by a dipeptide and by an amino acid, whereas nitrate was without influence (Miranda *et al.*, 2003; Chiang *et al.*, 2004).

1.3.4.2 Oligopeptide transporters (OPT)

The Oligopeptide Transporters (OPTs) comprises a group of membrane-localized proteins that have a broad range of substrate transport capabilities including small peptides (4–5 amino acids). They can also transport amino acid complexes with metals, and peptides containing modified amino acids (Curie *et al.*, 2001; Koh *et al.*, 2002; Bogs *et al.*, 2003). These integral membrane proteins are predicted to have 12 transmembrane domains and are characterized by several signature motifs. Two highly conserved motifs (NPG and KIPPR) have been found in many OPT family proteins (Koh *et al.*, 2002; Wiles *et al.*, 2006). It is widely accepted that OPTs are proton-coupled symporters that translocate their substrates in the cytosolic direction *(Bogs et al.*, 2003; Schaaf *et al.*, 2004; Osawa *et al.*, 2006; Lubkowitz, 2011).

The biochemical and physiological characteristics of several OPT homologues have been studied (Lubkowitz, 2006; Osawa *et al.*, 2006; Stacey *et al.*, 2008; Thakur *et al.*, 2008).

The OPT proteins belong to a small gene family in plants, which includes about 25 members in Arabidopsis and rice, 20 in poplar and 18 in Vitis (Cao e*t al.*, 2011).

Members of the OPT family were first characterized in yeast (Lubkowitz *et al.*, 1997, 1998), and since then they have also been found in archaea, bacteria and plants (Lubkowitz, 2011).

Phylogenetic analyses of plant OPT members have revealed two distant clades: the yellow stripe-like (YSL) proteins and the OPTs. The YSL transporters are involved in metal homeostasis through the translocation of metal-chelates (Lubkowitz, 2011). The OPT proteins likely do not have a common biological function and may be involved in four different processes: long-distance metal distribution (Stacey *et al.*, 2008), nitrogen

mobilization (Koh *et al.*, 2002; Cagnac *et al.*, 2004; Stacey *et al.*, 2006; Pike *et al.*, 2009), heavy metal sequestration (Cagnac *et al.*, 2004; Bogs *et al.*, 2003; Vasconcelos *et al.*, 2008; Pike *et al.*, 2009) and glutathione transport *(Cagnac et al., 2004; Bogs et al.*, 2003; Zhang *et al.*, 2004; Pike *et al.*, 2009). These processes may play a role in plant growth and development (Lubkowitz, 2006). Oligopeptide transport plays important roles in nitrogen storage and mobilization, quorum sensing, differentiation, sexual induction, mating and pheromone sensing. One of the yeast homologues is the sexual differentiation process (ISP4) protein of *Schizosaccharomyces pombe*. In plants, many OPTs appear to be plasma membrane–embedded proteins that import substrates from the apoplasm (the aqueous phase of the cell wall) and the external environment (Lubkowitz, 2011).

1.3.4.3 ATP binding cassette (ABC) transporters

ABC transporters constitute one of the largest protein families and are present in organisms ranging from bacteria to humans (Henikoff *et al.*, 1997). In most cases, functional ABC transporters act as ATP-driven pumps and consist of two transmembrane domains (TMD) hydrophobic domains, which constitute the membrane-spanning pore, and two cytosolic domains, which are referred to as the nucleotide-binding domains (NBD) or nucleotide- binding folds (NBF), as they contain the ATP-binding Walker A and B motifs (Martinoia *et al.*, 2002).

In plants, ABC proteins were originally identified as transporters involved in the final detoxification process, i.e., vacuolar deposition (Martinoia *et al.*, 2007). Since this finding, numerous reports have shown that the functions of this class of transporters extend far beyond detoxification. ABC transporters have frequently been shown to be involved in such diverse processes as pathogen response, surface lipid deposition, phytate accumulation in seeds, and transport of the phytohormones auxin and abscisic acid. Therefore, ABC transporters play an important role in organ growth, plant nutrition, plant development, response to abiotic stress, and the interaction of the plant with its environment (Kang *et al.*, 2011).

Classification into the ABC family depends on the presence of one or two ATP-binding domains consisting of a conserved Walker A motif (Gx4GK[S/T]), the Walker B motif ([R/K]x3Gx3L[hydrophobic]3) and the ABC signature or C motif ([L/I/V/M/F/Y]S[S/G]Gx3[R/K/A][L/I/V/M/Y/A]X[L/I/V/F/M][A/G]) (where X represents any amino acid) (Kretzschmar *et al.*, 2011). A complete survey of the

Arabidopsis genome revealed 129 open reading frames (ORFs) encoding ABC proteins with one or two ABC-binding domains, of which 103 appeared to encode intrinsic membrane proteins (Verrier *et al.*, 2008). However, only a proportion of these probably encode peptide transporters (Stacey *et al.*, 2002).

1.3.5 Mobilization of oil bodies

Lipid bodies are degraded during germination. Whereas some proteins, e.g. oleosins, are synthesized during the formation of lipid bodies of maturating seeds, a new set of proteins, including a specific form of lipoxygenase (LOX; EC 1.13.11.12), is detectable in lipid bodies during the stage of fat degradation in seed germination (Feussner *et al.*, 2001).

Storage oil (triacylglycerol [TAG]) breakdown plays an important role in the life cycle of many plants by providing carbon skeletons that support seedling growth immediately following seed germination and enable seedling establishment (Bewley and Black, 1994; Graham, 2008).

The mobilization of storage oil involves the coordinated induction of a number of biochemical pathways in different subcellular locations in order to convert fatty acids derived from TAG to 4-carbon compounds. The 4-carbon compounds in turn are converted to soluble sugars that are used to fuel seedling growth. Biochemical analysis has identified the main pathways involved in this process, including β-oxidation, the glyoxylate cycle, and gluconeogenesis (Theodoulou and Eastmond, 2012).

The first step in oil breakdown is catalyzed by lipases, which hydrolyze TAG to produce free fatty acids (FAs) and glycerol. The FAs then enter single membrane–bound organelles termed glyoxysomes where β-oxidation and part of the glyoxylate cycle occurs. Glyoxysomes are structurally similar but metabolically distinct from the more ubiquitous peroxisomes. Glyoxysomes contain two enzymes that are unique to the glyoxylate cycle, malate synthase (MLS) and isocitrate lyase (ICL) (Feussner *et al.*, 2001).

β-oxidation converts FAs to acetyl-CoA, which is subsequently condensed into 4-carbon compounds via the glyoxylate cycle. These 4-carbon compounds are then transported to the mitochondria, where they can either be converted to malate and transported to the cytosol for gluconeogenesis, or used as substrates for respiration. Traditional biochemical studies carried out on a number of oilseed species, in particular castor bean, resulted in the definition of the main pathways and enzymatic activities of

the storage oil mobilization process. More recently, these studies have been extended through the use of biochemical genetics in the model oilseed species Arabidopsis. These studies have also led to new insights into additional roles of peroxisomal β-oxidation, including, for example, synthesis of the wound signal jasmonic acid (JA), the synthesis of the auxin indole-3-acetic acid (IAA), and the breakage of seed dormancy (Fig.1.6) (Graham, 2008).

In most seeds and in maize scutellum, lipase activity is absent in the maturing and ungerminated seed, and appears 2 days after imbibition, lipase activity increased rapidly, concomitant with the decrease in storage in triacyglycerols. Since lipase catalyzes the first step in a long metabolic pathway, its reaction may be rate-limiting to the whole pathway and thus a desirable target of developmental and metabolic regulation. The mechanism which controls the appearance of lipase activity is unknown. This appearance may be due to *de novo* synthesis of the enzyme during seedling growth or an activation of an inactive enzyme synthesized during seed maturation. The lipase is tightly associated with the membrane of the lipid bodies during the active stage of lipolysis (Lin *et al.*, 1983; Wang and Huang, 1987; Barros *et al.*, 2010).

Oleosins play an indirect role in this process by keeping down the volume/surface of the oleosomes, which facilitates the action of lipases. Some lipases have been purified that are associated with oleosomes during germination. It is unclear if this interaction uses oleosins and other proteins. Electron microscopy observations suggest that during germination oleosomes contact the glyoxysomes and vacuoles and those invaginations occur that would allow the transport of TAGs to glyoxysomes. The mechanisms that control this process are unknown although it has been suggested that caleosins could be involved. Controlling the degradation of oil and FAs during embryo development could be a useful strategy to improve oil yield in existing crops or in those engineered to produce industrial FAs (Theodoulou and Eastmond, 2012).

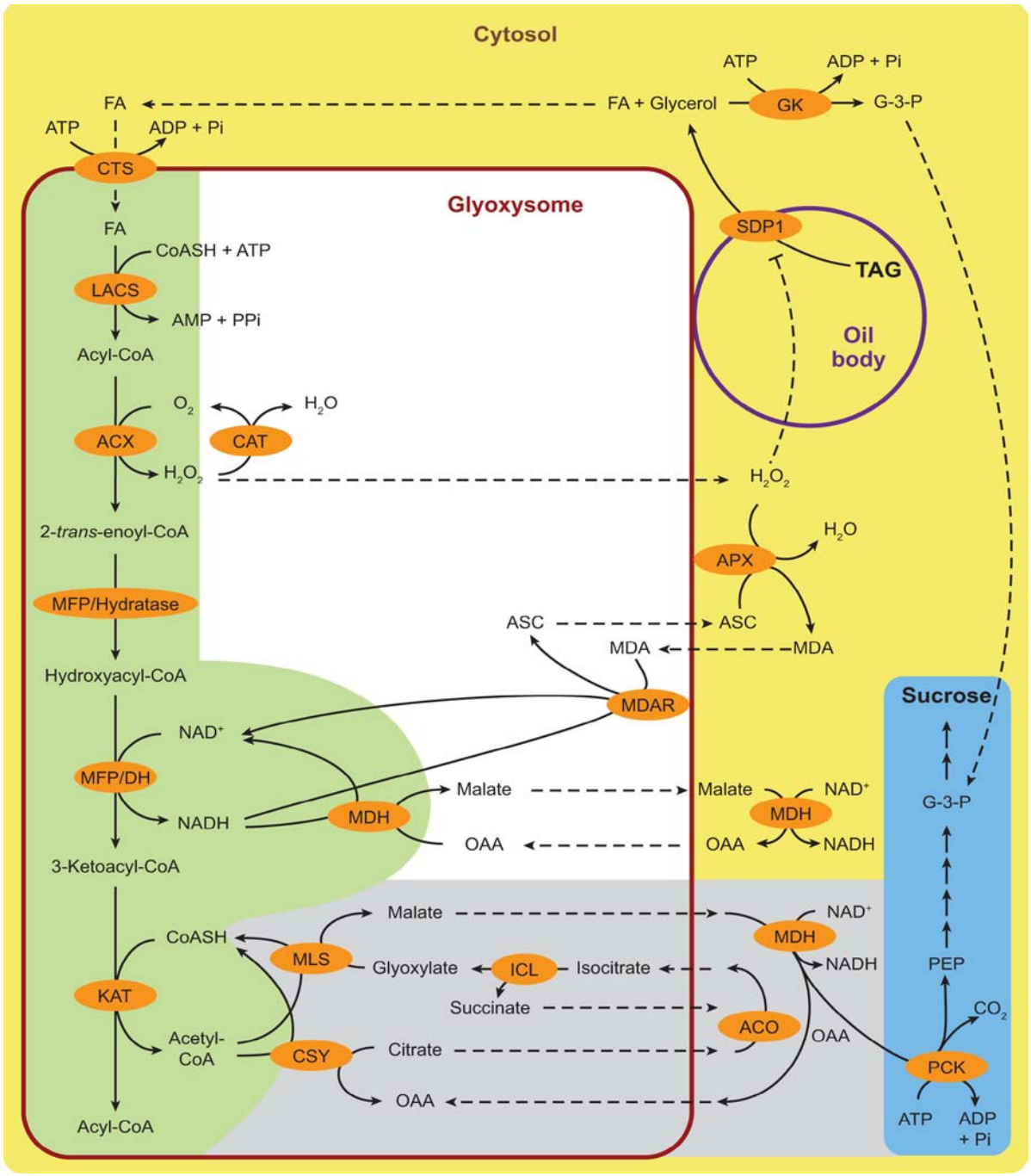


Figure 1.6. Pathways and processes involved in the mobilization of storage oil to sucrose that have been functionally characterized by molecular genetic analysis in Arabidopsis (Graham, 2008).

2. OBJECTIVES

2 OBJECTIVES

Seed germination is a complex multi-stage developmental process. Seed germination is accompanied with many cellular, physiological and fundamental metabolic activities such as respiration, RNA and protein synthesis machinery, as well many enzyme activities. Upon imbibition, the epithelial cells of maize scutellum elongate which leads to an increase in the contact area between the endosperm and the scutellum. Moreover, endosperm protein reserves are hydrolysed by the concerted action of proteinases and peptidases to form a pool of small peptides and amino acids that are translocated into the embryo by transporters localized in the scutellar epithelium.

The aim of the work is the determination of the cellular, physiological and fundamental metabolic activities of maize scutellum during early stages of germination. This involved:

1.- Control of the scutellar epithelial cell elongation during germination in maize.

2.-Identification of genes exclusively or predominantly expressed in the epithelium of maize scutellum during germination.

2.1 .- Construction of a cDNA library from external scutellum (scutellar epithelium and subepithelial parenchyma) a day after germination.

2.2 .- Printing and hybridization of macroarrays cDNA fragments. Selection of cDNAs that are expressed predominantly in the scutellar epithelium. Sequencing and identification of selected clones.

2.3 .- Analysis of the expression of some of the selected genes.

2.4.- Characterization of ZmPTR1, a peptide transporter expressed in the epithelial cells of the scutellum during germination.

3.-. Isolation of proteins associated with lipid bodies of maize scutellum.

3.1.- Protein composition analysis of oil bodies from maize embryos during germination.

3.2.- Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of maize.

3. RESULTS

3 RESULTS

3.1 Control of the scutellar epithelial cell elongation during germination in maize

The scutellum is the single cotyledon of the grass embryos. In mature seed it contains part of the storage nutrients of the seed, in special most of the storage lipids, but it also plays an important role during germination in the mobilization of the endosperm nutrients and, due to its intermediate position, their transport from the endosperm to the growing embryo axis. In this sense, the scutellar cells in direct contact with the endosperm are specialized and form the scutellar epithelium. In the mature seeds, epithelial cells are slightly elongated toward the outer layer and are densely packed without inter-cellular spaces. After imbibition, the epithelial cells suffer an elongation which leads to an increase of the contact area between the endosperm and the scutellum, thus providing greater exposure surface for secretion and absorption. Little is known about the physiological mechanisms that control epithelial cell elongation. The aim of this study was to determine the hormonal and environmental factors that regulate the elongation of scutellar epithelial cells in maize. Maize seeds were imbibed and subjected to different treatments and the elongation of the epithelial cells was measured. Our findings attest that scutellum epithelial cell elongation regulated by many factors. In general, factors inhibiting germination also inhibit cell elongation. We observed that elongation is inhibited by ABA and salicylic acid, basic and acid pH and high concentrations of sorbitol. Exogenous gibberellins stimulate elongation, but a reduction in gibberellin synthesis or perception does not inhibit elongation. Elongation is increased by sucrose, but not glucose. The application of transcription and translation inhibitors reduced elongation, so transcription and translation are necessary for the elongation process.

Tnani, H. ***et al.*** (2011). Control of the scutellar epithelial cell elongation during germination in maize (Zea mays L.). *Seed Science and Technology* **39**: 253–258.

Tnani, H., López, I. and Vicient, C.M. (2011), *Seed Sci. & Technol.,* **39**, 253-258

Research Note

Control of the scutellar epithelial cell elongation during germination in maize (*Zea mays* L.)

H. TNANI, I. LÓPEZ AND C.M. VICIENT*

Department of Molecular Genetics, Centre for Research in Agrigenomics CSIC-IRTA-UAB, Jordi Girona 18-26, Barcelona 08034, Spain (E-mail: cvsgmp@cid.csic.es)

(Accepted January 2011)

Summary

The scutellum is the single cotyledon of grass embryos. The cells in direct contact with the endosperm form the scutellar epithelium which plays a major role in controlling the mobilization of the endosperm nutrient reserves. After imbibition, the epithelial cells elongate which leads to an increase in the contact area between the endosperm and the scutellum. We studied which factors regulate epithelial cell elongation. Elongation was inhibited by ABA and salicylic acid, basic and acid pH and high concentrations of sorbitol. Exogenous gibberellins stimulated elongation, but a reduction in gibberellin synthesis or perception did not inhibit it. Elongation was increased by sucrose, but not glucose. The application of transcription and translation inhibitors reduced elongation.

Experimental and discussion

The scutellum is the cotyledon of the grass embryo and the part of the embryo in contact with the starchy endosperm. The scutellar cells in direct contact with the endosperm form the scutellar epithelium (also known as glandular layer or secretory layer). This is formed by a continuous layer of cylindrical cells and constitutes a secretory and absorptive organ that plays a major role in controlling the mobilization of the endosperm nutrient reserves. Once the seed starts imbibition, scutellar epithelial cells undergo a series of morphological changes. In the mature seeds, epithelial cells are slightly elongated towards the outer layer and are densely packed without inter-cellular spaces. During imbibition the epithelial cells lose their adhesion and elongate perpendicularly to the scutellar surface and form finger-like extensions that project into the starchy endosperm (Bewley and Black, 1994). This process contributes to an increase in the contact area between the endosperm and scutellum, thus providing the exposure of a greater surface for secretion and absorption. Little is known about the physiological mechanisms that control epithelial cell elongation.

* Author for correspondence

The aim of this study was to determine the hormonal and environmental factors that regulate the elongation of scutellar epithelial cells in maize.

Maize seeds were imbibed and subjected to different treatments and the elongation of the epithelial cells was determined by light microscopy on histological preparations. Maize (*Zea mays* L.) seeds were surface-sterilized for 20 min in 20% (v/v) commercial bleach and 0.1% Triton X-100 and rinsed four times with sterile distilled water. Sterile seeds were placed in moist rolled towels at 26°C in a 16h/8h light/dark photoperiod. After treatment, embryos were collected and fixed in an ethanol : formaldehyde : acetic acid (80 : 3.5:5 vol) fixative solution for one hour at room temperature, followed by one week at 4°C. Once fixed, the material was stored in 70% ethanol at 4°C. Paraffin inclusion, and sectioning were according to Fontanet and Vicient (2008). Sections were stained with toluidine blue (Peterson, 1988), viewed using bright field microscopy, the size of the cells determined and cell length to width ratio calculated. Each value was determined from the data of about 20 cells per seed, in three seeds from two independent treatments (about 120 cells in total). An example of the micrographs is shown in figure 1.

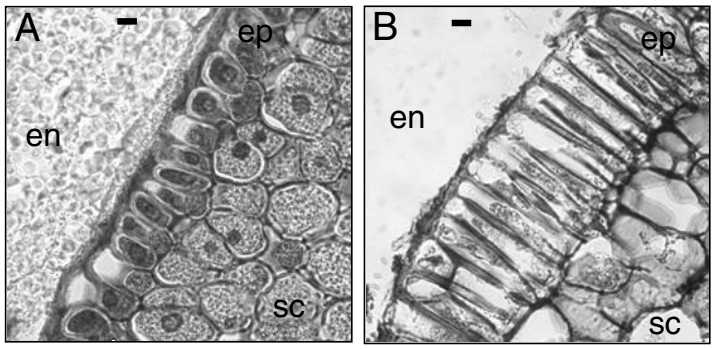
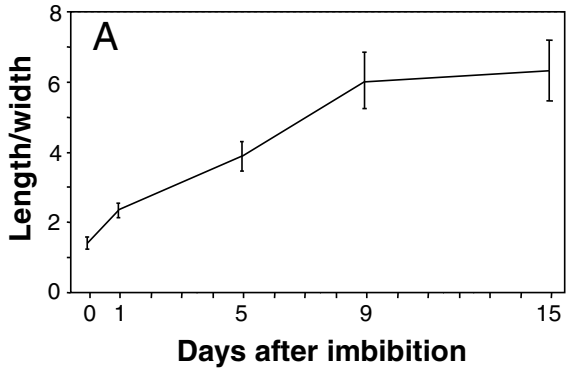
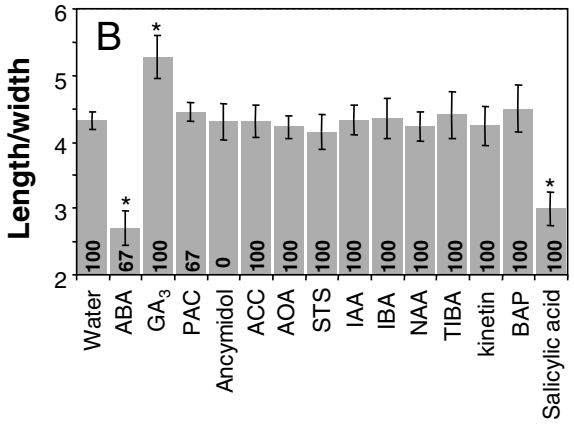


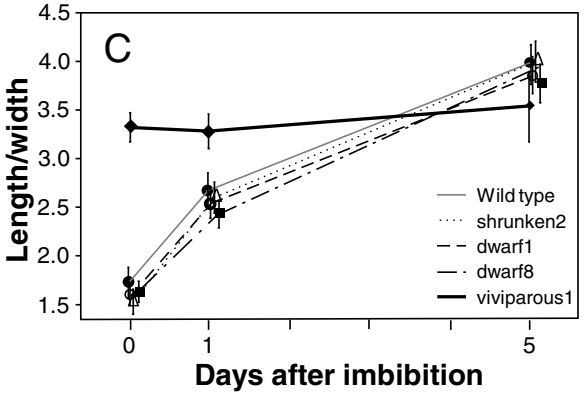
Figure 1. Changes in the length/width ratio of the scutellar epithelial cells during imbibition of maize wild type seeds. Light micrographs of dry seeds (A) and seeds 9 days after imbibition (B) stained with toluidine blue showing the scutellar epithelium (ep), the scutellar parenchyma (sc) and the endosperm (en). Scale bars: 10 µm.

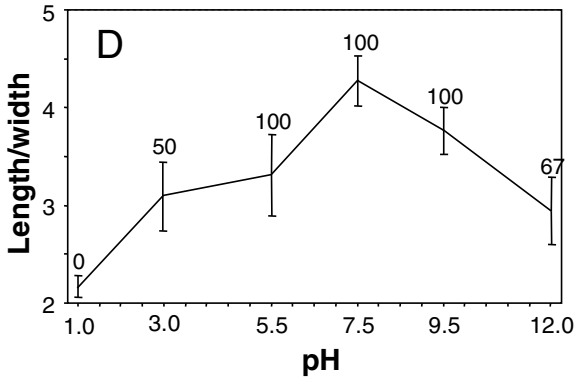
First, cell elongation was quantified for seeds incubated in water at different times after imbibition (figure 2a). About five days after imbibition the epithelial cells were twice as long as in mature unimbibed seeds, and about at 9 days they were three times longer. Little change occurred after 9 days. We also determined the dimensions of cells in the epidermis (adaxial layer, cells in contact with the aleurone) and no cell elongation was observed (data not shown).

Maize seeds were subsequently imbibed in the presence of different hormones and hormone inhibitors and cell elongation and the percentage germination (the proportion of seeds whose radicle emerged from the seed) determined (figure 2b). No significant effects in elongation or germination were observed in seeds treated with ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), ethylene synthesis inhibitor amino-oxyacetic acid (AOA), ethylene action blocker silver thiosulphate (STS), three compounds of the auxin family (indole acetic acid [IAA], indole-3-butyric acid [IBA] and naphtalen acetic acid [NAA]), with the inhibitor of polar auxin transport 2,3,5-triiodobenzoic acid (TIBA), and with the cytokinins kinetin and 6-benzylaminopurine (BAP). However, we observed a significant reduction of cell elongation in seeds treated with abscisic acid (ABA) and









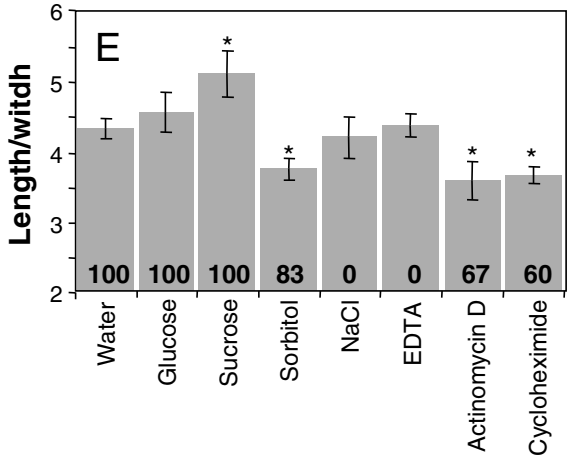


Figure 2. Changes in the length/width ratio of the scutellar epithelial cells during imbibition and in response to different treatments. Error bars represent the SE.

A) The length/width ratio of the epithelial cells at different times after imbibition.

B) The length/width ratio of the epithelial cells measured 5 days after imbibition. Numbers inside the bars are the percentage germination. ABA, abscisic acid 50 µM; GA_3, gibberellin A3 100 µM; PAC, paclobutrazol 100 µM; ancymidol 100 µM; ACC, 1-aminocyclopropane-1-carboxylic acid 10 µM; AOA, aminooxyacetic acid 100 µM; STS, silver thiosulfate 20 µM; IAA, indole acetic acid 1 mM; IBA, indole-3-butyric acid 1mM; NAA, naphtalen acetic acid 50 µM; TIBA, 2,3,5-triiodobenzoic acid 100 µM; kinetin 10 µM; BAP, 6-benzylaminopurine 50 µM; salicilic acid 1 mM. Asterisks indicate statistically significant changes compared to the water control.

C) The length/width ratio of the epithelial cells in different maize mutants.

D) The length/width ratio of the epithelial cells was measured 5 days after imbibition. Seeds were incubated on TrisH 10 mM adjusted to different pH. The numbers above the error bars are the percentage germination.

E) The length/width ratio of the epithelial cells measured 5 days after imbibition. Numbers inside the bars are the percentages of germination. The treatments were: glucose 200 mM, sucrose 200 mM, sorbitol 200 mM, NaCl 200 mM, EDTA 50 mM, actinomycin D 100 µg/ml and cycloheximide 10 µg/ml. Asterisks indicate statistically significant changes compared to the water control.

with salicylic acid. In contrast, the application of gibberellin (GA_3) produced an increase in the elongation rate, although the application of the gibberellin biosynthesis inhibitors paclobutrazol (PAC) and ancymidol did not change the elongation rate. While ABA and both gibberellin biosynthesis inhibitors reduced germination, salycilic acid did not.

Next, we examined the elongation of the epithelial cells in different maize mutants. Elongation was not significantly different to the wild type in the starch-accumulation deficient mutant shrunken2 (Sh2), the gibberellin synthesis deficient mutant dwarf1 and in the gibberellin signalling deficient mutant dwarf8 (figure 2c). However, viviparous1 (vp1), which is deficient in ABA signalling, showed contrasting cell elongation. No significant differences were observed between wild type and vp1 when comparing cells at 5 day (figure 2c). However, in dry seeds and at 1 day, the length/width ratio in the epithelial cells of vp1 was higher. In fact, no significant differences were observed between vp1 epithelial cells in dry seeds and after imbibition. We can conclude that in vp1 the epithelial cells are already elongated in dry seeds and no further changes are produced after imbibition.

The effect of acidity on epithelial cell elongation was examined by germinating seeds in the presence of a Tris-HCl 10 mM solution adjusted to different pH (figure 2d). The highest increase in length was observed with neutral solutions, whereas acid and basic solutions produced a reduction in both, elongation and germination.

Finally, we imbibed seeds in the presence of other substances (figure 2e). Sucrose (200 mM), but not glucose (200 mM), produced an increase in elongation. However incubation on 200 mM sorbitol significantly reduced both elongation and germination. Incubation on salt or in EDTA did not produce any significant differences in elongation, but totally inhibited germination. Actinomycin D, an inhibitor of transcription, and cycloheximide, an inhibitor of translation, produced a significant reduction in the elongation rate and a partial reduction in germination.

Scutellar epithelial cell elongation starts very early after imbibition. Only one day after imbibition epithelial cells increase their length/width ratio significantly. Cell elongation involves wall loosening and requires energy and synthesis of cell wall proteins and polysaccharides (Kutschera, 1990). Our data indicates that transcription and translation are at least partially necessary for cell elongation. ABA and GA are the key regulators of seed germination (Yamaguchi *et al*., 2007), but the influence is much less clear in cereals. For example, the seeds of the dwarf1 maize mutant normally germinate, despite containing a severely reduced level of GAs. We showed that ABA inhibits epithelium cell elongation and in vp1 mutant (which cannot respond to ABA) the elongation has occurred before seed desiccation. The application of exogenous gibberellin induces elongation. However, paclobutrazol and ancymidol did not produce a significant reduction in the elongation rate, despite reducing germination, and epithelial cells elongate in dwarf1 and dwarf8 mutants. Thus, although GAs stimulate cell elongation, they are not strictly necessary. In the view of these data, we hypothesize that the acceleration of the elongation produced by GAs could be an indirect consequence of the acceleration of germination.

Ethylene positively influences seed germination (Ogawa *et al*., 2003) and auxins are involved in embryo development (Chen and An, 2006). However, neither these hormones nor cytokinins, seem to be involved in the regulation of epithelial cell elongation. On

the other hand, 1 mM salicylic acid reduces cell elongation. Guan and Scandalios (1995) reported an increase in catalase activity in maize scutellum in response to salycilic acid, which may be one of the reasons of the decrease in cell elongation. However, SA at the concentration used here did not reduce germination, suggesting an at least partially different regulation of scutellar cell elongation and radicle extrusion. Cumulative evidences suggest a crosstalk between SA and ABA in the regulation of the responses to different stresses and in the regulation of other physiological processes like germination (Xie *et al*., 2007). SA inhibition of scutellar cell elongation could be an aspect of the SA and ABA crosstalk.

The pH in the endosperm is reduced to 4.9–5.1 during germination due to the release by the aleurone layer of organic and phosphoric acids into the starchy endosperm (Martínez-Camacho *et al*., 2004). The acidification process provides an optimum pH for several physiological events including phytate solubilization, hydrolytic activity of secreted enzymes, cell wall expansion and activity of nutrient transporters (Rayle and Cleeland, 1992). Cell elongation is inhibited in acid environment, but at the pH 5 the elongation is only reduced, not inhibited.

Cell water potential is involved in cell elongation. Application of 200 mM sorbitol reduces cell elongation probably reducing water potential. However, the application of the same concentration of glucose did not reduce cell elongation and sucrose increased elongation rate. Assimilable carbohydrates probably act as a source of energy and materials for cell wall synthesis. However, the source of carbohydrates should not be a limiting factor because cell elongation is not affected in the mutant Sh2 which contains about 25% of starch respect to wild type (Bhave *et al.*, 1990).

Scutellum epithelial cell elongation is a complex developmental process regulated by many factors. The findings presented here attest to this fact. Our data indicate that the germination process influences cell elongation, but their regulation are not completely correlated.

Acknowledgements

This work was carried out thanks to grants BIO2004-01577 and AGL2009-09151 from Plan Nacional de Investigación Científica y Técnica, by the Centre CONSOLIDER on Agrigenomics and by the Xarxa de Referencia en Biotecnologia of the Generalitat de Catalunya. H.T. was the recipient of a predoctoral I3P (CSIC).

References

Bewley, J.D. and Black, M. (1994). Seeds: Physiology of Development and Germination. Plenum Press, New York.

Bhave, M.R., Lawrence, S., Barton, C. and Hannah, L.C. (1990). Identification and molecular characterization of shrunken-2 cDNA clones of maize. *The Plant Cell,* **2**, 581-588.

Chen, K. and An, Y.Q.C. (2006). Transcriptional responses to gibberellin and abscisic acid in barley aleurone. *Journal of Integrative Plant Biology*, **48**, 591-612.

Fontanet, P. and Vicient, C.M. (2008). Maize Embryogenesis. *Methods in Molecular Biology*, **427**, 17-30.

Guan, L. and Scandalios, J.G. (1995). Developmentally related responses of maize catalase genes to salicylic acid. *Proceedings of the National Academy of Sciences U.S.A.*, **92**, 5930-5934.

Kutschera, U. (1990). Cell-wall synthesis and elongation growth in hypocotyls of *Helianthus annuus* L. *Planta*, **181**, 316-323.

Martínez-Camacho, J.L., La Vara, L.G., Hamabata, A., Mora-Escobedo, R. and Calderón-Salinas, V. (2004). A pH-stating mechanism in isolated wheat (Triticum aestivum) aleurone layers involves malic acid transport. *Journal of Plant Physiology*, **161**, 1289-1298.

Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y. and Yamaguchi, S. (2003). Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell*, **15**, 1591-1604.

Peterson, C.A. (1988). Exodermal Casparian bands: their significance for ion uptake by roots. *Physiologia Plantarum*, **72**, 204-208.

Rayle, D.L. and Cleland, R.E. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiology*, **99**, 1271-1274.

Xie, Z., Zhang, Z.L., Hanzlik, S., Cook, E. and Shen, Q.J. (2007). Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. *Plant Molecular Biology*, **64**, 293-303.

Yamaguchi, S., Kamiya, Y. and Nambara, E. (2007). Regulation of ABA and GA levels during seed development and germination in Arabidopsis. In *Seed Development, Dormancy and Germination*, (eds. Bradford, K. and Nonogaki, H.), pp. 224–247, Blackwell Publishing, Oxford.

3.2 Expression profile of maize scutellar epithelium during imbibition

Scutellar epithelium cells play specific roles during germination different to parenchymal cells. So, we expect some differences in the gene expression pattern of this tissue. That's why we construct a cDNA library using RNA extracted from scutellar epithelial cells 1 day after imbibition and selected them using array hybridization comparing the mRNA accumulated in epithelial cells with the mRNA accumulated in the other scutellar tissues. We identified 30 genes up-regulated in the epithelium. A high proportion of these genes are involved in metabolic processes, the production of energy or in the transport of peptides into the embryo. These genes are not expressed exclusively in the epithelium but their selection indicates that the metabolism of the epithelial cells increases more rapidly after imbibition than in the parenchymal cells. Some other identified genes are involved in the synthesis or modification of cell walls, which may be related to the changes in the shape of the cells and in the composition of the cell walls observed during imbibition. The other genes identified are involved in protein processing which may be related to storage protein mobilization during germination. The roles of more than 40% of the identified genes remain undetermined, indicating the level of ignorance we still have in a process of such importance such as grass seed germination.

Tnani, H. ***et al.*** (2012). Expression profile of maize (Zea mays) scutellar epithelium during imbibition. *Journal of Plant Physiology* **in press**.

Title Page:

Expression profile of maize (*Zea mays*) scutellar epithelium during imbibition

Authors:

Hedia Tnani, Nora García-Muniz, Carlos M Vicient, Ignacio López-Ribera

Author's academic address:

Department of Molecular Genetics, Centre for Research in Agrigenomics CSIC-IRTA-UAB-UB. Campus UAB; Bellaterra (Cerdanyola del Vallès, Barcelona)

SUMMARY

The scutellum is a shield-shaped structure surrounding the embryo axis in grass species. The scutellar epithelium (Sep) is a monolayer of cells in contact with the endosperm. The Sep plays an important role during seed germination in the secretion of gibberellins and hydrolytic enzymes and the transport of the hydrolized products to the growing embryo. We identified 30 genes predominantly expressed after imbibition in the Sep as compared to other parts of the scutellum. A high proportion of these genes is involved in metabolic processes. Some other identified genes are involved in the synthesis or modification of cell walls, which may be reflected in the changes of cell shape and cell wall composition that can be observed during imbibition. One of the genes encodes a proteinase that belongs to a proteinase family typical of carnivorous plants. Almost nothing is known about their role in other plants or organs, but the scutellar presence may point to a "digestive" function during germination. Genes involved in the production of energy and the transport of peptides were also identified.

Keywords:

epithelium, germination, imbibition, maize, scutellum

Abbreviations:

Sep, Scutellar epithelium.

INTRODUCTION

The mature embryo in grasses comprises the embryonic axis and the scutellum. The scutellum is the single cotyledon in the monocotyledoneus embryos. The scutellum functions as a storage organ, accumulating 90% of the storage lipids in the kernel and 19% of the storage proteins, but it also plays an important role in endosperm digestion during germination. In addition to the aleurone layer, the scutellum secretes enzymes to digest the endosperm starch and provides the pathway along which these food reserves move to the growing axis during germination. The scutellar cells in direct contact with the endosperm form the scutellar epithelium (Sep) (Tnani et al., 2011).

During imbibition the scutellum enlarges and the fresh weight increases and the vascular bundles quickly differentiate (Negbi, 1984). The cells of the Sep lose their adhesion and elongate perpendicularly to the scutellar surface and form finger-like extensions that project into the starchy endosperm (Tnani et al, 2011). This process contributes to increase the contact area between the endosperm and scutellum, thus providing the exposure of a greater surface for secretion and absorption.

During germination, the epithelium is the source of the initial complement of hydrolytic enzymes causing the breakdown of the endosperm, previous to the activation of the aleurone cells (Ganglui and Sen-Mandi, 1993; Mikkonen et al., 1996) and secretes gibberellins into the endosperm (Stoddart et al., 1973). The abscissic acid (ABA), an inhibitor of gibberellin-induced synthesis of hydrolases in the aleurone, does not inhibit the production of α-amylases and cell wall hydrolases by the barley scutellum (Gibbons, 1981).

Sugars, small peptides and amino acids are translocated across the scutellum to supply the growing embryo. Sucrose transporters are expressed in the Sep (Aoki et al., 2006) and the promoter of rice *OsENT2*, encoding a nucleoside transporter, drove the expression of the beta-glucuronidase reporter

gene in the scutellum during germination (Hirose et al., 2005). Barley peptide transporter HvPTR1, is localized in the plasma membrane of the Sep cells during germination (Waterworth et al., 2000) and its activity is inhibited at the post-translational level by phosphorylation in response to rising levels of amino acids emanating from the endosperm as a result of storage protein breakdown (Waterworth et al., 2005).

Transcriptomics has proved to be a powerfull tool to analyze specific processes or developmental stages of living organisms and, particularly, in maize (Strable et al., 2008; Jimenez-Lopez et al., 2011; Sánchez-Pons et al., 2011). We identified, using a transcriptomic approach, genes predominantly expressed in the Sep during germination.

MATERIALS AND METHODS

Tissue collection and RNA extraction

Maize seeds (*Zea mays* L. W64A) were surface sterilized and placed on wet vermiculite at 28ºC. After one day, the scutellum was manually dissected into epithelium and the internal tissues under a microscope. Total RNA was extracted using TRIzol (Invitrogen), treated with DNAseI (Promega) and further purified using RNAeasy Plant Mini Kit (Qiagen).

cDNA library construction

A cDNA library was constructed using total RNA and SMART™ cDNA Library Construction Kit (Clonthech) with oligo dT priming. The resulted cDNA fragments were cloned into pCR®II-TOPO (Invitrogen). The cDNA inserts were amplified using primers: 5'-GGAAACAGCTATGACCATGATTACG-3' and 5'–GTCACGACGTTGTTAAACGACGGC–3'. The PCR products were analyzed by agarose-gel electrophoresis and only the colonies containing single inserts longer than 150 bp were selected for further analysis.

Construction and hybridization of a cDNA macroarray and data analysis

PCR amplified cDNAs fragments (100 ng/µL) were denatured (NaOH 0.2 N final concentration). Spotting was done with a 96 pin Multi-Print Arrayer (V&P Scientfic) using 200 nL hanging pins and Hybond-N^+ nylon membranes (Amersham). For probe labelling, 10 µg of total RNA was annealed with 1 µg oligo$(dT)_{12\text{-}18}$ (Invitrogen) and incubated in presence of [α-^{32}P]dCTP and Supercript II RT (Invitrogen) for 70 min at 42ºC. cDNA-RNA hybrid was hydrolyzed by 1 µL 5M NaOH (37°C, 15 min) and neutralized by adding 1 µL 5M HCl and 5 µL 1M Tris-HCl (pH 7.0). Probes were purified using Bio-Spin P-30 columns (Amersham). Pre-hybridization was done with MicroHyb hybridization buffer (Invitrogen), 10 % dextrane sulphate and 100 µg/mL denatured salmon sperm DNA (42ºC, 2 h). Hybridization in the same buffer (18 h at 42ºC). Washing was done in 2x SSC/0.1 % SDS (twice 65ºC, 30 min). Filters were exposed to phosphorImager screens (3d) and scanned in a phosphorImager (Molecular Imager, Bio-Rad). Hybridizations were done in triplicate.

The image data were imported into Quantity-One program (Bio-Rad) and signal intensities normalized based on the average intensities of seven housekeeping genes: actin (AM937802), Hsp70 (AM939105), elongation factor 1-alpha (AM938565), translation initiation factor 5A (AM937959), polyubiquitin containing seven ubiquitin monomer (AM938744), beta-7 tubulin; (AM938025) and ubiquitin extension protein 2 (AM938244).

Sequencing, EST clustering and sequence analysis

Sequencing was carried out using an automatic fluorescent sequencer ABI PRISM 377 (Applied Biosystems) with a sequencing primer (5'–GTATCAACGCAGAGTCG–3') designed to obtain sequences from the 5'-end. EST sequences were compared against public databases using BLASTN algorithm against NCBI DNA database (http://www.ncbi.nlm.nih.gov/BLAST/).

Quantitative Real-time RT-PCR

Quantitative real-time RT-PCR (qPCR) was done using 2 µg of total RNA were reverse transcribed with Omniscript RT kit (Qiagen) using an oligo-dT primer. qPCR was carried out in a LightCycler 480 system (Roche Diagnostics, Penzberg, Germany) using SYBR® Premix Ex Taq™ (TaKaRa) and the qPCR conditions included an initial denaturation step at 95ºC for 30s, followed by 50 cycles of 95 ºC for 5s, 60°C for 20s and 72 °C for 20s. Melting curve analysis was performed on each sample to ensure single amplicon specificity. Gene-specific primers were designed using Primer Express software (Applied Biosystems, http://www.appliedbiosystems.com/). Actin was checked among six housekeeping genes as the better one (BestKeeper and Normfinder software) and used as reference endogenous control for normalization purposes. Relative quantification was performed using standard curves from serial dilutions of cDNA. The efficiency tested was >99 %. PCR reactions were performed in triplicate for each RNA sample.

Digital expression analysis

The relative abundance (digital expression) of ESTs was obtained based on the Unigene data available for maize (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4577) as previously described (Vicient, 2010).

RESULTS

Construction of cDNA library and identification of up-regulated genes

A cDNA library was constructed with RNA extracted from the Sep one day after imbibition. After a first clone selection based on the size of the insert (>150 bp), a total of 864 cDNA clones were selected, their inserts amplified and used to print nylon macroarray membranes. Total RNAs from the Sep and from the internal region of the scutellum were radioactivelly labelled and used to

hybridize replicates of the nylon macroarray membranes. Significant differential hybridization intensities (≥2-fold; p<0,05) were observed in 31 spots showed and the sequences of the cDNAs were obtained (Table 1). They corresponded to 30 maize genes (Table 1). The role of 43% of the genes remains undetermined, 27% is involved in metabolic processes, 13% in protein synthesis or processing and 7% in cell structure (Table 2).

Expression of the selected genes in other tissues and conditions

The transcriptional levels of four of the selected genes were analized in the scutellum at different times after imbibition using real-time qRT-PCR (Figure 1). For all them, the expression increases only one day after imbibition compared with dry seeds, and continue increasing except for the beta-hexosaminidase subunit B2, that decreases after 3 days of imbibition.

The expression of the selected genes was also examined in other organs using a virtual expression approach based on the EST abundance (Supplementary table S1), as described earlier (Vicient, 2010). The patterns of expression are diverse and, in general, the selected genes are not only expressed in the embryo.

DISCUSSION

Our analyses showed that the expression of the genes predominantly expressed in the Sep increase quickly after imbibition, which is probably necessary to fuel the rapid morphological and metabolic changes that occur in these cells. Horning and Petrie (1927) observed that the number of mitochondria increase in the Sep cells during germination and, accordingly, the electron transfer flavoprotein subunit alpha is predominantly expressed in Sep cells.

Four of the identified genes are involved in carbohydrate metabolism. During germination starch in the endosperm is broken down by the action of α- and β-amylases, predominantly into glucose, that move into the scutellum, where it is converted to sucrose, the form in which the

carbohydrate is then transported to the growing embryo axis (Aoki et al., 2006). Chávez-Bárcenas et al. (2000) showed that sucrose-phosphate synthase expression is localized in the scutellum of germinating rice grains and that the activity significantly increases in early germination. Daussant et al. (1982) observed a transient increase in the number of amyloplasts in Sep cells during early germination, suggesting that part of the carbohydrates absorbed from the endosperm are accumulated in the form of starch during the first days of imbibition before they can be mobilized towards the embryo axis. This may explain the high expression of carbohydrate metabolism related genes in the Sep cells during early germination.

Seed storage proteins are degraded into small peptides during germination and peptide transporters have been shown to be involved in uptake of peptides from the endosperm into the scutellum (Salmenkallio and Sopanen, 1989). Barley *HvPTR1* is predominantly expressed in the Sep during germination (West et al., 1998) and the protein is located in the plasma membrane of Sep cells (Waterworth et al., 2000). We have also observed a predominant expression of a gene encoding a peptide transport protein in maize Sep cells during imbibition.

Sep cells elongate and separate ones from the others during imbibition (Tnani et al., 2011), which involves changes in their cell walls. Interestingly, two of the detected genes are involved in cell wall synthesis. The β-N-acetylhexosaminidase catalyzes the hydrolysis of N-acetyl-D-hexosamine and is involved in cell wall modifications. The activity of this enzyme is increased in corn seeds during germination, mostly in the scutellum (Oikawa et al., 2003). The GDP-D-mannose 4,6 dehydratase is the first enzyme in the biosynthetic pathway of GDP-L-fucose, a monosaccharide which is part of the plant cell walls (Bonin et al., 1997).

Protease activities have been detected in the scutellum during germination: carboxypeptidases (Doan and Fincher, 1988), aspartic proteases (Tamura et al., 2007), serin proteases (Dominguez et

al., 2002), cysteine proteases (Mikkonen et al., 1996) and thiol proteases (Cejudo et al., 1992). One of the selected genes encodes an aspartic proteinase nepenthesin II, which belongs to a proteinase family typical of carnivorous plants (Takahashi et al., 2005). Almost nothing is known about their role in other plants or organs, but the presence of this enzyme enhances the possible "digestive" function of the scutellum during germination.

Allene oxide synthase catalyzes the first step in the biosynthesis of jasmonic acid (JA). JA is involved in the regulation of seed germination (Linkies and Leubner-Metzger, 2012). High levels of jasmonate have been measured in the barley scutellum (Hause et al., 1996) and several jasmonate-related genes are induced in the barley coleorhiza during imbibition, including the allene oxide synthase (Barrero et al. 2009; Maucher et al., 2000). They propose that the coleorhiza may serve as a germination-regulating tissue by preventing root emergence in dormant seeds and in relation to ABA contents and sensitivity. Our findings suggest that Sep may also be involved in a similar role.

Finally, a high proportion of the identified genes encode proteins of unknown function which indicates our widespread ignorance of the genetic and metabolic processes taking place in the Sep during imbibition and germination.

ACKNOWLEDGMENTS

We thanks Mercè Miquel and Mireia Sancenon for their help in DNA sequencing at CRAG DNA sequencing Service. This work was funded by the Spanish Ministry of Science and Innovation (BIO2007-64791 and 200720I037), the CONSOLIDER-INGENIO programme (CSD2007-00036), and the Xarxa de Referencia en Biotecnologia of the Government of Catalonia. H.T. was the recipient of a predoctoral I3P (CSIC).

208 **REFERENCES**

209 Aoki N, Scofield GN, Wang XD, Offler CE, Patrick JW, Furbank RT. Plant Physiol
210 2006;141:1255-63.

211 Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F. Plant Physiol 2009;150:1006–21.

212 Bonin CP, Potter I, Vanzin GF, Reiter WD. Proc Natl Acad Sci USA 1997;94:2085-90.

213 Cejudo FJ, Murphy G, Chinoy C, Baulcombe DC. Plant J 1992;2:937-48.

214 Chávez-Bárcenas AT, Valdez-Alarcón JT, Martínez-Trujillo M, Chen L, Xoconostle-Cázares B,
215 Lucas WJ, et al. Plant Physiol 2000;124:641-53.

216 Daussant J, Renard HA, Skakon A. Electrophoresis 1982;3:99-101

217 Doan NP, Fincher GB. J Biol Chem 1988;263:11106-10.

218 Dominguez F, Gonzalez MC, Cejudo FJ. Planta 2002;215:727-34.

219 Ganglui S, Sen-Mandi S. Ann Bot 1993;71:411-6.

220 Gibbons GC. Carlsberg Res Commun 1981;46:215-25.

221 Hause B, Demus U, Teichmann C, Parthier B, Wasternack C. Plant Cell Physiol 1996 37,641-49.

222 Hirose N, Makita N, Yamaya T, Sakakibara H. Plant Physiol 2005;138:196-206.

223 Horning ES, Petrie AHK. Proc R Soc London Ser B 1927;102:188-206.

224 Jiménez-López S, Mancera-Martínez E, Donayre-Torres A, Rangel C, Uribe L, March S, et al.
225 Plant Cell Physiol 2011;52:1719-33.

226 Linkies A, Leubner-Metzger G. Plant Cell Rep 2012;31:253–70.

227 Maucher H, Hause B, Feussner I, Ziegler J, Wasternack C. Plant J 2000;21:199-213.

228 Mikkonen A, Porali I, Cercos M, Ho TH. Plant Mol Biol 1996;31:239-54.

229 Negbi FLSM. Bot J Linnean Soc 1984;88:205-22.

230 Oikawa A, Itoh E, Ishihara A, Iwamura H. J Plant Physiol 2003;160:991-9.

Salmenkallio M, Sopanen T. Plant Physiol 1989;89:1285-91.

Stoddart JL, Thomas H, Robertson A. Planta 1973;112:309-21.

Strable J, Borsuk L, Nettleton D, Schnable PS, Irish EE. Plant J 2008;56:1045-57.

Takahashi K, Athauda SB, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, et al. Curr Protein Pept Sci 2005;6:513-25.

Tamura T, Terauchi K, Kiyosaki T, Asakura T, Funaki J, Matsumoto I, et al. J Plant Physiol 2007;164:470-7.

Tnani H, López I, Vicient CM. Seed Sci Tech 2011;39:253-8.

Vicient CM. BMC Genomics 2010;11:601.

Waterworth WM, West CE, Bray CM. J Exp Bot 2000;51:1201-9.

Waterworth WM, Ashley MK, West CE, Sunderland PA, Bray CM. J Exp Bot 2005;56:1545-52.

West CE, Waterworth WM, Stephens SM, Smith CP, Bray CM. Plant J 1998; 15:221–9.

Table 1.- Genes predominantly transcribed in the Sep one day after pollination compared to the rest of the scutellum.

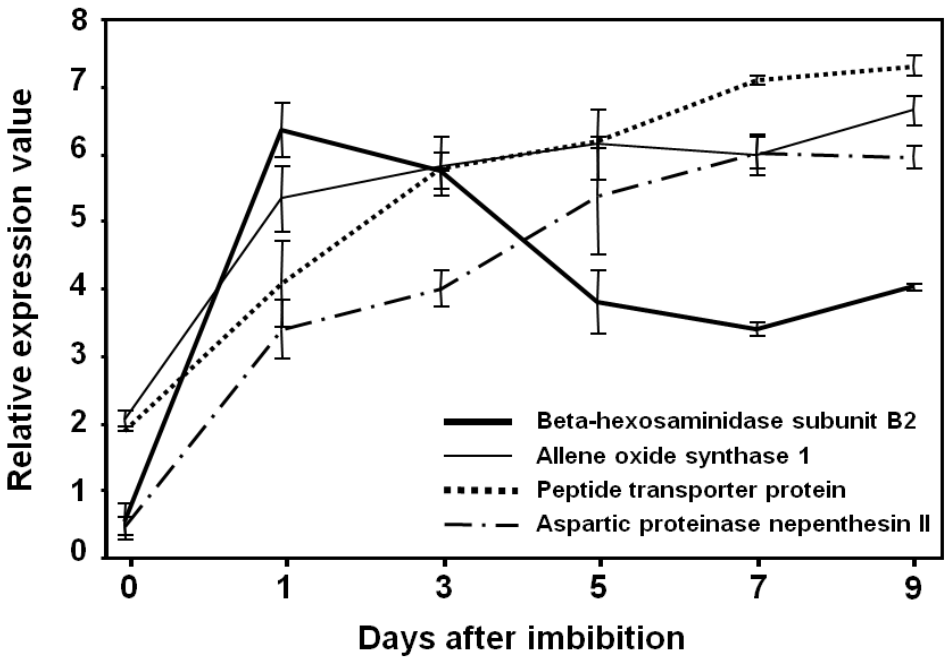
GeneBank	Unigene	Definition	Functional category	Fold change[1]
JK784372/ JK340356	Zm.6258	PP2Ac-5 - Phosphatase 2A isoform 5	Protein modification	4,11[2]
JK340357	Zm.23253	Histone H4	Cell structure	4,08
JK784373	Zm.74344	D-TDP-glucose dehydratase	Carbohydrate metabolism	3,9
JK340358	Zm.84828	EGG APPARATUS-1 protein	Undetermined	3,78
JK784374	Zm.74153	Hypothetical protein	Undetermined	3,32
JK340359	Zm.9255	Electron transfer flavoprotein subunit alpha	Energy	2,88
JK784375	Zm.84849	Glutathione S-transferase theta-1	Response to stress	2,83
JK340361	Zm.35297	Pentatricopeptide repeat protein	Undetermined	2,67
JK340360	Zm.502	GDP-D-mannose 4,6 dehydratase 2	Carbohydrate metabolism[3]	2,67
JK784371	Zm.4578	DEAD/DEAH box RNA helicase	Transcriptional regulation	2,63
JK340363	Zm.6780	Phosphoglycerate kinase, cytosolic	Carbohydrate metabolism	2,57
JK784376	Zm.1053	Hypothetical protein	Undetermined	2,54
JK784377	Zm.98214	Hypothetical protein	Undetermined	2,51
JK340364	Zm.85894	Peptide transporter protein	Transport	2,42
JK784378	Zm.1802	Hypothetical protein	Undetermined	2,42
JK784379	Zm.94967	Armadillo/beta-catenin-like repeat-containing protein	Undetermined	2,36
JK784380	Zm.84126	Protein phosphatase 2C	Protein processing	2,35
JK784381	Zm.3467	Beta-hexosaminidase subunit B2-like	Carbohydrate metabolism[3]	2,32
JK784382	Zm.79291	Benzoxazinone synthesis 2	Secondary metabolism	2,28
JK784383	Zm.138535	Hypothetical protein	Undetermined	2,26
JK784384	Zm.27614	Hypothetical protein	Undetermined	2,26
JK784385	Zm.84763	Aspartic proteinase nepenthesin II	Protein processing	2,24
JK784386	Zm.77560	Hypothetical protein	Undetermined	2,23
JK784387	Zm.34537	tRNA-specific adenosine deaminase	Translation	2,12
JK784388	Zm.93878	Allene oxide synthase 1	Secondary metabolism	2,10
JK784389	Zm.94054	Hypothetical protein	Undetermined	2,10
JK784390	Zm.14469	Anthranilate synthase alpha subunit	Amino acids metabolism	2,08
JK784391	Zm.85050	Autophagocytosis protein	Cell structure	2,07
JK784392	Zm.6941	RNA-binding protein	Undetermined	2,07
JK784393	Zm.72678	Hypothetical protein	Undetermined	2,01

1. Hybridization intensity in Sep cells compared to the rest of the scutellum.
2. It was calculated according to the hybridization average of the two spots corresponding to the same gene
3. Genes encoding enzymes involved in the modification of cell wall components.

Table 2.- Functional categories of the genes predominantly transcribed in the Sep one day after pollination compared to the rest of the scutellum.

Functional category	**Number of genes**	**% genes**
Cell structure	**2**	**7**
Metabolism	**8**	**27**
- Carbohydrate	4	13
- Amino acids	1	3
- Secondary metabolism	2	7
- Energy	1	3
Protein synthesis and processing	**4**	**13**
- Translation	1	3
- Protein processing	3	10
Response to stress	**1**	**3**
Transport	**1**	**3**
Regulation of transcription	**1**	**3**
Undetermined	**13**	**43**

290 **LEGENDS OF FIGURES**



291

292 Figure 1.- Real time quantitative RT-PCR analyses of four selected genes in maize scutellum at
293 different times after imbibition. Relative expression values were calculated using actin as
294 housekeeping gene for normalization. Relative expression values were calculated from three
295 independent biological replicates. The standard errors are indicated (error bars).

296

297 **SUPPLEMENTARY DATA**

298

299 Supplementary Table S1.- Patterns of EST presence in different maize organs. Lines represent
300 different genes and columns different organs. Numbers are the normalized values of EST
301 abundance.

302

303

Supplementary

Table S1.- Patterns of EST presence in different maize organs. Lines represent different genes and columns different organs. Numbers are the normalized values of EST abundance.

	Cell culture	Ear	Embryo	Endosperm	Leaf	Meristem	Ovary	Pericarp	Pollen	Root	Shoot	Tassel	Total ESTs				
Total ESTs in the analysis	13.389	35.904	19.894	55.333	14.689	156.197	22.917	9.594	17.165	15.670	93.897	23.798					
PP2Ac-5 - Phosphatase 2A isoform 5	149	111	50	72	0	89	0	25	0	191	42	42	0				
Histone H4	2.165	12.310	1.809	1.012	68	8.316	1.134	0	3.204	638	6.613	20.547	7.639				
D-TDP-glucose dehydratase	0	222	251	90	1.565	115	130	416	116	574	53	378	278				
EGG APPARATUS-1 protein	0	0	753	0	0	0	261	0	0	0	42	0	364				
Hypothetical protein (Zm.74153)	74	55	0	18	68	0	87	521	0	0	10	0	67				
Electron transfer flavoprotein subunit alpha	0	27	150	72	0	44	0	0	58	63	10	84	98				
Glutathione S-transferase theta-1	448	0	50	0	0	6	218	0	116	63	0	0	43				
Pentatricopeptide repeat protein	0	0	0	0	0	0	0	0	0	0	0	0	10				
GDP-D-mannose 4,6 dehydratase 2	0	55	50	18	0	51	174	208	0	0	21	0	66				
DEAD/DEAH box RNA helicase	0	55	50	36	0	102	130	0	0	0	53	84	83				
Phosphoglycerate kinase, cytosolic	298	640	2.965	1.807	0	224	829	0	58	191	95	462	606				
Hypothetical protein (Zm.1053)	0	0	201	0	0	44	0	0	0	1.403	0	0	189				
Hypothetical protein (Zm.98214)	0	222	0	18	0	70	174	0	0	0	85	0	147				
Peptide transporter protein	0	0	0	36	0	6	0	0	0	0	42	0	64				
Hypothetical protein (Zm.1802)	0	27	50	108	0	153	87	729	58	0	53	210	199				
Armadillo/beta-catenin-like repeat-containing protein	1.045	167	301	18	0	153	87	104	0	255	255	546	202				
Protein phosphatase 2C	0	55	100	108	0	25	43	0	0	0	42	336	101				
Beta-hexosaminidase subunit B2-like	0	0	50	185	0	19	43	0	0	127	0	42	32				
Benzoxazinone synthesis 2	0	0	0	0	0	6	0	0	0	0	0	0	325				
Hypothetical protein (Zm.138535)	0	0	0	0	272	0	43	0	0	0	31	0	15				
Hypothetical protein (Zm.27614)	0	55	0	0	68	19	130	0	58	0	0	0	51				
Aspartic proteinase nepenthesin II	448	0	50	0	68	19	43	104	0	63	21	84	70				
Hypothetical protein (Zm.77560)	0	0	50	18	0	57	43	0	0	63	42	42	109				
tRNA-specific adenosine deaminase	0	0	0	0	0	6	0	0	0	0	0	0	26				
Allene oxide synthase 1	0	27	0	54	0	0	261	104	0	127	0	0	287				
Hypothetical protein (Zm.94054)	298	27	100	18	0	25	0	0	0	63	31	42	132				
Anthranilate synthase alpha subunit	224	0	100	72	0	83	0	416	116	255	74	126	148				
Autophagocytosis protein	0	55	0	72	0	76	130	0	174	510	0	42	121				
RNA-binding protein	0	222	100	0	0	160	87	0	58	0	95	0	122				
Hypothetical protein (Zm.72678)	448	111	0	36	0	51	174	104	0	446	53	0	198				

3.3 *ZmPTR1, a peptide transporter expressed in the epithelial cells of the scutellum during germination*

Plants have evolved different transport systems to facilitate nitrogen uptake and reallocation. Besides uptake of inorganic nitrogen (nitrate and ammonium), plants also take up and transport organic nitrogen in the form of amino acids, peptides and proteins. Transport of peptides across membranes is mediated by three types of transporters ATPbinding cassette (ABC) proteins, oligopeptide transporters (OPT) and peptide transporters (PTR/NTR1). Members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family in plants transport a variety of substrates such as nitrate, di- and tripeptides, auxin and carboxylates. The high number of genes in plant genomes together with the diversity of substrates and cell locations suggest that PTR/NTR1 may be involved in a wide range of cellular processes in plants, however, the physiological functions for most of them remain unknown. Plant roots can take up small peptides from soil and some PTR/NTR1 genes are transcribed in roots. It has also been suggested that the PTR family is important for the mobilization of nitrogen during germination and in the long-distance transport of peptides to growing tissues. Seed storage proteins are degraded into small peptides during germination to support growth of the seedling and peptide transporters have been shown to be involved in uptake of peptides from the endosperm into the scutellum. We isolated and characterized ZmPTR1 a non-characterized maize peptide transporter. *ZmPTR1* encodes a 587 amino acid protein with a predicted molecular mass of 64.52 kDa, which displayed 69% identity to the *Arabidopsis thaliana* peptide transporter AtPTR2-B (At2g02040), 59% identity to AtPTR4 (At2g02020) and 62% identity to AtPTR6 (At1g62200). Moreover, ZmPTR1 includes the conserved motif and the transmembrane domains typical of these peptide transporter proteins. Furthermore, the C-terminal fusion of ZmPTR1 with YFP showed fluorescence at the tonoplast. This tonoplast localization was verified when the same construct was transiently introduced in tobacco BY-2 cells. Plants overexpressing *ZmPTR1* performed better than control *Arabidopsis thaliana* plants when grown on a medium with Ala-Ala dipeptide as the unique N source. *ZmPTR1* is expressed in the scutellar epithelium during germination and, to a less extent, in the radicle and the hypocotyl. Our studies suggest that *ZmPTR1* plays an important role in transport into the embryo of the small peptides produced during enzymatic hydrolysis of the storage proteins in the endosperm. **Submitted Planta (2012)**

Author's names:
Hedia Tnani*, Ignacio López-Ribera*, Nora García-Muniz, Carlos M Vicient
*These authors contributed equally to this work

Title: ZmPTR1, a maize peptide transporter expressed in the epithelial cells of the scutellum during germination

Affiliation and address of the authors
Department of Molecular Genetics, Centre for Research in Agrigenomics CSIC-IRTA-UAB-UB. Campus Universitat Autònoma de Barcelona; Bellaterra (Cerdanyola del Vallès, Barcelona), 08193 Barcelona

e-mail, telephone and fax of the corresponding author
Carlos M Vicient
carlos.vicient@cragenomica.es
Tel. +34 935636600
Fax +34 935636601

Abstract
In plants, peptide transporter/nitrate transporter 1 (PTR/NRT1) family proteins transport a variety of substrates such as nitrate, di- and tripepetides, auxin and carboxylates across membranes. We isolated and characterized ZmPTR1, a maize member of this family. ZmPTR1 protein sequence is highly homologous to the previously characterized di- and tripeptide Arabidopsis transporters AtPTR2, AtPTR4 and AtPTR6. *ZmPTR1* gene is expressed in the cells of the scutellar epithelium during germination and, to a less extent, in the radicle and the hypocotyl. *Arabidopsis thaliana* lines overexpressing ZmPTR1 performed better than control plants when grown on a medium with Ala-Ala dipeptide as the unique N source. ZmPTR1 is predominantly located in the tonoplast. Our studies suggest that ZmPTR1 plays a role in transport into the embryo of the small peptides produced during enzymatic hydrolysis of the storage proteins in the endosperm.

Keywords: Arabidopsis - Dipeptide - Embryo - Organic nitrogen - Zea mays

Abbreviations
- *ABA*
Abscisic acid
- *ABC*
ATP-binding cassette
- *Ala*
Alanine
- *CaMV-35S*
Cauliflower mosaic virus 35S promoter
- *dai*
Days after imbibition
- *Eph*
Epithelium
- *GA*
Gibberellin
- *N*
Nitrogen
- *NTR*
Nitrate transporter
- *OPT*
Oligopeptide transporter
- *PAC*
Paclobutrazol
- *PTR*
Peptide transporter
- *qRT-PCR*
Quantitative reverse transcriptase – polymerase chain reaction
- *SDS*
Sodium dodecyl sulfate
- *YFP*
Yellow fluorescent protein

Introduction

Plants have evolved different transport systems to facilitate nitrogen uptake and reallocation (Tsay et al. 2007). Besides uptake of inorganic nitrogen (nitrate and ammonium), plants also take up and transport organic nitrogen in the form of amino acids, peptides and proteins (Näsholm et al. 2009). Transport of peptides across membranes is mediated by three types of transporters (Rentsch et al. 2007): ATP-binding cassette (ABC) proteins, oligopeptide transporters (OPT) and peptide transporters (PTR/NTR1). ABC proteins can transport a wide range of substrates, from small ions to large macromolecules, including peptides ranging from 6–59 amino acids (Sánchez-Fernández et al. 2001; Ramos et al. 2011). The *Arabidopsis thaliana* genome contains 129 genes coding for ABC proteins (Sánchez-Fernández et al. 2001), although probably only a proportion of these genes encode proteins with peptide transport activity. The OPT proteins can transport tetra- and pentapeptides (Gomolplitinant and Saier 2011; Lubkowitz 2011), and are also involved in long-distance metal distribution (Stacey et al. 2008), heavy metal sequestration (Vasconcelos et al. 2008; Pike et al. 2009) and glutathione transport (Bogs et al. 2003; Cagnac et al. 2004; Zhang et al. 2004). Nine members of the OPT family have been found in *Arabidopsis* and rice (Koh et al. 2002; Vasconcelos et al. 2008), 20 in *Populus* and 18 in *Vitis* (Cao et al. 2011). Members of the PTR/NTR1 family transport di- and tripeptides (Chiang et al. 2004; Tsay et al. 2007; Komarova et al. 2008) but they have also been shown to transport other substrates, including nitrate, auxin, amino acids and carboxylates (Frommer et al. 1994; Zhou et al. 1998; Tsay et al. 2007; Krouk et al. 2010). Plant genomes contain a large number of genes coding for PTR/NRT1 proteins: 53 in *Arabidopsis*, divided in four subfamilies (Tsay et al. 2007), and 84 in rice, divided in five subfamilies (Zhao et al. 2010), although functional peptide transport activity and substrate selectivity has only been demonstrated for a few of them (Hammes et al. 2010). PTR/NTR1 proteins have also been described in bacteria, yeasts and animals (Daniel et al. 2006).

PTR/NTR1 proteins are located in different cellular membranes (Weichert et al. 2012). For example, AtPTR1, AtPTR5, HaPTR12 and HvPTR1 are localized at the plasma membrane (Waterworth et al. 2000; Dietrich et al. 2004; Komarova et al. 2008; Paungfoo-Lonhienne et al. 2009), whereas AtPTR2, AtPTR4, AtPTR6, *Hakea actites* HaPTR4 and HvPTR2 from barley are located in the tonoplast (Carter et al. 2004; Shimaoka et al. 2004; Dunkley et al. 2006; Endler et al. 2006; Paungfoo-Lonhienne et al. 2009; Weichert et al. 2012; Komarova et al., 2012).

The high number of genes in plant genomes together with the diversity of substrates and cell locations suggest that PTR/NTR1 may be involved in a wide range of cellular processes in plants, however, the physiological functions for most of them remain unknown (Stacey et al. 2002). Plant roots can take up small peptides from soil (Schimel and Chapin, 1996) and some PTR/NTR1 genes are transcribed in roots (*Arabidopsis AtPTR1* and *AtPTR2*, and *Hackea HaPTR4*, *HaPTR5* and *HaPTR12*). This suggests that they may be involved in the uptake of small peptides from soil (Song et al. 1996; Komarova et al. 2008; Paungfoo-Lonhienne et al. 2009), but they may also be involved in their distribution to other parts of the plant. PTR/NTR1s also play a role in flower establishment. AtPTR5 facilitates uptake of di- and tripeptides by germinating pollen and ovules (Komarova et al. 2008), *AtPTR2* antisense plants exhibit delayed flowering (Song et al. 1997), *AtPTR1* expression is present in the style and stigma (Dietrich et al. 2004) and *AtPTR6* is expressed in pollen (Weichert et al. 2012). PTRs are also involved in seed development, processes which require the mobilization of large quantities of nitrogen. The Arabidopsis *AtPTR2* gene is expressed during seed development (Rentsch et al. 1995; Song et al., 1996), and seed development is partially arrested in antisense *AtPTR2* lines (Song et al. 1997). *Arabidopsis AtPTR1* and *AtPTR5* genes are expressed in seeds (Dietrich et al., 2004; Komarova et al., 2008), faba bean *VfPTR1* is expressed in the epidermis at early stages of cotyledon development, and at later stages in cells surrounding the vasculature, suggesting that VfPTR1 is involved in the uptake of peptides from the seed apoplast (Miranda et al. 2003). PTR/NTR1s may also be involved in other physiological processes. For example, *AtPTR3* is expressed in response to salt stress, mechanical wounding and pathogen defense (Karim et al. 2005, 2007), and NaNTR1, from the carnivorous plant *Nepenthes alata*, is probably involved in the phloem loading of peptide nitrogen exported from the pitcher, the prey-trapping mechanism of the plant (Schulze et al., 1999). *AtPTR6* is highly expressed in senescent leaves (Weichert et al. 2012) and it has been suggested that AtPTR6 is involved in the translocation of the resulting breakdown products (peptides and amino acids) to other parts of the plant.

It has also been suggested that the PTR family is important for the mobilization of nitrogen during germination and in the long-distance transport of peptides to growing tissues. Seed storage proteins are degraded into small peptides during germination to support growth of the seedling (Herman and Larkins 1999), and peptide transporters have been shown to be involved in uptake of peptides from the endosperm into the scutellum (Sopanen et al. 1977; Higgins and Payne 1981; Salmenkallio and Sopanen 1989). One of the best-characterized plant PTRs, the barley scutellar peptide transporter HvPTR1, is responsible for the mobilization of peptides across the scutellum during germination (West et al. 1998). *HvPTR1* expression is seed-specific, with transcripts detected only in scutellar epithelial cells during germination and in barley grain from the earliest stages of grain development (Waterworth et al. 2003). HvPTR1 has been localized in the plasma membrane of scutellar epithelial cells (West et al. 1998; Waterworth et al. 2000). Other PTR genes that seem to be important for seed germination are *VfPTR1* and *VfPTR2* which seem to have a role in faba bean similar to that in barley *HvPTR1* (Miranda et al. 2003). In addition, several *Arabidopsis* peptide transporters have been proposed as important for seed germination and establishment of the seedling. *AtPTR2* has high mRNA expression levels in three-day germinating seed (Rentsch et al. 1995) with antisense plants exhibiting arrested seed development (Song et al.

1996, 1997). Reduced total N levels and reduced shoot growth has been found in Atptr1 mutant seedlings, whereas seedlings of p35S::AtPTR5 lines displayed enhanced shoot growth and increased N content (Komarova et al. 2008).

In the present study we studied a maize peptide transporter we called ZmPTR1. *ZmPTR1* expression was found in the scutellar epithelial cells during seed imbibition, but it is also expressed, at a lower level, in shoots and roots of germinating seedlings. Transgenic *Arabidopis* plants over-expressing ZmPTR1 can uptake the di-peptide Ala-Ala from the growing media. ZmPTR1 is located in the tonoplast. The possible physiological roles of ZmPTR1 are discussed.

Materials and methods

Plant material

Maize (*Zea mays* L.) of the pure inbred line W64A was used, originally obtained from the Aula Dei Experimental Station (CSIC, Zaragoza, Spain). For germination studies, seeds were surface sterilized with 50% (v/v) bleach for 15 min and then washed 3-4 times in water.
Seeds of *Arabidopsis* (Col-0 and transgenic lines) were surface sterilized in 70% (v/v) ethanol for 1 min followed by a solution containing 50% (v/v) bleach and 0.5% (w/v) SDS for 5 min, then 3-4 rinses in sterile water. The seeds were stratified for 3 days at 4ºC in the dark. After stratification, they were transferred to growth media and placed in a growth cabinet at 22ºC under 16 h light with 120 µmol m^{-2} s^{-1} light intensity. The growth media consisted of AM medium (2.16 g L^{-1} Murashige and Skoog salts [Duchefa]), the N medium (half strength Murashige and Skoog medium) with NH_4NO_3 and KNO_3 replaced by KCl, and -N medium containing 20 mM Ala-Ala as the sole nitrogen source. All media were adjusted to pH 5.8, 1% (w/v) sucrose added, and solidified with agar (Oxoid).
Tobacco BY-2 cell cultures were obtained from Toshiyuki Nagata (Tokyo University, Japan) and cultivated as previously described (Hemmerlin and Bach, 2000)

Phylogenetic and sequence analysis

Three different programs, with similar results, were used for transmembrane predictions: DAS-Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/), Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). ClustalW was used for multiple sequence alignment and to generate the unrooted phylogenetic tree based on the neighbor-joining method using TREECON to generate the tree (Van de Peer and Wachter, 1997). Bootstrap values were based on 1,000 replicates.

RNA extraction and expression analyses

Total RNA was extracted using TRI®zol Isolation Reagents (Invitrogen), treated with RNAse-free DNAseI (Qiagen), and further purified using the RNAeasy Plant Mini Kit (Qiagen), all according to the manufacturers' instructions. Northern-blot analyses were performed as described in Ausubel et al. (1994) according to the formaldehyde/formamide protocol using 15µg of total RNA and *ZmPTR1* cDNA as probe. For quantitative real-time RT-PCR, 2µg of total RNA were reverse transcribed with the Omniscript RT kit (Qiagen) using an oligo-dT primer. Quantitative real time PCR (qPCR) was carried out in a LightCycler 480 system (Roche Diagnostics, Penzberg, Germany) using SYBR® Premix Ex Taq™ (TaKaRa). Gene-specific primers were designed using the Primer Express software (Applied Biosystems, http://www.appliedbiosystems.com/). For qPCR, 2µl of template cDNA and 200nM of each primer were used, made up to a volume of 20µl. The PCR conditions included an initial denaturing step at 95ºC for 30s followed by 50 cycles of 95 ºC for 5s, 60°C for 20s and 72°C for 20s. Melting curve analysis was performed on each sample to ensure single amplicon specificity. Actin was used as the reference endogenous control for normalization purposes. Standard curves from serial dilutions of cDNA were used for relative quantification, using the LightCycler 480 software. The efficiency tested was >99 %. PCR reactions were in triplicate in three RNA samples. The mRNA *in situ* hybridizations were performed as previously indicated (Fontanet and Vicient 2008; Miquel et al. 2011). Probe labeling was with the DIG RNA Labeling Kit (SP6/T7) (Roche), and for negative controls the same protocol was used for SP6 transcribed sense probes. Photomicrographs were taken on a Zeiss Axiophot microscope.

Plant transformation

Standard DNA manipulation methods (PCR, cloning procedure, transformation of bacteria, plasmid preparation, and DNA cleavage with restriction enzymes) were as previously described (Ausubel et al. 1994). The ORF of *ZmPTR1* cDNA was PCR amplified using the primers AttB1-ZmPTR1 start (AttB1-GCGGCCGCATGGCCGG) and AttB2-ZmPTR1 Stop (CCGCGGTCAAGCTGCCTTCTT). The PCR product was amplified with the universal U3-stop primer 5'AGATTGGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATC3' and the U5 primer 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG3', and cloned into the Gateway®binary vector pYL-YFPct (Rubio et al., 2005).
The floral-dip method was used for Arabidopsis transformation (Clough and Bent 1998). Transgenic *Arabidopsis* plants

seeds were observed with a FV 1000 confocal microscope (Olympus, Tokyo, Japan). YFP fluorescence was excited with a 514 nm argon laser, and emission was detected with a 535 to 590 nm band pass filter combination.
Nicotiana tabacum L., cv Bright Yellow 2 cells spread on filter papers were transformed by microprojectile bombardment as described by Kost et al (1998) using gold particles and an helium-driven particle accelerator (PDS-1000/He; BIO-RAD). Bombarded BY-2 cells were allowed to transiently express gene products at room temperature in NT medium in covered Petri dishes at 28°C for 18–42 h.

Results

Cloning and sequence analysis of the *ZmPTR1* gene

The *ZmPTR1* gene was isolated from a cDNA library prepared from *Zea mays* scutellar epithelium extracted one day after imbibition (GeneBank accession number JK340364; gene code GRMZM5G867390; locus LOC100280282) (Tnani *et al*., 2012, in press). The predicted ZmPTR1 protein has 587 amino acids with a calculated molecular mass of 64.52 kDa. Bioinformatic analysis of the ZmPTR1 amino acid sequence predicts 10 conserved transmembrane domains (amino acids 125-147, 157-179, 204-226, 233-255, 347-369, 397-418, 432-453, 480-502, 522-544 and 557-579) (Fig. 1). A homology search against the Gene-Bank database showed that ZmPTR1 was homologous to several proteins annotated as peptide transporters in plant species. When aligned to some well-characterized members of the PTR/NRT1 family, ZmPTR1 has high sequence conservation, especially with regard to the three conserved motifs defined for PTR proteins (Zhao et al. 2010). The number and approximate position of transmembrane domains is well conserved in all the aligned proteins (Fig. 1).

Plant genomes contain several genes encoding PTR/NRT1 proteins (Tsay et al. 2007). Phylogenetic analyses has shown that rice PTR/NRT1 genes can be divided in five subfamilies (Zhao et al. 2010). To investigate the evolutionary relationship of ZmPTR1 with the other members of the PTR/NRT1 family, a phylogenetic tree was constructed (Fig. 2). Given the relatively close evolutionary relationship between maize and rice, the comparison was made using all the sequences of the PTR/NRT1 proteins encoded in the rice genome. In the analysis, we also included protein sequences of well characterized members of the PTR/NRT1 family from other species. The results revealed that ZmPTR1 belongs to the subfamily I (group I-1) with high sequence similarity to AtPTR2, AtPTR4, AtPTR6, VfPTR1, PaPTR2, LeNTR1, HaPTR4 and rice OsPTR2 (Os03g51050). The highest similarity was observed against OsPTR2 (86 %). Interestingly, ZmPTR1 contains an N-terminal sequence extension which is also present in AtPTR2, AtPTR4 and AtPTR6, but not in AtPTR1, AtPTR3 and AtPTR5. This extension contains a possible dileucine motif which is involved in tonoplast protein targeting (Komarova et al., 2012).

Pattern of *ZmPTR1* expression

We analyzed the levels of mRNA accumulation using three techniques: northern-blot, *in situ* hybridization and real-time quantitative RT-PCR. Although we cannot discard the possibility of cross-hybridization with other members of the family in the northern-blot and *in situ* hybridization analyses, the fact that northern-blot and real-time qRT-PCR gave similar results suggests that any cross-hybridization probably does not seriously interfere with the interpretation of the results. The levels of mRNA accumulation in the two main tissues of the scutellum during germination showed a higher expression in the epithelial cells compared with the parenchyma at 3 days after imbibition (Fig. 3a). Using northern-blot hybridization for a time course analysis of *ZmPTR1* mRNA accumulation in scutellum during imbibition, no hybridization signal was observed in dry seeds, but was clearly detected one day after imbibition, increasing during the first seven days (Fig. 3b). *ZmPTR1* mRNA was also accumulated in shoots and roots of seedlings seven days after imbibition, but the expression levels were weaker than in the scutellum. For the qRT-PCR analyses, RNA was extracted from scutella dissected at different times, from dry seeds to nine days after imbibition (Fig. 3c). The results were in agreement with the northern-blot analyses: *ZmPTR1* expression was detectable from one day after imbibition, peaked at seven days and then decreased. We also carried out northern-blot analysis to examine the spatial distribution of the *ZmPTR1* mRNA in shoots and roots of germinating seedlings seven days after imbibition (Fig. 3d). In the shoot, hybridization signals were detected in the dissected leaves, node and hypocotyl, with the highest intensity in the hypocotyl, followed by the node. Roots were serially dissected along the axis as root-tip, elongation zone and mature root. The highest level of hybridization was observed in the mature part, with a reduction in the elongation zone and a very low level in the root-tip. Finally, we analyzed the response of *ZmPTR1* expression to different hormones. Seeds were imbedded for seven days in the presence of abscisic acid (ABA), gibberellin (GA3) and paclobutrazol, an inhibitor of gibberellin biosynthesis. Whereas *ZmPTR1* expression was repressed by ABA, gibberellin produced a significant increase in *ZmPTR1* expression (Fig. 3e). The application of paclobutrazol drastically reduced the expression of *ZmPTR1* compared to control seeds imbedded in water. From this, we conclude that gibberellin induces and is necessary for the induction of *ZmPTR1* transcription.

No signal was obtained using the ZmPTR1 antisense RNA probe for in situ hybridization in cross-sections of the scutellum of dry seeds (Fig. 4a), but prominent signals were observed in epithelial cells of cross-sections from seeds 3 days after imbibition (Fig. 4b). No signal was observed with the sense RNA probe as control (Fig. 4c).

Sub-cellular localization of ZmPTR1

To assess the intracellular localization of ZmPTR1, *Arabidopsis* plants were stably transformed with a ZmPTR1/YFP fusion construct and analyzed using confocal laser scanning microscopy (Fig. 5a,b,c). The C-terminal fusion of ZmPTR1 with YFP showed fluorescence at the tonoplast (Fig. 2a,b). The tonoplast localization was verified when the same construct was transiently introduced in tobacco BY-2 cells (Fig. 5d,e,f). In both cases the fusion protein is localized predominantly on the tonoplast, but we also observed fluorescence associated with some vesicular structures.

ZmPTR1 mediates the uptake of Ala-Ala into roots

Arabidopsis plants were transformed with the 35S::ZmPTR1 construct harboring the coding region of *ZmPTR1* under the control of the CaMV-35S promoter. Three independently transformed lines were used to analyze the ability of ZmPTR1 to transport dipeptides from growth media to the plants compared to wild type (Col-0). No phenotypic difference were detected when control and 35S::ZmPTR1 lines were cultured in AM complete medium (Fig. 6), nor when seeds were cultured on a media without nitrogen (-N). In -N medium control and transformed plants died short after imbibition. In contrast, differences were observed when plants were grown on –N medium supplemented with 20 mM of Ala-Ala dipeptide as the sole N source: 35S::PTR1 transgenic plants had green cotyledons whereas control plants died shortly after germination. These data indicate that ZmPTR1, when ectopically expressed, is able to transport the Ala-Ala dipeptide from the media to the *Arabidopsis* root cells.

Discussion

In this study, a new member of the PTR/NTR1 family was analyzed. Sequence comparisons showed a high amino acid identity to different members of the PTR/NTR1 family, including the conserved motif and the transmembrane domains typical of these peptide transporter proteins (Fig. 1). This indicates that *ZmPTR1* encodes a PTR/NTR1 transporter, so functional tests were carried out with *Arabidopsis* over-expressing ZmPTR1. Experiments showed that ZmPTR1 restores growth of the Arabidopsis plants when cultivated on growth medium where the dipeptide Ala-Ala was the only source of N, implying that ZmPTR1 mediates the uptake of this dipeptide. It has been previously demonstrated that PTR proteins have the capacity for uptake of dipeptides into roots when ectopically expressed, for example, a similar role has been observed for Arabidopsis AtPTR5 (Komarova et al. 2008). Although the substrate range recognized by ZmPTR1 remains to be investigated, we can conclude that it is a functionally active peptide transporter.

Peptide transporters have been found in different membranes in the plant cell. Stable expression of ZmPTR1-YFP in *Arabidopsis* showed that ZmPTR1 is mainly located in the tonoplast (Fig. 5). Phylogenetic analysis revealed that ZmPTR1 belongs to the PTR/NTR1 subfamily I (group I-1) (Fig. 2), which includes functionally characterized peptide transporters (AtPTR2, AtPTR4, AtPTR6, HvPTR2 and HaPTR4) located in tonoplast (Carter et al. 2004; Shimaoka et al. 2004; Dunkley et al. 2006; Endler et al. 2006; Paungfoo-Lonhienne et al. 2009; Zhao et al. 2010; Weichert et al. 2012; Komarova et al., 2012). N terminal ZmPTR1 contains a peptide similar to the dileucine motif described for tonoplast localization (Komarova *et al*., 2012).

ZmPTR1-YFP seems also to be located in some kind of vesicles (Fig. 5). These spherical structures (“bulbs”) are derived from the tonoplast and have also been observed in other cases. For example, shortly after transfection, DMP1-eGFP induces in tobacco epidermal cells similar spherical structures (Kasaras et al., 2012), and similar structures have also been observed in the cotyledons of young Arabidopsis seedlings (Saito et al., 2002), and, under stress conditions, in different tissues at various developmental stages, in different plant species, suggesting additional functions (Saito et al., 2011). The possible roles of ZmPTR1 in these structures remains to be determined but we can not discard that this location is artefactual as a consequence of the stress produced during bombardment or due to the overexpression of the protein.

The temporal and spatial expression patterns of the *ZmPTR1* gene provide useful information for establishing its putative function. Expression is induced after germination and this induction is dependent on gibberellin and repressed by ABA. Maturing maize embryos require GA for germination and the expression of many germination-related genes is regulated by ABA and GAs (White et al. 2000; An and Lin, 2011). Moreover, the expression of the *ZmPTR1* gene was higher in the scutellar epithelium during imbibition. In grasses, the embryo cells in direct contact with the endosperm form the scutellar epithelium, which plays a major role in the uptake of the endosperm nutrient reserves by the embryo in order to provide energy and materials for seedling growth (Tnani et al. 2011). Degradation of the major storage proteins in maize endosperm, zeins and glutelins, begins the second day of imbibition, and protease activity in the endosperm is first detected two days after imbibition, reaching maximum activity at about eight days (Harvey and Oaks 1974). In germinating barley grains, the peptide pool of the endosperm increases during the first three days of germination and subsequently decreases (Higgins and Payne 1981). An increase in peptide transporter activities during early germination has been reported in the scutellum of different grass species, including maize (Sopanen et al. 1977; Salmenkallio and Sopanen 1989). Interestingly, the maximum hydrolytic enzyme activity coincides with the maximum

expression of *ZmPTR1*. These data indicate a possible role of ZmPTR1 in remobilizing small peptides, produced by the enzymatic hydrolysis of storage protein in the endosperm during germination, to the growing seedling. A similar pattern of expression and role have been described for the barley HvPTR1 (Waterworth et al. 2000, 2001, 2005). The fact that the expression of *ZmPTR1* is regulated by gibberellins is compatible with this role.

ZmPTR1 is also expressed, at lower levels, in seedling roots and shoots (Fig. 3). Active peptide uptake by roots has been demonstrated and seems to have certain nutritional importance (Schimel and Chapin 1996). This view is supported by the expression of a number of the PTR genes (AtPTR1, AtPTR2, HaPTR4, HaPTR5 and HaPTR12) characterized in roots (Song et al. 1996; Komarova et al. 2008; Paungfoo-Lonhienne et al. 2009). The highest *ZmPTR1* expression in the root was observed in the mature part of the seminal root, which contains root hairs and is responsible for most of the water and nutrient uptake. These data suggest that ZmPTR1 may play a role in the uptake of peptides (and/or nitrate) by roots. Here we demonstrate that, when over-expressed in *Arabidopsis*, it is able to transport the Ala-Ala dipeptide from the growing media to the plant. *ZmPTR1* is also expressed in the aerial part of the seedling, particularly in the hypocotyl and the node, suggesting that it may also be involved in the transport of peptides, resulting from the breakdown of the seed storage proteins, to the growing leaves (Kuang et al. 1996; Dietrich et al. 2004).

In summary, *ZmPTR1* encodes a peptide transporter that probably mediates peptide transport from the endosperm to the growing embryo, and may also be involved in the uptake of peptides by roots.

Acknowledgements

We thank Dr. Vicente Rubio for providing the pVR-YFPCt vector. We thank Pilar Fontanet and Montse Amenós for their help and valuable inputs. This work was funded by the Spanish Ministry of Science and Innovation (BIO2007-64791 and BIO2007-20I037), the Consolider-Ingenio program (CSD2007-00036), and the Xarxa de Referencia en Biotecnologia of the Government of Catalonia. H.T. was the recipient of a predoctoral I3P (Consejo Superior de Investigaciones Científicas).

References

Almagro A, Lin SH, Tsay YF (2008) Characterization of the Arabidopsis nitrate transporter NRT1.6 reveals a role of nitrate in early embryo development. Plant Cell 20:3289-3299

An YQ, Lin L (2011) Transcriptional regulatory programs underlying barley germination and regulatory functions of Gibberellin and abscisic acid. BMC Plant Biol 11:105

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A, Struhl K (1994) Current Protocols in Molecular Biology. John Wiley & Sons, New York

Bogs J, Bourbouloux A, Cagnac O, Wachter A, Rausch T, Delrot S (2003) Functional characterization and expression analysis of a glutathione transporter, BjGT1, from Brassica juncea: evidence for regulation by heavy metal exposure. Plant Cell Environ 26:1703-1711

Cagnac O, Bourbouloux A, Chakrabarty D, Zhang MY, Delrot S (2004) AtOPT6 transports glutathione derivatives and is induced by primisulfuron. Plant Physiol 135:1378-1387

Campalans A, Pagès M, Messeguer R (2001) Identification of differentially expressed genes by the cDNA-AFLP technique during dehydration of almond (*Prunus amygdalus*). Tree Physiol 21:633-643

Cao J, Huang J, Yang Y, Hu X (2011) Analyses of the oligopeptide transporter gene family in poplar and grape. BMC Genomics 12:465

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16:3285-3303

Chiang CS, Stacey G, Tsay YF (2004) Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2. J Biol Chem 279:30150-30157

Chiu CC, Lin CS, Hsia AP, Su RC, Lin HL, Tsay YF (2004) Mutation of a nitrate transporter, AtNRT1:4, results in a reduced petiole nitrate content and altered leaf development. Plant Cell Physiol 45:1139-1148

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743

Daniel H, Spanier B, Kottra G, Weitz D (2006) From bacteria to man: archaic proton-dependent peptide transporters at work. Physiology 21:93-102

Dietrich D, Hammes U, Thor K, Suter-Grotemeyer M, Fluckiger R, Slusarenko AJ, Ward JM, Rentsch D (2004) AtPTR1, a plasma membrane peptide transporter expressed during seed germination and in vascular tissue of Arabidopsis. Plant J 40:488-499

Dunkley TP, Hester S, Shadforth IP, Runions J, Weimar T, Hanton SL, Griffin JL, Bessant C, Brandizzi F, Hawes C, Watson RB, Dupree P, Lilley KS (2006) Mapping the Arabidopsis organelle proteome. Proc Natl Acad Sci USA 103:6518-6523

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E, Schmidt UG (2006) Identification of a vacuolar sucrose transporter in barley and Arabidopsis mesophyll cells by a tonoplast

proteomic approach. Plant Physiol 141:196-207
Fontanet P, Vicient CM (2008) Maize embryogenesis. Methods Mol Biol 427:17-29
Frommer WB, Kwart M, Hirner B, Fischer WN, Hummel S, Ninnemann O (1994) Transporters for nitrogenous compounds in plants. Plant Mol Biol 26:1651-1670
Gomolplitinant KM, Saier MH Jr (2011) Evolution of the oligopeptide transporter family. J Membr Biol 240:89-110
Hammes UZ, Meier S, Dietrich D, Ward JM, Rentsch D (2010) Functional properties of the Arabidopsis peptide transporters AtPTR1 and AtPTR5. J Biol Chem 285:39710-39717
Harvey BM, Oaks A (1974) The Hydrolysis of Endosperm Protein in *Zea mays*. Plant Physiol 53:453-457
Hemmerlin A, Bach TJ (2000) Farnesol-induced cell death and stimulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in tobacco cv Bright Yellow-2 cells. Plant Physiol 123:1257–1268
Herman EM, Larkins BA (1999) Protein storage bodies and vacuoles. Plant Cell 11:601-614
Higgins CF, Payne JW (1981) The Peptide pools of germinating barley grains: relation to hydrolysis and transport of storage proteins. Plant Physiol 67:785-792
Huang NC, Liu KH, Lo HJ, Tsay YF (1999) Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell 11:1381-1392
Jeong J, Suh S, Guan C, Tsay YF, Moran N, Oh CJ, An CS, Demchenko KN, Pawlowski K, Lee Y (2004) A nodule-specific dicarboxylate transporter from alder is a member of the peptide transporter family. Plant Physiol 134: 969-978
Karim S, Lundh D, Holmstrom KO, Mandal A, Pirhonen M (2005) Structural and functional characterization of AtPTR3, a stress-induced peptide transporter of Arabidopsis. J Mol Model 11:226-236
Karim S, Holmstrom KO, Mandal A, Dahl P, Hohmann S, Brader G, Palva ET, Pirhonen M (2007) AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in Arabidopsis. Planta 225:1431-1445
Kasaras A, Melzer M, Kunze R (2012) Arabidopsis senescence-associated protein DMP1 is involved in membrane remodeling of the ER and tonoplast. BMC Plant Biol 12:54
Koh S, Wiles AM, Sharp JS, Naider FR, Becker JM, Stacey G (2002) An oligopeptide transporter gene family in Arabidopsis. Plant Physiol 128: 21-29
Komarova NY, Thor K, Gubler A, Meier S, Dietrich D, Weichert A, Suter Grotemeyer M, Tegeder M, Rentsch D (2008) AtPTR1 and AtPTR5 transport dipeptides in planta. Plant Physiol 148:856-869
Komarova NY, Meier S, Suter Grotemeyer M, Rentsch D (2012) Determinants for Arabidopsis peptide transporter targeting to the tonoplast or plasma membrane. Traffic, in press.
Kost, B., Spielhofer, P., and Chua, N.-H. (1998). A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the. References actin cytoskeleton in growing pollen tubes. Plant J. 16, 393–401.
Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K, Zazimalova E, Benkova E, Nacry P, Gojon A (2010) Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Dev Cell 18:927-937
Kuang A, Xiao Y, Musgrave ME (1996) Cytochemical localization of reserves during seed development in Arabidopsis thaliana under spaceflight conditions. Ann Bot 78:343-351
Li JY, Fu YL, Pike SM, Bao J, Tian W, Zhang Y, Chen CZ, Zhang Y, Li HM, Huang J, Li LG, Schroeder JI, Gassmann W, Gong JM (2010) The Arabidopsis nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. Plant Cell 22:1633-1646
Lin SH, Kuo HF, Canivenc G, Lin CS, Lepetit M, Hsu PK, Tillard P, Lin HL, Wang YY, Tsai CB, Gojon A, Tsay YF (2008) Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. Plant Cell 20:2514-2528
Lubkowitz M (2011) The Oligopeptide Transporters: A Small Gene Family with a Diverse Group of Substrates and Functions? Mol Plant 4:407-441
Miquel M, López-Ribera I, Ràmia M, Casillas S, Barbadilla A, Vicient CM (2011) MASISH: a database for gene expression in maize seeds. Bioinformatics 27:435-436
Miranda M, Borisjuk L, Tewes A, Dietrich D, Rentsch D, Weber H, Wobus U (2003) Peptide and amino acid transporters are differentially regulated during seed development and germination in faba bean. Plant Physiol 132:1950–1960
Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31-48
Ouyanga J, Caia Z, Xiaa K, Wangc Y, Duana J, Zhanga M (2010) Identification and analysis of eight peptide transporter homologs in rice. Plant Sci 179:374-382
Paungfoo-Lonhienne C, Lonhienne TGA, Rentsch D, Robinson N, Christie M, Webb RI, Gamage HK, Carroll BJ, Schenk PM, Schmidt S (2008) Plants can use protein as a nitrogen source without assistance from other organisms. Proc Natl Acad Sci USA 11:4524–4529
Paungfoo-Lonhienne C, Schenk PM, Lonhienne TG, Brackin R, Meier S, Rentsch D, Schmidt S (2009) Nitrogen affects cluster root formation and expression of putative peptide transporters. J Exp Bot 60:2665-2676
Pike S, Patel A, Stacey G, Gassmann W (2009) Arabidopsis OPT6 is an oligopeptide transporter with exceptionally broad substrate specificity. Plant Cell Physiol 50:1923-1932
Ramos MS, Abele R, Nagy R, Grotemeyer MS, Tampé R, Rentsch D, Martinoia E (2011) Characterization of a transport activity for long-chain peptides in barley mesophyll vacuoles J Exp Bot 62:2403-2410

Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S, Frommer WB (1995) NTR1 encodes a high affinity oligopeptide transporter in Arabidopsis. FEBS Lett 370:264-268
Rentsch D, Schmidt S, Tegeder M (2007) Transporters for uptake and allocation of organic nitrogen compounds in plants. FEBS Lett 581:2281-2289
Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW (2005) An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. Plant J 41:767-778
Saito C, Ueda T, Abe H, Wada Y, Kuroiwa T, Hisada A, Furuya M, Nakano A (2002) A complex and mobile structure forms a distinct subregion within the continuous vacuolar membrane in young cotyledons of Arabidopsis. Plant J 2002, 29:245–255.
Saito C, Uemura T, Awai C, Tominaga M, Ebine K, Ito J, Ueda T, Abe H, Morita MT, Tasaka M, et al (2011) The occurrence of bulbs, a complex configuration of the vacuolar membrane, is affected by mutations of vacuolar SNARE and phospholipase in Arabidopsis. Plant J 2011, 68:64–73
Salmenkallio M, Sopanen T (1989) Amino acid and peptide uptake in the scutella of germinating grains of barley, wheat, rice, and maize. Plant Physiol 89:1285–1291
Sánchez-Fernández R, Davies TG, Coleman JO, Rea PA (2001) The Arabidopsis thaliana ABC protein superfamily, a complete inventory. J Biol Chem 276:30231–30244
Schimel JP, Chapin III FS (1996) Tundra plant uptake of amino acid and NH4+ nitrogen in situ: plants compete well for amino acid N. Ecology 77:2142–2147
Schulze W, Frommer WB, Ward JM (1999) Transporters for ammonium, amino acids and peptides are expressed in pitchers of the carnivorous plant Nepenthes. Plant J 17:637–646
Shimaoka T, Ohnishi M, Sazuka T, Mitsuhashi N, Hara-Nishimura I, Shimazaki K, Maeshima M, Yokota A, Tomizawa K, Mimura T (2004) Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of *Arabidopsis thaliana*. Plant Cell Physiol 45:672-683
Song W, Steiner HY, Zhang L, Naider F, Stacey G, Becker JM (1996) Cloning of a second Arabidopsis peptide transport gene.Plant Physiol 110:171-178
Song W, Koh S, Czako M, Marton L, Drenkard E, Becker JM, Stacey G (1997) Anti-sense expression of the peptide transport gene AtPTR2-B delays flowering and arrests seed development in transgenic Arabidopsis plants. Plant Physiol 114:927–935
Sopanen T, Burston D, Matthews DM (1977) Uptake of small peptides by the scutellum of germinating barley. FEBS Lett 79:4-7
Stacey G, Koh S, Granger C, Becker JM (2002) Peptide transport in plants. Trends Plant Sci 7:257-263
Stacey MG, Patel A, McClain WE, Mathieu M, Remley M, Rogers EE, Gassmann W, Blevins DG, Stacey G (2008) The Arabidopsis AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. Plant Physiol 146:589-601
Tnani H, Lopez I, Vicient CM (2011) Control of the scutellar epithelial cell elongation during germination in maize (*Zea mays* L.). Seed Sci Technol 39:253-258
Tnani H, García-Muniz N, Vicient CM, López-Ribera I (2012) Expression profile of maize (*Zea mays*) scutellar epithelium during imbibition. J. Plant Phys, in press.
Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK (2007) Nitrate transporters and peptide transporters. FEBS Lett 581:2290–2300
Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72:705-713
Van de Peer Y, De Wachter R (1997) Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. Comput Appl Biosci 13:227-230
Vasconcelos MW, Li GW, Lubkowitz MA, Grusak MA (2008) Characterization of the PT clade of oligopeptide transporters in rice. Plant Genome 1:77–88
Waterworth WM, West CE, Bray CM (2000) The barley scutellar peptide transporter: biochemical characterization and localization to the plasma membrane. J Exp Bot 51:1201–1209
Waterworth WM, West CE, Bray CM (2001) The physiology and molecular biology of peptide transport in seeds. Seed Sciece Res 11:275-284
Waterworth WM, Ashley MK, West CE, Bray CM (2003) Peptide transport in the developing barley grain. In: Nicolas G, Bradford KJ, Come D, Pritchard HW (eds) The biology of seeds, CABI International, Wallingford, pp 85–92
Waterworth WM, Ashley MK, West CE, Sunderland PA, Bray CM (2005) A role for phosphorylation in the regulation of the barley scutellar peptide transporter HvPTR1 by amino acids. J Exp Bot 56:1545-1552
Weichert A, Brinkmann C, Komarova NY, Dietrich D, Thor K, Meier S, Suter Grotemeyer M, Rentsch D (2012) AtPTR4 and AtPTR6 are differentially expressed, tonoplast-localized members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family. Planta 235: 311-323
West CE, Waterworth WM, Stephens SM, Smith CP, Bray CM (1998) Cloning and functional characterization of a peptide transporter expressed in the scutellum of barley grain during the early stages of germination. Plant J 15:221–229
White CN, Proebsting WM, Hedden P, Rivin CJ: Gibberellins and Seed Development in Maize. I. Evidence That Gibberellin/Abscisic Acid Balance Governs Germination versus Maturation Pathways. Plant Physiol 2000, 122(4):1081-1088

Zhang MY, Bourbouloux A, Cagnac O, Srikanth CV, Rentsch D, Bachhawat AK, Delrot S: A novel family of transporters mediating the transport of glutathione derivatives in plants. Plant Physiol 2004, 134: 482-491

Zhao X, Huang J, Yu H, Wang L, Xie W (2010) Genomic survey, characterization and expression profile analysis of the peptide transporter family in rice (*Oryza sativa* L.). BMC Plant Biol 10:92

Zhou JJ, Theodoulou FL, Muldin I, Ingemarsson B, Miller AJ (1998) Cloning and functional characterization of a *Brassica napus* transporter that is able to transport nitrate and histidine. J Biol Chem 273:12017-12023

Figures

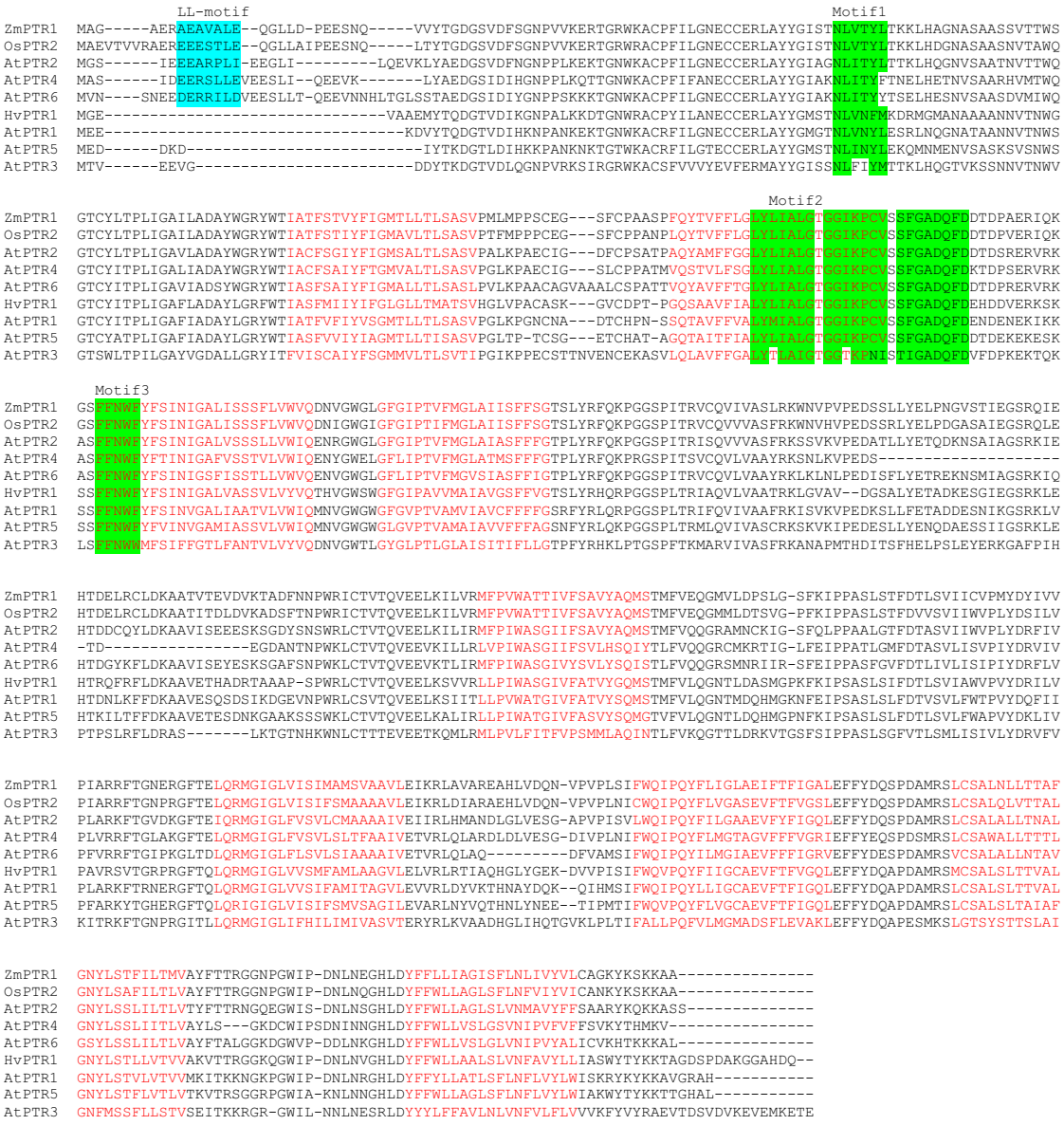


Fig. 1 Multiple sequence alignment of amino acid sequences of ZmPTR1 with other members of the PTR/NRT1 protein family. Sequences were aligned using ClustalW, and gaps were introduced to maximize alignment. The conserved amino acid motif typical of plant peptide transporter proteins are shaded in green (Zhao *et al*., 2010) and the predicted transmembrane domains are indicated with red letters. The alignment includes characterized protein sequences of members of the PTR/NRT1 protein family: rice OsPTR2 (AK068351; Ouyang et al. 2010), barley HvPTR1 (AAC32034; West et al. 1998), and *Arabidopsis thaliana* AtPTR1 (At3g54140; Dietrich et al. 2004), AtPTR2 (At2g02040; Frommer et al. 1994; Rentsch et al. 1995; Song et al. 1996), AtPTR3 (At5g46050; Karim et al. 2007), AtPTR4 (At2g02020; Weichert et al., 2012), AtPTR5 (At5g01180; Komarova et al. 2008) and AtPTR6 (At1g62200; Weichert et al., 2012). In blue, the possible dileucine tonoplast localizing motif (Komarova et al., 2012).

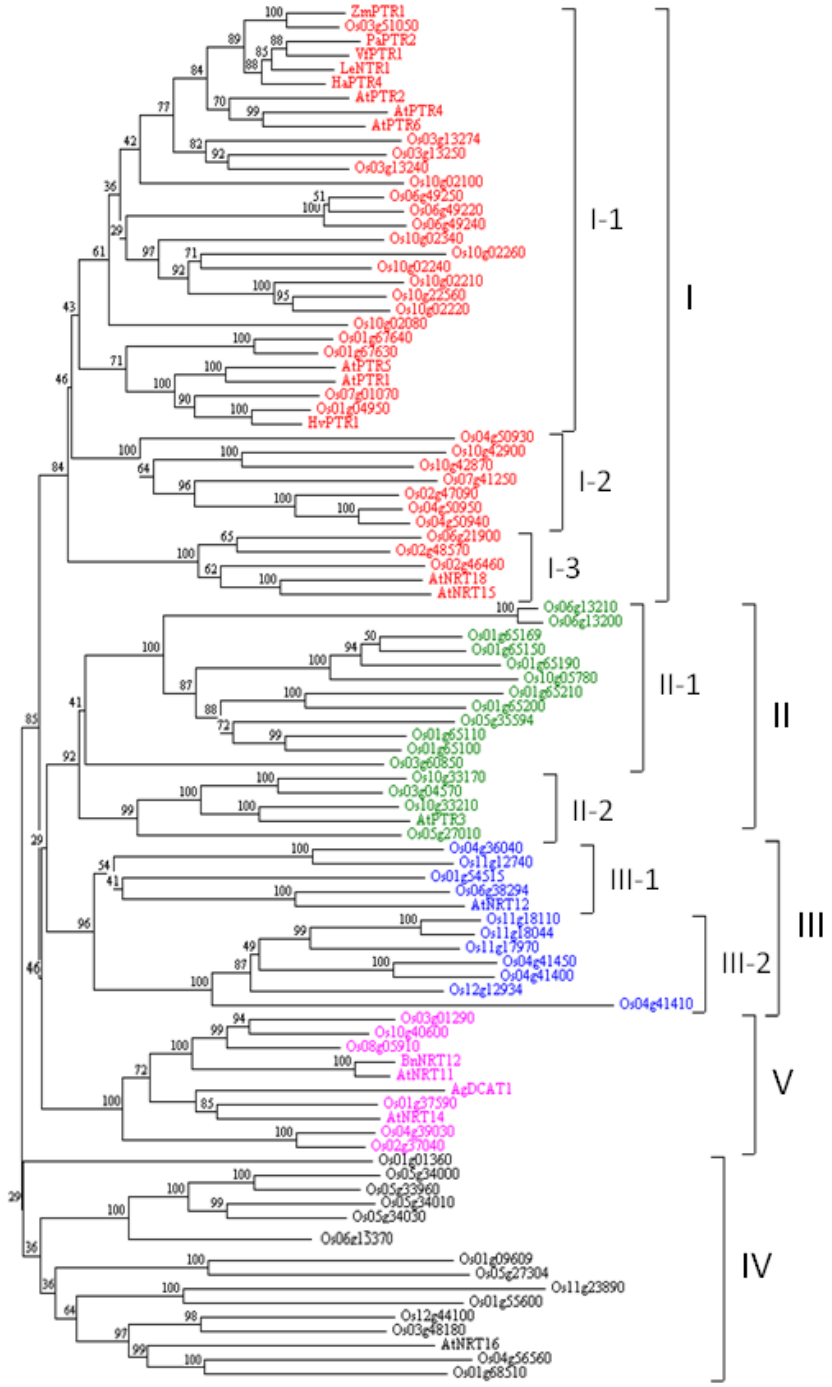


Fig. 2 Phylogenetic relationship of ZmPTR1 with members of the PTR/NRT1 protein family. The analysis was performed using the aligned protein sequences of members of the PTR/NRT1 protein family in rice (Zhao et al. 2010) and characterized members of the family from *Arabidopsis thaliana*: AtPTR1 (At3g54140; Dietrich et al. 2004), AtPTR2 (At2g02040; Frommer et al. 1994; Rentsch et al. 1995; Song et al. 1996), AtPTR3 (At5g46050; Karim et al. 2007), AtPTR4 (At2g02020), AtPTR5 (At5g01180; Komarova et al. 2008), AtPTR6 (At1g62200), AtNRT1.1 (At1g12110; Tsay et al. 1993), AtNRT1.2 (At1g69850; Huang et al. 1999), AtNRT1.4 (At2g26690; Chiu et al. 2004), AtNRT1.5 (At1g32450; Lin et al. 2008), AtNRT1.6 (At1g27080; Almagro et al. 2008) and AtNRT1.8 (At4g21680; Li et al. 2010). Other sequences used were AgDCAT1, from *Alnus glutinosa* (CAD32549; Jeong et al. 2004), BnNRT1.2 from *Brassica napus* (U17987; Zhou et al. 1998), HaPTR4 from *Hakea actites* (ABR32183; Paungfoo-Lonhienne et al. 2009), HvPTR1 from *Hordeum vulgare* (AAC32034; West et al. 1998), PaPTR2 from *Prunus amygdalus* (AF213936; Campalans et al. 2001), LeNTR1 from *Solanum lycopersicum* (AF016713), and VfPTR1 from *Vicia faba* (AY289622; Miranda et al. 2003). The protein sequences were aligned using ClustalW followed by neighbor-Joining analysis in TREECON to generate the tree (Van de Peer and Wachter 1997). Clades were named according to Zhao et al. (2010). Bootstrap values from 1,000 replicates are indicated at each node.

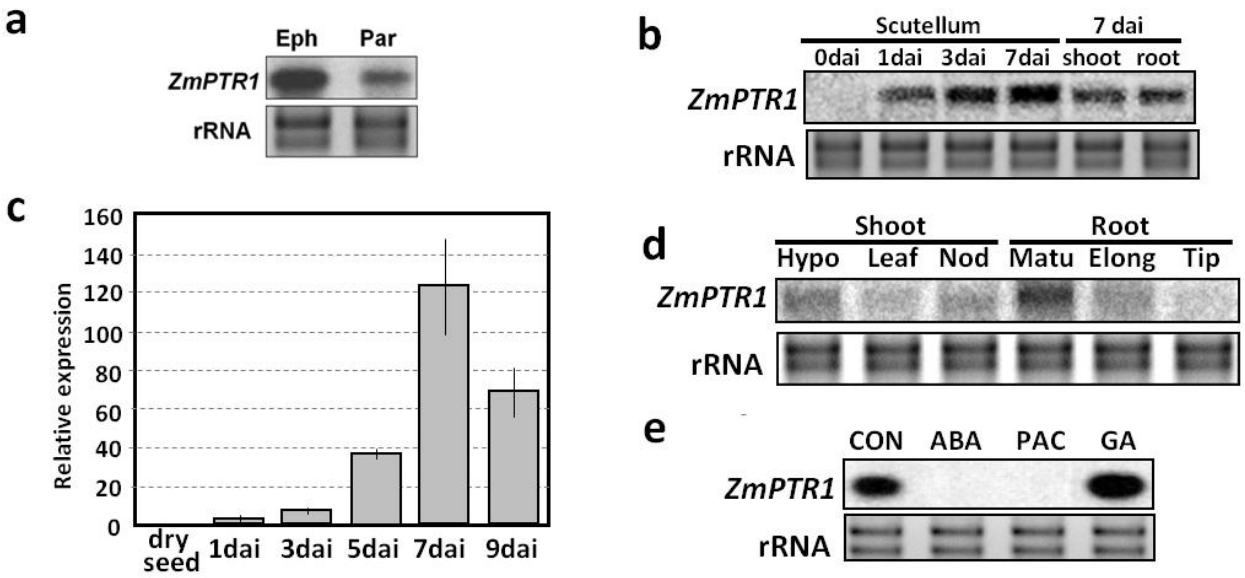


Fig. 3 Expression patterns of the *ZmPTR1* gene in maize seeds and seedlings during imbibition. *ZmPTR1* mRNA accumulation was analyzed by northern-blot hybridization, using the full-length cDNA as probe (**a**, **b**, **d**, **e**), and by quantitative real-time RT-PCR (**c**). **a** Total RNAs were extracted from the epithelium (Eph) and the parenchyma (Par) of the scutellum 3 days after imbibition. **b** Total RNAs were extracted from the scutellum at different days after imbibition (dai) and from shoots and roots 7 dai. **c** Quantitative real-time RT-PCR analysis in dissected scutella at different times after imbibition. Transcript levels relative to actin are presented for each sample. Three fully independent biological repeats and three technical repeats were included, and the mean values and standard errors are shown (error bars). **d** Total RNAs were extracted from different parts of the shoot and root of 7 dai seedlings: hypocotyl (Hypo), leaf (Leaf), node (Nod), mature part of the root (Matu), elongation part of the root (Elong), root-tip (Tip). **e** Total RNAs were extracted from seedlings imbibed for 7 days in the presence of water (CON), 50µM abscisic acid (ABA), 100µM gibberellin A3 (GA) and 100µM paclobutrazol (PAC).

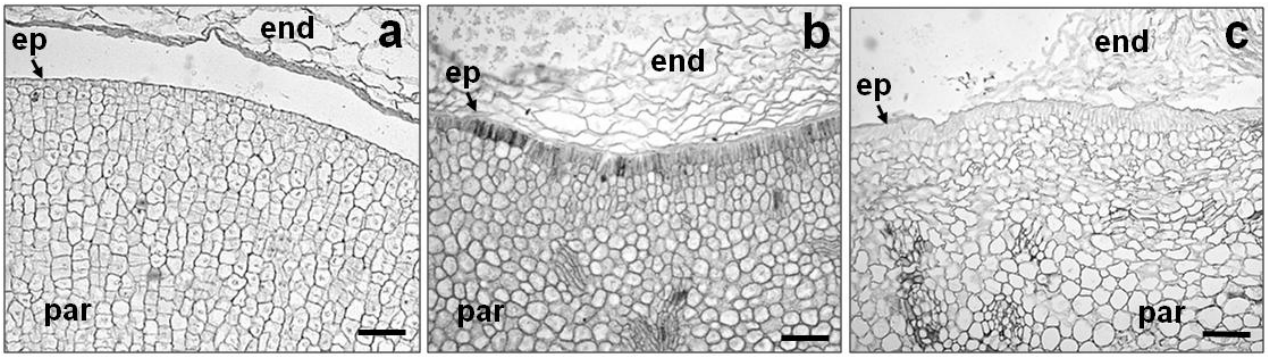


Fig. 4 *ZmPTR1* in situ hybridization in maize scutellum of dry seed (**a**) or seeds 3 days after imbibition (**b**, **c)**. Images of in situ hybridization on cross sections using antisense-RNA (**a**, **b**) or sense-RNA (**c**) probes. The hybridization signal appears dark inside the cells. Abbreviations: scutellar epithelium (**ep**), scutellar parenchyma (**par**) and endosperm (**end**). Scale bars 100 μm.

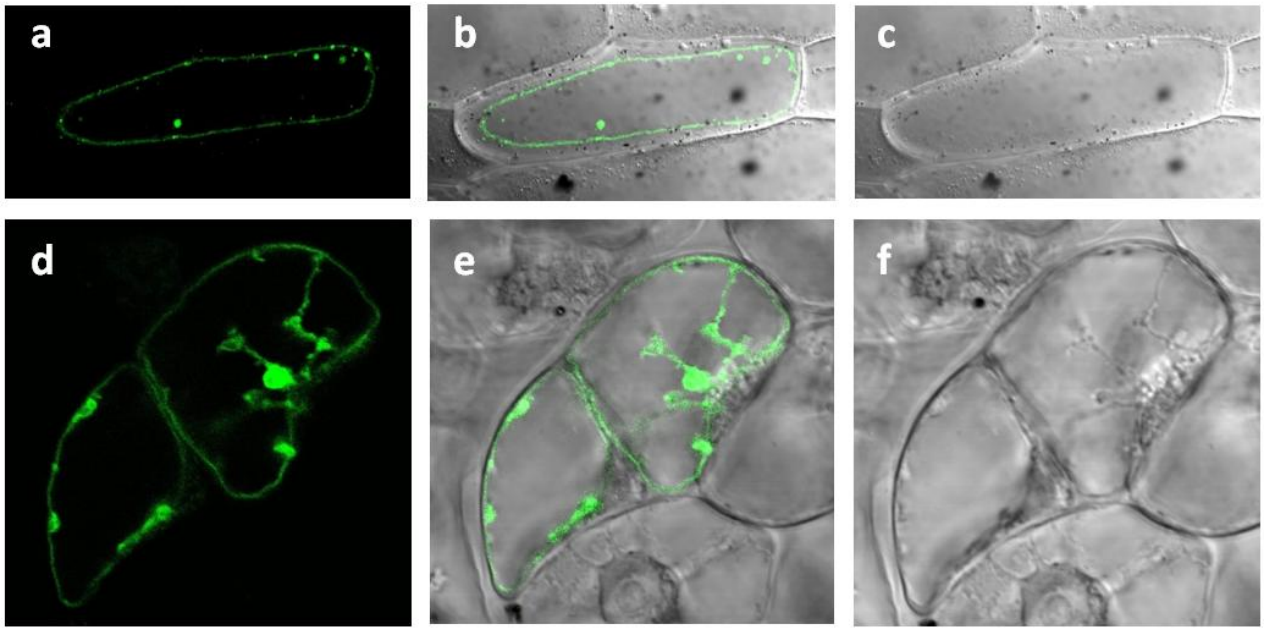


Fig. 5 Subcellular Localization of ZmPTR1/YFP fusion protein. Confocal laser-scanning microscope images (**a**, **d**), corresponding bright-field images (**c, f**) and merged images (**b**, **e**) in root cells of Arabidopsis plants stably expressing fusion protein of ZmPTR1/YFP (**a**, **b**, **c**) or in tobacco BY-2 cells transiently transformed expressing the ZmPTR1/YFP fusion protein (**d**, **e**, **f**).

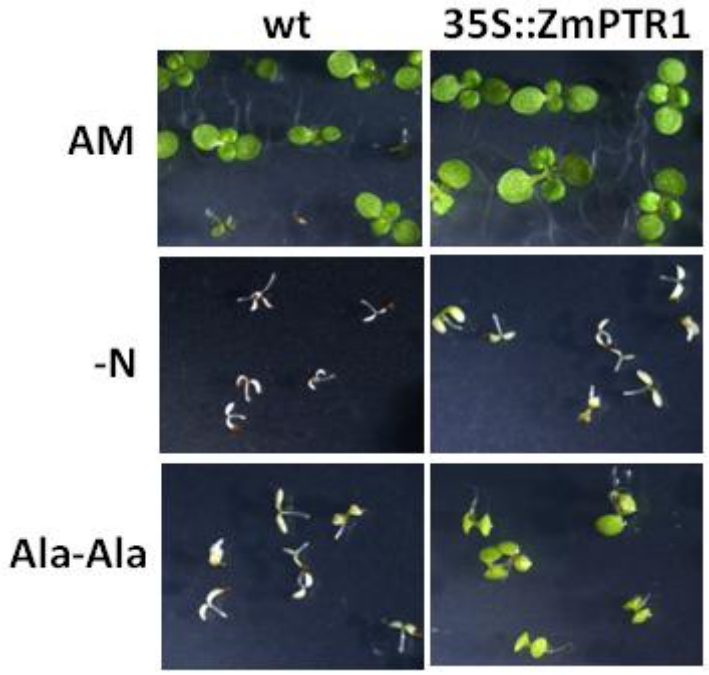


Fig. 6 Growth of Arabidopsis wild-type (wt) and 35S::ZmPTR1 seedlings on medium containing Ala-Ala as the sole N source. Col-O (wt) and transgenic seeds containing the 35S::ZmPTR1 construct were germinated for one week on different growth media: AM medium; -N, one-half strength MS medium with NH_4NO_3 and KNO_3 replaced by KCl; Ala-Ala, –N medium containing 20 mM of the di-peptide Ala-Ala. Data shown are representative for the wild type and three independent overexpressing *Arabidopsis* lines.

3.4 Isolation of proteins associated with oil bodies of maize scutellum

Seed germination is a complex process that requires cell division, expansion and differentiation. It involves the activation of many metabolic pathways and signal transduction processes, which require a great quantity of energy and stored materials which are provided by seed reserves (oil, storage proteins and starch) and the synthesis and/or activation of many proteins. Plant seeds store triacylglycerols (TAGs) into oil bodies (OBs), specialized organelles which serve as an energy reserve during germination and post-germinative growth. The oil bodies (OBs) are composed of a neutral lipid core surrounded by a phospholipid monolayer embedded with proteins that confer stability to the oleosome and may also be involved in the interactions with other organelles. In plants, the most abundant described OB-associated proteins are oleosins. The oleosins possess a long central hydrophobic domain anchoring the protein into the lipid core, and two hydrophilic N- and C-terminal domains which are localized at the surface. Oleosins are believed to form a steric barrier to prevent coalescence of OBs. Other described OB-associated proteins in plants are caleosins and steroleosins. Storage oil breakdown following seed imbibition provides energy and carbon skeletons that support seedling growth immediately following germination. This metabolic process is initiated by lipases, which catalyze the hydrolysis of triacylglycerols (TAGs) to release free fatty acids and glycerol. Different proteomic studies in plant seeds have identified OB-associated proteins. In addition to the already known proteins, some less abundant OB-associated proteins have been identified including enzymes, proteins involved in membrane traffic and proteins of unknown function.

3.4.1 Protein composition analysis of oil bodies from maize embryos during germination

In the first paper we examined the protein composition of the OBs during germination, the scutellum was dissected from maize seeds 2 days after imbibition and the proteins extracted from the OB fraction. High resolution 2-DE analysis of OB-proteins revealed several protein spots. 17 spots were excised from gel, digested with trypsin and analyzed by LC–MS/MS, allowed us to identify 11 of the 17 spots. Identified proteins included an oleosin, a caleosin and a steroleosin in their predicted positions. In addition to the previously characterized OB-associated proteins we identified other proteins of diverse function: an embryonic protein DC-8, a globulin-2 (both are embryo-specific proteins of unknown function), four proteins with enzymatic activities (protein disulfide isomerase, xylose isomerase, strictosidine synthase and ATP synthase beta chain), a protein similar to karyopherin-beta-3 and a stress-induced membrane pore protein (the last two identified proteins involved in membrane transport).

Tnani, H. *et al.* (2011). Protein composition analysis of oil bodies from maize embryos during germination. *Journal of plant physiology* **168**: 510-513.

Journal of Plant Physiology 168 (2011) 510–513

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Short communication

Protein composition analysis of oil bodies from maize embryos during germination

Hedia Tnani[a], Ignacio López[a], Thierry Jouenne[b,c], Carlos M. Vicient[a,*]

[a] Department of Molecular Genetics, Centre for Research in Agrigenomics CSIC-IRTA-UAB, Jordi Girona 18-26, Barcelona 08034, Spain
[b] European Institute for Peptide Research, University of Rouen, 76821 Mont-Saint-Aignan, France
[c] UMR 6270 CNRS, University of Rouen, 76821 Mont-Saint-Aignan, France

ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 4 August 2010
Accepted 9 August 2010

Keywords:
Oil body
Zea mays
Proteome

ABSTRACT

Seed oil bodies (OBs) are intracellular particles that store lipids. In maize embryos, the oil bodies are accumulated mainly in the scutellum. Oil bodies were purified from the scutellum of germinating maize seeds and the associated proteins were extracted and subjected to 2-DE analysis followed by LC–MS/MS for protein identification. In addition to the previously known oil body proteins oleosin, caleosin and steroleosin, new proteins were identified.

Introduction

Oil bodies (OBs) are small spherical organelles present in a wide range of species. OBs are composed of a neutral lipid core surrounded by a phospholipid monolayer embedded with proteins (Purkrtova et al., 2008). OB-associated proteins confer stability to the organelle and facilitate lipolysis during germination. The set of proteins associated with a particular OB depends on the species, the cell type and the developmental stage (Murphy, 2005). In plants, the most abundant OB-associated proteins are oleosins (Capuano et al., 2007), caleosins (Purkrtova et al., 2007) and steroleosins (Lin et al., 2002).

OBs are especially abundant in the embryos of oleaginous seeds, which provide energy to the vigorous seedling growth during early steps of germination. In mature maize embryos, cells are full of OBs, especially in the scutellum (cotyledon). Lipid hydrolysis involves the action of different enzymes as lipases, phospholipases, lipoxygenases and enzymes involved in the conversion of fatty acids to succinate in the glyoxysome (Bhatla et al., 2009; Yang et al., 2009). Some of these enzymes are associated with OBs during germination. It has been proposed that lipases attach the OBs through their interaction with oleosins or other OB-associated proteins. Some studies have shown the possibility of a direct interaction between OBs and glyoxisomes and vacuoles during germination (Graham, 2008). Caleosins may be involved in the OB-vacuole interaction (Poxleitner et al., 2006).

Proteomic studies in *Brassicaceae* seeds have identified, in addition to the known OB-associated proteins, some less abundant OB-associated proteins, including enzymes, proteins involved in membrane traffic and proteins of unknown function (Jolivet et al., 2004, 2009; Katavic et al., 2006). Little information is available, however, about the protein composition of OBs in other plant species. In the present work, we aimed to identify proteins associated with OBs extracted from the scutellum of maize germinating seeds.

Materials and methods

Isolation of OB associated proteins from germinating seeds

Maize seeds (*Zea mays* L. W64A) were surface sterilized and placed on wet vermiculite at 28 °C. After two days, the scutellum was manually dissected and frozen. OB-associated proteins were purified by floating extraction (Katavic et al., 2006).

1-DE SDS-PAGE electrophoresis and Western blot

1-DE SDS-PAGE electrophoresis was performed using 15% SDS polyacrylamide gels and a molecular weight marker (Marker Wide Range, Sigma M4038). Gels were stained with Coomassie blue and photographed with a LAS-3000 Luminescent Image Analyzer (Fujifilm). Western blot was carried out as previously described (Niogret et al., 1996). The antibody against 2,3-bisphosphoglycerate-independent phosphoglycerate mutase was kindly provided by Dr. D. Ludevid and the antibody against

Abbreviation: OB, oil body.

* Corresponding author. Tel.: +34 934006100; fax: +34 932045904.
E-mail address: cvsgmp@cid.csic.es (C.M. Vicient).

0176-1617/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2010.08.020

Sar1 was purchased from Agrisera Antibodies (Sweden; Ref. AS08 326).

2-D electrophoresis

2-D electrophoresis was performed in the Proteomics Service of CRAG using 550 μg of proteins in 450 μl of urea re-hydration buffer (7 M urea, 2 M thiourea, 18 mM Tris–HCl pH 8.0, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer in same range as the IPG strip, and 0.002% bromophenol blue) containing 1.6% (v/v) DeStreak reagent (GE Healthcare). Samples were loaded onto pH 4–7, 24 cm immobilized pH gradient (IPG) strips (Immobiline DryStrips, GE Healthcare) for the first dimension. IEF was performed at 50 V for 10 h (re-hydration), 500 V in gradient for 1 h 30 min, 1000 V in gradient for 1 h 30 min, 2000 V in gradient for 1 h 30 min, 4000 V in gradient for 1 h 30 min, 8000 V in gradient for 2 h and 8000 V holding for 6 h, using EttanTM IPGphorTM Isoelectric Focusing System (Amersham, Biosciences). Prior to second dimension, IPG strips were equilibrated for 15 min with 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS, a trace of bromophenol blue and 10 mg/ml DTT, followed by a second equilibration step with the same buffer containing 25 mg/ml iodoacetamide instead of DTT, for an additional 15 min with gentle shaking. For the second dimension, the strips were loaded onto SDS-PAGE 12% polyacrylamide gels (26 cm × 20 cm × 0.1 cm) using Ettan DALTsix System (Amersham Biosciences) for 30 min at 2.5 W/gel, followed by 20 W/gel during 4 h. Proteins were visualized with Coomassie R-250 staining (Bio-Rad). 2-DE gels were scanned using an ImageScanner desktop instrument (Amersham, Biosciences) and images were acquired and analyzed using ImageMaster™ 2-D Platinum 5.0 Software (Amersham, Biosciences). Gels were made two times using two independently obtained samples with similar results.

Protein identification by LC–MS/MS

Selected spots were excised and trypsin digested overnight with a MultiPROBE II (PerkinElmer). Gel fragments were incubated twice for 15 min in a H_2O/CH_3CN solution. Peptide extracts were dried and dissolved in 3% CH_3CN and 0.1% HCOOH in water. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, MA, USA). For protein identification, MS/MS peak lists were extracted and compared with the protein database using MASCOT Daemon (version 2.1.3; Matrix Science, London, UK). The searches were performed with no fixed modification and with variable modifications for oxidation of methionine residues, and with a maximum of one missed cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 for MS/MS fragments. The LC–MS/MS data were converted into DTA-format files, which were further searched for proteins with MASCOT Daemon. Measured peptides were searched in the NCBInr-protein sequence database Viridiplantae.

Results and discussion

To examine the protein composition of the OBs during germination, the scutellum was dissected from maize seeds 2 days after imbibition and the proteins extracted from the OB fraction. Total and OB proteins extracted from an equal quantity of dissected scutella were analyzed in parallel using SDS-PAGE (Fig. 1). OB proteins showed a simple band pattern with only three prominent bands compared to the more than 20 bands visible in the total protein extract. The most prominent band in the OB extract with an apparent molecular weight of about 18 kDa

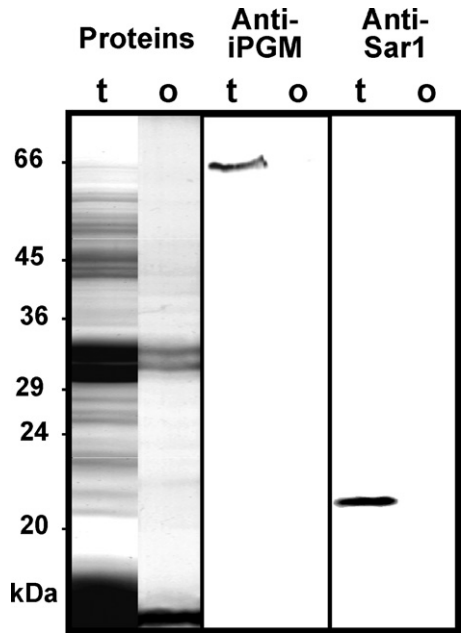


Fig. 1. Analyses of total proteins extracts from maize scutellum (t) and proteins from the oil body fraction (o). SDS-PAGE gel stained with Coomassie blue (proteins). The positions of the molecular mass markers are indicated. Immunodetection of the cytoplasmatic enzyme 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (anti-iPGM). Immunodetection of the endoplasmic reticulum marker protein Sar1 (anti-Sar1).

may correspond to the oleosins. The OB protein extract purity was confirmed with Western blotting using antibodies against 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGM), an enzyme located mainly in the cytoplasm (Graña et al., 1989), and against the small GTPase Sar1, mainly located in the endoplasmic reticulum (Pimpl et al., 2000). No hybridization was observed in the OB-protein fraction (Fig. 1).

High resolution 2-DE analysis of OB-proteins revealed several protein spots (Fig. 2). Although the spot pattern was not identical, similar complexity was obtained using *Brassica* embryo OB proteins

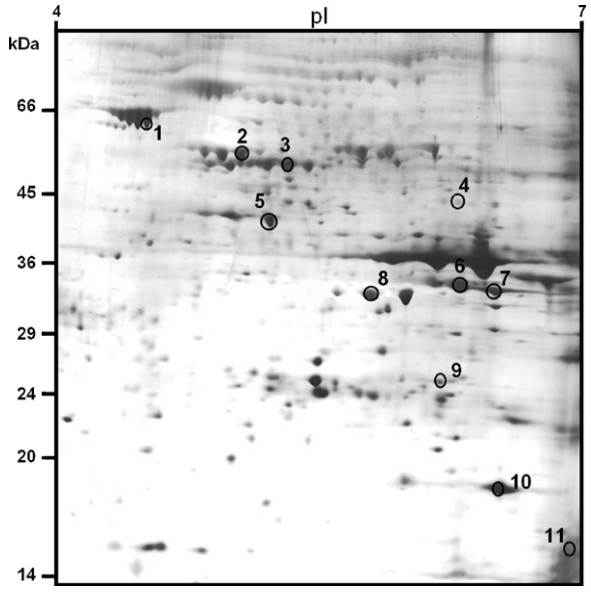


Fig. 2. Analysis of proteins isolated from maize oil bodies by 2-DE in combination with Coomassie R-250 staining. Proteins (550 μg) were analyzed using IPG strips with pH range from 4 to 7. pI and molecular mass (in kDa) are noted. Protein spots identified by LC–MS/MS are circled and numbered in accordance with Table 1.

Table 1
Protein assignments from LC–MS/MS analyses of 2-D gel spots from maize purified oil body proteins.

Sp[a]	Protein identification (source)	Acc. number	Mr[b]/Mr[c]	pI[b]/pI[c]	Peptides	Cov[d]
1	Protein disulfide isomerase (*Zea mays*)	NP_001105754	57.3/56.9	4.6/5.0	K.FIDASTIPR.V K.AAAAEPVKDEL.- R.EADGIVDYLKK.Q K.ETAGAATTTTQAPPASEK.A R.SDYDFGHTLHANHLPR.G	12
2	ATP synthase beta chain (*Zea mays*)	ACG28992	52.6/59.0	5.1/5.9	K.VVDLLAPYQR.G R.TIAMDGTEGLVR.G R.IINVIGEPIDEK.G R.VLNTGSPITVPVGR.A R.FTQANSEVSALLGR.I R.LVLEVAQHLGENMVR.T R.QISELGIYPAVDPLDSTSR.M R.EAPAFVEQATEQQILVTGIK.V R.IPSAVGYQPTLATDLGGLQER.I	27
3	Xylose isomerase (*Zea mays*)	ACG35698	50.1/54.1	5.3/5.3	K.VYAYAAAQVK.K R.GTGADPFGAPTK.V K.LIEDGSLDALVR.K K.NGGLAPGGFNFDAK.L R.YQSFDSEIGALIEAGK.G K.TLEETNANLDEIVELAK.Q	16
4	Globulin 2 (*Zea mays*)	1802402A	44.5/49.9	6.2/6.2	R.LLDMDVGLANIAR.G K.LLAFGADEEQQVDR.V R.FTHELLEDAVGNYR.V K.GEITTASEEQIRELSR.S R.AFLQPSHYDADEVMFVK.E R.AFLQPSHYDADEVMFVKEGEGVIVLLR.G	18
5	Strictosidine synthase 1 precursor (*Zea mays*)	ACG46069	40.9/39.5	5.2/5.2	R.SGETSVVLDR.L R.LGFANGVALPR.D K.GAMVAQVSEDGTILR.V R.VGDEGVTLLASEVEGSPIR.F	17
6	Similar to karyopherin-beta-3 (*Zea mays*)	ACN36862	34.2/38.2	6.2/4.8	R.QAAVYGIGICAECGGSAFRPHTGEALSR.L	9
7	Steroleosin (*Zea mays*)	ACG24673	33.3/38.6	6.4/6.0	R.ASLALVAR.R R.HVADRALELGAR.D R.DAQIGLFPVEYAK.N	11
8	Embryonic protein DC-8 (*Zea mays*)	ACG37499	32.9/33.6	5.7/6.2	R.VAGPDVAAVDGK.G K.AQETLSQTADAAAEK.A K.AGYAKETAGDAAAGASNK.A K.AQETLSQTADAAAEKAGTAK.D	18
9	Caleosin (*Zea mays*)	ACF82687	25.6/26.4	6.1/6.5	R.VYDGSLFEYVER.Q R.FMPVNFENLFSK.Y R.GDLEEHLPKPYLAR.A K.DSQTIVALQSPVTVMRPVR.G	20
10	Stress-inducible membrane pore protein (*Zea mays*)	ACG40477	18.2/17.8	6.5/6.1	R.SLDAVVKTAGK.E R.EAYVVTVEGLSGDSSGLDADGGKR.S	22
11	Oleosin 18 kDa (*Zea mays*)	ACG48654	15.2/17.6	6.8/9.7	R.TPDYVEEAR.R R.SGIYGGGAYGQQQGGGRPMGEQVK.G	21

[a] Spot according to Fig. 2.
[b] Experimental data.
[c] Theoretical data.
[d] Percentage of coverage.

(Katavic et al., 2006). These differences could not only be explained by the phylogenetic differences between species, but also because we were using dissected scutellum and not the whole embryo. 17 spots were excised from gel, digested with trypsin and analyzed by LC–MS/MS. These included spots located at the predicted positions for oleosin, caleosin and steroleosin, some of the most intense spots and some other randomly selected less intense spots. LC–MS/MS allowed us to identify 11 of the 17 spots (Table 1). Identified proteins included oleosin, caleosin and steroleosin in their predicted positions. It was surprising that oleosin, the most abundant OB associated protein, appeared in a spot that was not very intense (spot 11, Fig. 2). This is because the predicted pI for oleosin is 9.7 and the pH gradient (IPG) strips used here ranged from pH 4 to 7. Consequently, an important part of the oleosins was lost during first dimension IEF. However, better resolution was obtained for the observed protein spots.

In addition to the previously characterized OB-associated proteins, we identified other proteins of diverse function. Two of them (DC-8 and globulin 2) are embryo-specific proteins of unknown function, and four correspond to proteins with enzymatic activity (protein disulfide isomerase, xylose isomerase, strictosidine synthase 1 precursor and ATP synthase beta chain). Some of them are known to be located on organelles like the ER (protein disulfide isomerase). However, we showed that there was not strong contamination of ER proteins in our extract (Fig. 1). A possible explanation of such an apparent contradiction may be that OB-associated proteins interact specifically with some proteins in the reticulum and only these, and not all the ER proteins, were carried away in the process of OB purification. The *in vivo* interaction of OBs with glyoxysomes, protein storage vacuoles and small Golgi vesicles has been reported during pollen germination of *Magnolia x soulangeana* (Dinis and Coutinho, 2009). It has been suggested that protein storage vesicles from *Brassica* adhere to OBs (Katavic et al., 2006). A similar scenario could be at work for the ATP synthase beta chain, usually located in mitochondria and the strictosidine synthase 1, identified in the *Arabidopsis* plasma membrane proteome (Marmagne et al., 2007). The OB-associated proteins identified here may reflect these organelle interactions.

Two of the identified proteins are involved in membrane transport (Karyopherin-beta (Kap), and a stress-induced membrane pore protein). Kap proteins transport molecules through the pores of the nuclear envelope (Mosammaparast and Pemberton, 2004). Kap3 have been previously shown to be associated with lipid droplets in animals (Cermelli et al., 2006). Human kap3 interacts with a yeast apolipoprotein A-I (apo A-I), a secretion protein with a primary function in cholesterol transport (Chung et al., 2008). Interestingly, steroleosins contain a sterol-binding domain and free sterols constitute about 0.5% of the total lipids in the OBs of maize scutellum (Dyas and Goad, 1994). This suggests that sterol transport from OB to the nucleus is part of the normal hydrolysis of the OBs. A possible role of lipid droplets in inter-membrane lipid traffic has been proposed (Zehmer et al., 2009). Stress-induced membrane pore proteins encode a transmembrane protein whose expression is induced not only in the seeds, especially in the aleurone layer, but also in the embryo (NCBI Unigene Zm.22773). The presence of membrane traffic proteins in OBs has previously been described in *Arabidopsis* and *Brassica* (Jolivet et al., 2004; Katavic et al., 2006) and in mammalian lipid droplets (Than et al., 2003; Liu et al., 2004).

In conclusion, we identified a series of proteins associated with OBs. In addition to the characterized proteins oleosin, caleosin and steroleosin, we identified other proteins that have not been previously described and may represent new aspects of the biological functions of the OBs during germination.

Acknowledgements

We thank Sami Irar (proteomics service of CRAG) and Jose Luis La Paz for technical assistance with the 2D gels. This work was carried out with grants BIO2004-01577 and AGL2009-09151 from Plan Nacional de Investigación Científica y Técnica, by the Centre CONSOLIDER on Agrigenomics and by the Xarxa de Referencia en Biotecnologia of the Generalitat de Catalunya. H.T. was the recipient of a predoctoral I3P (CSIC). We thank Dolores Ludevid for providing us with the iPGM antibody.

References

Bhatla SC, Vandana S, Kaushik V. Plant Signal Behav 2009;4:176–82.
Capuano F, Beaudoin F, Napier JA, Shewry PR. Biotechnol Adv 2007;25:203–6.
Cermelli S, Guo Y, Gross SP, Welte MA. Curr Biol 2006;16:1783–95.
Chung KM, Cha SS, Jang SK. Mol Cells 2008;26:291–8.
Dinis AM, Coutinho AP. Protoplasma 2009;238:35–46.
Dyas L, Goad LJ. Plant Phys Biochem 1994;32:799–805.
Graham IA. Annu Rev Plant Biol 2008;59:115–42.
Graña X, Ureña J, Ludevid D, Carreras J, Climent F. Eur J Biochem 1989;8(186):149–53.
Jolivet P, Roux E, D'Andrea S, Davanture M, Negroni L, Zivy M, et al. Plant Physiol Biochem 2004;42:501–9.
Jolivet P, Boulard C, Bellamy A, Larré C, Barre M, Rogniaux H, et al. Proteomics 2009;9:3268–84.
Katavic V, Agrawal GK, Hajduch M, Harris SL, Thelen JJ. Proteomics 2006;6:4586–98.
Lin LJ, Tai SS, Peng CC, Tzen JT. Plant Physiol 2002;128:1200–11.
Liu P, Ying Y, Zhao Y, Mundy DI, Zhu M, Anderson RGW. J Biol Chem 2004;279:3787–92.
Marmagne A, Ferro M, Meinnel T, Bruley C, Kuhn L, Garin J, et al. Mol Cell Proteomics 2007;6:1980–96.
Mosammaparast N, Pemberton LF. Trends Cell Biol 2004;14:547–56.
Murphy DJ.Murphy DJ, editor. Plant lipids: biology, utilisation and manipulation. Florida: CRC-Press; 2005. p. 226–69.
Niogret MF, Culiáñez-Macià FA, Goday A, Mar Albà M, Pagès M. Plant J 1996;9:549–57.
Pimpl P, Movafeghi A, Coughlan S, Denecke J, Hillmer S, Robinson DG. Plant Cell 2000;12:2219–36.
Poxleitner M, Rogers SW, Lacey Samuels A, Browse J, Rogers JC. Plant J 2006;47:917–33.
Purkrtova Z, d'Andrea S, Jolivet P, Lipovova P, Kralova B, Kodicek M, et al. Arch Biochem Biophys 2007;464:335–43.
Purkrtova Z, Jolivet P, Miquel M, Chardot T. CR Biol 2008;331:746–54.
Than NG, Sumegi B, Bellyei S, Berki T, Szekeres G, Janaky T, et al. Eur J Biochem 2003;270:1176–88.
Yang MF, Liu YJ, Liu Y, Chen H, Chen F, Shen SH. J Proteome Res 2009;8:1441–51.
Zehmer JK, Huang Y, Peng G, Pu J, Anderson RGW, Liu P. Proteomics 2009;9:914–21.

3.4.2 Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of *Zea mays*

In the second paper, we performed a comparative proteomic analysis using 2-D electrophoresis of OB-associated proteins extracted from maize scutellum at two different developmental stages: dry seeds and 2 days after germination (dag). Protein identification by tandem mass spectrometry allowed us to identify proteins differentially accumulated in association to OBs in these two developmental stages. Statistical analysis showed that 26 protein spots were significantly different between the two samples. We found 19 spots with a higher accumulation in dry seeds and 7 in 2 dag seeds. We identified proteins that have not been previously described as OB-associated. The 19 spots exhibiting higher accumulation in dry seeds corresponded to 11 different proteins, and protein similarities were found for nine of them: five spots corresponded to two different oleosins, four spots corresponded to different forms of seed specific protein cupin, two are different protein disulfide isomerases, and one spot corresponded to triosephosphate isomerase, a nucleoside phosphate kinase, a class IV heat shock protein, the embryonic protein DC-8, a 60S acidic ribosomal protein P0 and the rubber elongation factor. The 7 spots exhibiting an accumulation in 2 dai scutellum corresponded to four polypeptides: two of them were different oleosins, two corresponded to the mitochondrial protein Tim17, and two spots corresponded to prohibitin-2. The last spot corresponded to a manganese superoxide dismutase. In conclusion, we identified a series of proteins associated with OBs. In addition to the characterized proteins oleosin, caleosin and steroleosin, we identified other proteins that have not been previously described and may represent new aspects of the biological functions of the OBs during germination.

Tnani, H. *et al.* (2012). Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of Zea mays. *Plant Science* **191-192**: 1-7.

Plant Science 191–192 (2012) 1–7

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of *Zea mays*

H. Tnani [a], I. López [a], T. Jouenne [b], C.M. Vicient [a,*]

[a] CRAG – Center for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Campus UAB, Bellaterra (Cerdanyola del Vallés), 08193 Barcelona, Spain
[b] Laboratoire Polymères, Biopolymères, Surfaces, UMR CNRS 6270, Plate-forme Protéomique PISSARO, Faculté des Sciences de Rouen, 76821 Mont-Saint-Aignan, France

ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form 22 February 2012
Accepted 23 February 2012
Available online 22 March 2012

Keywords:
Oil body
Scutellum
Maize
Imbibition
Proteomic

ABSTRACT

Oil bodies (OBs) were purified from the scutellum of mature maize embryos and from embryos 2 days after imbibition and their associated proteins were extracted and separated by 2-DE. Eighteen proteins were shown to be differentially accumulated, thirteen showed a higher accumulation in mature scutellum and five were highly accumulated in the germinating scutellum. Proteins were identified using LC–MS/MS. Besides previously known oil body protein oleosin, other proteins were identified in this study. Among accumulated proteins during imbibition are prohibitin 2, stress-inducible membrane pore protein Tim17 and manganese superoxide dismutase. Among the proteins whose amount decreases during imbibition are cupin 2, two different protein disulfide isomerases, a triosephosphate isomerase, a class IV heat shock protein, the embryonic protein DC-8, the 60S ribosomal protein P0, a nucleoside-diphosphate kinase, and a rubber elongation factor protein. Some of the identified proteins were previously located in organelles other than oil bodies, suggesting that OBs may interact with these organelles. We also suggest that OBs may act as transient storage depots for proteins that are temporally in excess.

1. Introduction

The storage lipids of seeds are predominantly accumulated in specialized cytoplasmic organelles termed lipid bodies, oil bodies or oleosomes [1]. The oil bodies (OBs) are composed of a neutral lipid core surrounded by a phospholipid monolayer embedded with proteins that confer stability to the oleosome and may also be involved in the interactions with other organelles [2,3]. The set of proteins associated with an OB depends on the species [4]. In plants, the most abundant described OB-associated proteins are oleosins [5]. The oleosins possess a long central hydrophobic domain anchoring the protein into the lipid core, and two hydrophilic N- and C-terminal domains which are localized at the surface. Oleosins are believed to form a steric barrier to prevent coalescence of OBs. Other described OB-associated proteins in plants are caleosins and steroleosins. Caleosins constitute a minor group of integral lipid body proteins found in a wide range of plants that share with oleosins a similar three-domain structure and possess a half-calcium binding site [6]. On the other hand, the steroleosins contain a NADPH and a sterol-binding domain and seems to be involved in signal transduction regulated by sterols [7].

Storage oil breakdown following seed imbibition provides energy and carbon skeletons that support seedling growth immediately following germination. This metabolic process is initiated by lipases, which catalyze the hydrolysis of triacylglycerols (TAGs) to release free fatty acids and glycerol. Phospholipases, lipoxygenases and other enzymes are also involved in the conversion of fatty acids to succinate in the glyoxysome [8,9]. It has been proposed that lipases attach the OBs through their interaction with oleosins or other OB-associated proteins. Some studies have shown the possibility of a direct interaction between OBs and glyoxysomes or vacuoles during germination [10]. Caleosins may be involved in the OB-vacuole interaction [11].

Different proteomic studies in plant seeds have identified OB-associated proteins. In addition to the already known proteins, some less abundant OB-associated proteins have been identified including enzymes, proteins involved in membrane traffic and proteins of unknown function [12–15]. Here, we performed a comparative proteomic analysis of OB-associated proteins extracted from maize scutellum (the modified cotyledon of grass species) at two different developmental stages, i.e., dry seeds and 2 days after germination. Protein identification by tandem mass spectrometry allowed us to identify proteins differentially accumulated in association to OBs in these two developmental stages.

2. Materials and methods

2.1. Plant material and protein extraction

Maize seeds (*Zea mays* L. W64A) were surface sterilized and placed on wet vermiculite at 28 °C. After two days, the scutella were

Abbreviation: OB, oil body.
* Corresponding author.

0168-9452/$ – see front matter © 2012 Published by Elsevier Ireland Ltd.
doi:10.1016/j.plantsci.2012.02.011

manually dissected and frozen. The scutellum was also dissected from mature seeds.

2.2. Isolation of oil bodies from scutellum of maize embryo

Oil bodies were isolated according to Tzen et al. [16]. 25 g of maize seeds were ground in 15 mL cold (4 °C) grinding medium one (GMI: 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 0.6 M sucrose, 0.15 M tricine–KOH, pH 7.5) using a mortar and pestle. Crude homogenates were filtered through two layers of Miracloth and added to 15 mL cold (4 °C) flotation medium one (FMI; the same composition as GMI with the addition of 0.4 M sucrose). The samples were centrifuged at 10,000 × *g* for 30 min. The top oleaginous layers were removed using a spatula and resuspended in grinding medium two (GMII; the same composition as GMI with the addition of 2 M NaCl) using a 50 mL glass Dounce homogenizer. The suspensions were added to 15 mL of flotation medium two (FMII; the same composition as FMI plus 2 M NaCl), and centrifuged at 10,000 × *g* for 30 min. The top layers were resuspended in 15 mL GMI, added to 15 mL FMI and centrifuged at 10,000 × *g* for 30 min. The procedure was repeated and the final oil body layers were resuspended in 3 mL GMI.

2.3. Protein isolation from oil body preparations

Oil body associated proteins were isolated according to the method by Katavic et al. [13]. 0.5 mL hexane were mixed with 0.5 mL of isolated oil bodies and centrifuged 5 min at 13,000 × *g*. After centrifugation, the interfacial layer and bottom aqueous phase were sparged with nitrogen gas to remove any remaining hexane. 0.75 mL chloroform/methanol (2:1, v/v) was mixed with the interfacial layer and centrifuged for 5 min at 13,000 × *g*. The protein rich interfacial layer was resuspended in 0.25 mL water, mixed with 0.75 mL chloroform/methanol (2:1, v/v) and centrifuged for 5 min at 13,000 × g. The procedure was repeated two more times. After washing, oil body protein pellet was resuspended in 0.5 mL water, sonicated for 5 min, precipitated in 4 volumes of cold 100% acetone for 16 h at −20 °C and resuspended in urea re-hydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 0.5% Biolyte (pH 3–10), 0.75% DTT, 0.002% Bromophenol Blue). Protein concentration was determined using protein assay from BioRad (Hercules, CA) based upon modified Bradford procedure [17].

2.4. 2-DE gel electrophoresis

For the first dimension (IEF), linear IPG gel strips (pH 3–11, 18 cm, GE Healthcare) were passively rehydrated with IEF buffer and 500 μg aliquots of OB proteins were applied by cup loading. The IEF was performed using the following focusing method: active rehydration for 12 h at 50 V, 250 V for 15 min, gradient from 250 to 10,000 V for 3 h and final focusing for 12 h at 10,000 V. The strips containing focused proteins were stored at −20 °C. After IEF, the strips were placed in equilibration buffer (50 mM Tris, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) containing 1% dithiothreitol for 10 min. A second equilibration step was performed in the same equilibration buffer containing 4% iodoacetamide for 10 min, rinsed in SDS running buffer and loaded into 12.5% acrylamide vertical gel (16 cm width, 20 cm length, 0.75 cm thickness). Experiments were carried out using the Protean II Xi vertical system (BioRad) at 60 mA/gel for 2 h. After electrophoresis, gels were washed three times in deionized water before silver staining. For each biological sample six experimental replicates were performed.

2.5. Image and data analysis

Gel images were acquired using a fluoroimager (ProXpress, PerkinElmer). Images were saved as Tag Image File Format (TIFF) and analyzed using the ImageMaster™ 2-D Platinum 5.0 Software

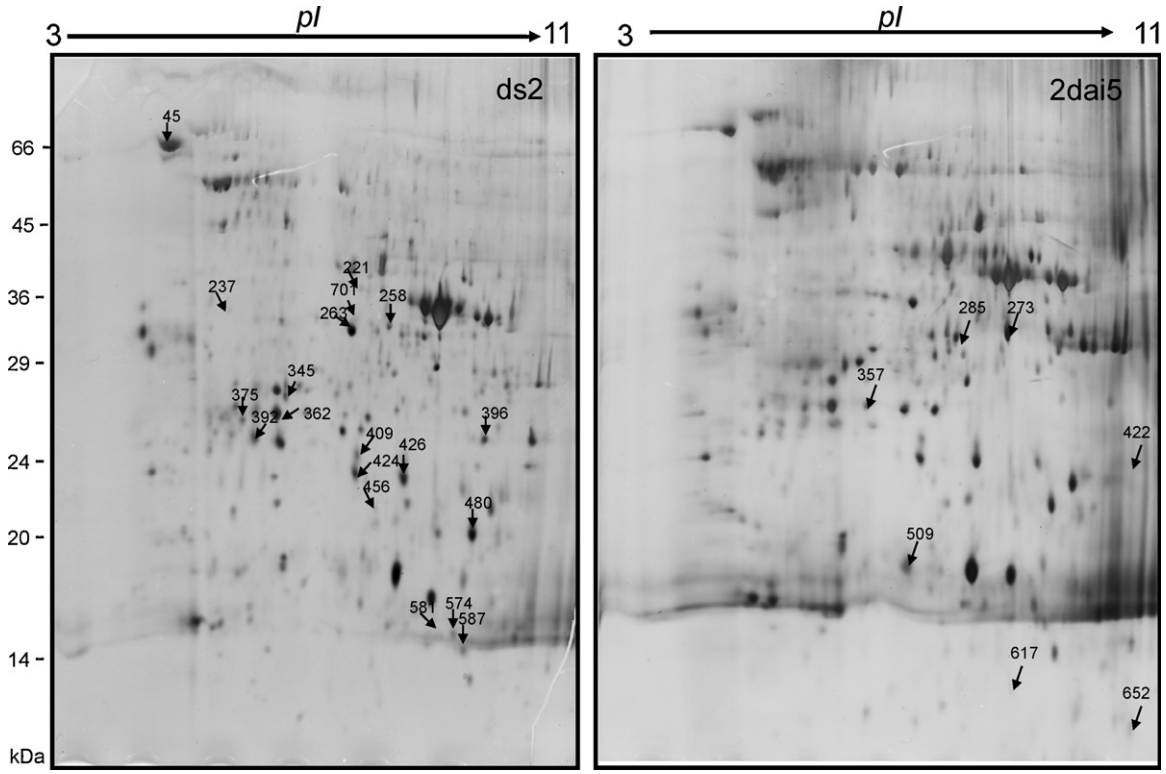


Fig. 1. 2-DE maps of the maize scutellum OB-associated proteins of dry seeds (ds) and seeds 2 days after imbibition (2 dai). Arrows indicate the 26 protein spots with differences in intensity higher than 2-folds. The identified proteins are indicated in Tables 1 and 2 with the same code number as in the figure. These images are representative gels of six biological replicates.

(GE Healthcare). After automatic spot detection, manual spot editing was carried out. Careful visual inspection was performed to confirm correct spot matching. To evaluate protein expression differences among gels, relative spot volume (% Vol) was used. This is a normalized value and represents the ratio of a given spot volume to the sum of all spot volumes detected in the gel. Spots showing a quantitative variation ≥1.5 and positive GAP (Statistical parameters ImageMaster™ 2-D Platinum 5.0 Software) were selected as differentially expressed. Statistically significant protein abundance variation was validated by Student's *t*-test ($p < 0.05$).

2.6. Protein identification

The selected protein spots were manually excised from the gels and destained in 30 mM potassium ferricyanide and 100 mM sodium thiosulfate at room temperature for 10 min. Gel fragments were then incubated twice for 15 min in a H_2O/CH_3CN solution and dried using a SpeedVac centrifuge for a few minutes. Trypsin digestion was performed using an automatic digester (MultiPROBE II, PerkinElmer). After lyophilization, the peptide extracts were resuspended in 10 μL of 0.1% formic acid/3% acetonitrile. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, MA, USA). For protein identification, MS/MS peak lists were extracted and compared with the protein database using MASCOT Daemon (version 2.1.3; Matrix Science, London, UK). Searches were performed with no fixed modification and allowed for carbamidomethylation, oxidation and a maximum of one missed cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 for MS/MS fragments. The LC–MS/MS data were converted into DTA-format files, which were further searched for proteins with MASCOT Daemon. Measured peptides were searched in the NCBInr-protein sequence database *Viridiplantae*.

3. Results

3.1. Protein profiles

Two-dimensional gel electrophoresis (2-DE) was used to compare the OB-associated proteins present in the scutellum of maize mature dry seeds (ds) and germinating seeds 2 days after imbibition (2 dai). The OB-associated proteins were purified by floating extraction. The extracted proteins were then separated by SDS-PAGE 2-DE in denaturing conditions in a range of pHs 3–11 (Fig. 1; Supplementary Figs. 1 and 2). About 400 protein spots were detected in each gel ranged from 12 to 66 kDa. The spot patterns were similar in both samples but a quantitative analysis of the images using ImageMaster™ 2-D Platinum software revealed some differences. Thus, four spots were exclusively present in 2 dai gels and six spots were exclusively present in ds gels. On the other hand, the intensities of 3 additional spots were significantly higher in 2 dai gels compared to ds gels (at least 2 folds increase, $p < 0.05$). On the contrary, the intensities of 13 additional spots were significantly higher in ds gels using the same criteria.

3.2. Protein identification and functional categorization

All the spots showing significant differences between ds and 2 dai gels were excised, subjected to trypsin hydrolysis and the resulting peptides were identified using LC–MS/MS. The identified proteins (which fulfilled the criteria chosen for mass spectrometry identification) are given in Tables 1 and 2. Among them, five different polypeptides were accumulated in germinating seeds

Table 1
Protein spots identified by LC–MS/MS which exhibit a higher accumulation 2 days after imbibition (2 dai) compared to dry seeds (ds).

Band no.	Accession no.	Protein	Mascot score	SC%	Theor. Mw/pI	Obs. Mw/pI	Matched peptide	Peptide sequence	Variation
422	GI:195658109	Oleosin 18 kDa	71	23	18/9.7	23/9.8	2	R.TPDYVEEAR.R	2 dai
								R.SGIYGGGAYGQQQQGGRPMGEQVK.G	
652	GI:226503851	Oleosin Zm-II	114	15	17/11.1	12/10.2	1	R.QGEGGGVTGMVVQPLDQGSKR.H	2 dai
357	GI:601869	Manganese superoxide dismutase	111	14	25/6.5	26/5.6	2	K.ALEQLDAAVAK.G	2 dai
								K.LSVETTANQDPLVTK.G	
617	GI:226492587	Stress-inducible membrane pore protein Tim17	150	32	18/6.4	13/7.6	2	K.NSAIAGAIAGAAVALTGDAGGHSDK.L	2 dai
								R.EAYVVTVEGLSGDSSGLDADGGKR.S	
509	GI:226492587	Stress-inducible membrane pore protein Tim17	234	47	18/6.4	17/6.1	4	R.VADSFIR.A	2 dai > ds
								R.TLKDELTSMDR.K	
								K.NSAIAGAIAGAAVALTGDAGGHSDK.L	
								R.EAYVVTVEGLSGDSSGLDADGGKR.S	
273	GI:162462211	Prohibitin-2	522	40	30/6.6	30/7.6	5	K.VLPSIGNEVLK.A	2 dai > ds
								R.SPNVAYIPSGENGK.M	
								K.VAAGLGAAASLASASLYTVDGGER.A	
								K.AVVAQFNADQLLTDRPHVSALVR.D	
								R.LLSRPDVQHLPTIFTSLGLEYDDK.V	
285	GI:162462211	Prohibitin-2	380	45	30/6.6	30/6.5	6	K.EIAAELAR.S	2 dai > ds
								K.DLQMVNLTLR.L	
								R.LISEATAMAGTGLIELR.R	
								K.VAAGLGAAASLASASLYTVDGGER.A	
								K.AVVAQFNADQLLTDRPHVSALVR.D	
								R.LLSRPDVQHLPTIFTSLGLEYDDK.V	

Table 2
Protein spots identified by LC–MS/MS exhibiting a higher accumulation in dry seeds (ds) compared to 2 days after imbibition (2 dai).

Band no.	Accession no.	Protein	Mascot score	SC%	Theor. Mw/pI	Obs. Mw/pI	Matched peptide	Peptide sequence	Variation
345	GI:224028729	Triosephosphate isomerase	168	33	27/5.5	27/5.6	4	R.ALLGESNEFVGDK.V R.EAGSTMDVVAAQTK.A K.VATPAQAQEVHASLR.D K.TLNEGQVPPSDVVEVVVSPPYVFLPVVK.S	ds
456	GI:226501206	Class IV heat shock protein	55	11	25/6.7	21/6.4	1	R.LPENADLDSVGASLDNGVLTVR.F	ds
581	GI:223974989	Nucleoside-diphosphate kinase	86	12	26/9.0	14/7.8	2	R.GDLAIVVGR.N R.TFIAIKPDGVQR.G	ds
701	GI:226497424	Embryonic protein DC-8	516	45	33/6.2	34/6.2	8	K.ISEGLGLK.H K.DKISEGLGLK.H R.VAGPDVAAVDGK.G K.DAAWETVEAAK.E K.AQETLSQTADAAAEK.A K.EAAEAASESGAEAHER.S K.TKEAAEAASESGAEAHER.S K.TDVHDVAEESNKDEESWTGWAK.D	ds
237	GI:162460698	60S acidic ribosomal protein P0	144	13	34/5.19	35/5.2	2	K.VGSSESALLAK.L K.TGNHTFDPLMDLLVGNVGLIFTK.G	ds
221	GI:162461925	Protein disulfide isomerase 7	118	13	40/6.3	36/6.3	2	R.SVEALAEFVNSEAGTNVK.I K.IAAIPSSVVVLTSETFDSIVLDETK.D	ds
45	GI:162461063	Protein disulfide isomerase	1278	76	56/5.0	67/4.0	22	K.HDPPIVLAK.V K.DFDVAALMK.F K.FIDASTIPR.V K.DFDVAALMK.F K.AAAAEPVKDEL. R.TADDIVDFIK.K K.YEIQGFPTIK.I R.EADGIVDYLKK.Q R.TADDIVDFIKK.S K.SPEDATALIDDK.K K.SEPIPEVNNEPVK.V K.VVVADNVHDFVFK.S R.LLKPFDELVVDSK.D K.VHVEADQIVAWLK.E R.KSEPIPEVNNEPVK.V K.EDQTPLILIQDGDSK.K K.ETAGAATTTTTQAPPASEK.A K.SKETAGAATTTTTQAPPASEK.A K.FLIGDIEASQGAFQYFGLK.E K.LAPILDEAATTLQSDEEVVIAK.M K.KLAPILDEAATTLQSDEEVVIAK.M R.LLKPFDELVVDSKDFDVAALMK.F	ds > 2 dai
587	GI:195658177	Oleosin 16 kDa	56	21	24/9.6	14/8.5	2	R.GATGGGGGYGDLQR.G R.LCVVLCVCMLVSGGMQCSAVLYAVCVSL	ds > 2 dai
574	GI:162461691	Oleosin Zm-I	87	11	16/9.6	14/8.0	1	R.GATGGGGGYGDLQR.G	ds > 2 dai
480	GI:162461691	Oleosin Zm-I	70	11	16/9.6	20/8.8	1	R.GATGGGGGYGDLQR.G	ds > 2 dai
396	GI:195658177	Oleosin 16 kDa	63	20	24/9.6	25/9.0	2	R.GATGGGGGYGDLQR.G R.LCVVLCVCMLVAGGMQCSAVLYAVCVSL	ds > 2 dai
375	GI:162461691	Oleosin Zm-I	58	11	16/9.6	26/5.3	1	R.GATGGGGGYGDLQR.G	ds > 2 dai
424	GI:226509468	Cupin 2	222	11	56/6.1	23/6.2	3	K.VGYIHKDELVER.K K.TLFVPQYLDSSITLFVQR.G K.MGDVLHIDAGSTFYMVNPGK.G	ds > 2 dai
392	GI:226509468	Cupin 2	166	10	56/6.1	25/5.4	3	K.VGYIHKDELVER.K R.EGLMHIGFITMEPK.T K.TLFVPQYLDSSITLFVQR.G	ds > 2 dai

Table 2 (*Continued*)

Band no.	Accession no.	Protein	Mascot score	SC%	Theor. Mw/pI	Obs. Mw/pI	Matched peptide	Peptide sequence	Variation
409	GI:226509468	Cupin 2	251	21	56/6.1	24/6.2	6	R.GQPWPPASFACR.E R.VEKVVESEGGQVR.V K.VGYIHKDELVER.K R.EGLMHIGFITMEPK.T K.TLFVPQYLDSSITLFVQR.G K.MGDVLHIDAGSTFYMVNPGK.G	ds > 2 dai
426	GI:226509468	Cupin 2	245	17	56/6.1	23/6.6	5	R.GLFLLHR.V K.VGYIHKDELVER.K R.EGLMHIGFITMEPK.T K.TLFVPQYLDSSITLFVQR.G K.MGDVLHIDAGSTFYMVNPGK.G	ds > 2 dai
263	GI:219887411	Rubber elongation factor protein	433	37	27/6.0	33/6.2	5	R.FGGLPLDVLAFVDR.K R.YEPAAEHLAVSAWR.S R.FGGLPLDVLAFVDRK.V K.DHAGPLRPSVDAVESAVK.G K.VDDTVHEVDKHLPGALK.A	ds > 2 dai
362	GI:242041881	Unknown protein	154	8	75/6.2	26/5.6	3	K.GFETDVLR.Q R.QGFGVKAEVVEAIK.S R.IYAIFTSEGINADDPSKPK.V	ds > 2 dai
258	GI:226507242	Unknown protein	325	26	38/6.3	34/6.5	6	R.FGGGGEPTVR.I K.ADEGFSATVR.N R.LNFDAFHGDK.G R.DGNVVLAPANPR.D R.NGAVCLAPTNPR.D K.DEEGYPAFALVNR.V	ds > 2 dai

(Table 1). Two of them were identified as oleosins (spots 422 and 652), the most abundant protein associated to plant oil bodies. Two spots were identified as the mitochondrial protein Tim17 (spots 509 and 617), involved in protein import across the mitochondrial membrane [18]. Two spots corresponded to prohibitin-2 (spots 273 and 285), another mitochondrial protein involved in different processes, including the regulation of the mitochondria ultra-structure, respiratory complexes, cellular senescence and NO responses [19,20]. The last spot (spot 357) corresponded to a manganese superoxide dismutase, Mn-SOD, which is usually located in the chloroplasts and, in a minor proportion, in the mitochondria [21]. Due to that the maize scutellum does not contain chloroplast, this protein is more likely located in the mitochondria.

The 19 spots exhibiting an accumulation in the scutellum of dry seeds corresponded to 13 different proteins and protein similarities were found for 11 of them (Table 2). Five spots corresponded to two oleosins exhibiting differences in their molecular weights (spots 375, 396, 480, 574 and 587). Four spots corresponded to different forms of the seed specific protein cupin 2 (spots 392, 409, 424 and 426), which is predicted to be located in the vacuole [22]. Cupins are a very diverse super-family of proteins which are implicated in many biological processes including plant growth and development. The other identified spots encoded a variety of proteins with different functions: two different protein disulfide isomerases (spots 45 and 221), a triosephosphate isomerase (spot 345), a nucleoside phosphate kinase (spot 581), a class IV heat shock protein (spot 456), the embryonic protein DC-8 (spot 701), a 60S acidic ribosomal protein P0 (spot 237) and a rubber elongation factor protein (spot 263).

4. Discussion

Seed germination is a complex process that requires cell division, expansion and differentiation. It involves the activation of many metabolic pathways and signal transduction processes, which require a great quantity of energy and stored materials which are provided by seed reserves (oil, storage proteins and starch) and the activation and accumulation of many genes and proteins. In maize, the starch accumulated in the endosperm provides an important part of the energy required during germination, but the oil accumulated in the embryo, mainly in the scutellum, also provides an important proportion of the required energy. Oil is stored inside the cells in small spherical organelles termed oil bodies. Storage lipid breakdown is necessary for the maize seedling growth during first steps of germination involving the action of phospholipases, lipases, lipoxygenases and other enzymes.

We observed that the intensity of 26 protein spots changed when comparing ds and 2 dai gels. Some proteins were found as mass isoforms. Those isoforms exhibited different apparent molecular masses and pI values, witness of posttranslational modifications included oxidation, phosphorylation, deamidation, acetylation, and truncation. A gradual degradation of oleosins has been described during imbibition in maize [23]. However, not all the oleosins are degraded at the same rate and, for example, mobilization of the maize 17.5-kDa oleosin is faster than that of the 20-kDa [24]. A similar situation was observed in sunflower, safflower and castor bean, suggesting that one or more proteases may act at specific exposed portions of the oleosins to generate intermediate forms. These differential rates of oleosin degradation might explain the presence of an oleosin spot with a higher intensity in 2 dai compared to ds. Cupin 2 is also found in different spots exhibiting a high discordance between theoretical and experimental masses. The cupins are a functionally diverse superfamily of proteins that share a beta-barrel structural core domain to which the term cupin (Latin cupa, barrel) was given [22,25]. A

common feature that allowed the unification in the cupin superfamily of proteins, though diverse functions, amino acid sequences and domain organization, was the presence of a β-barrel containing the nodular sequence motifs characteristic of germins in their structure [26]. Results suggest here proteolysis of cupin during early steps of germination.

The intensity of a spot corresponding to a protein involved in embryo specific processes (DC-8) decreased during the first steps of imbibition, as well as the intensity of a spot corresponding to the triosephosphate isomerase. On the contrary, we observed an increase in the intensity of spots corresponding to Tim17, prohibitin-2 or manganese superoxide dismutase. Tim17 is involved in membrane transport [27]. Prohibitins are an extensively studied family of proteins that are highly conserved between animals and plants [28,29]. Prohibitins serve many functions in such diverse processes as apoptosis and aging, cell cycle progression, tumorigenesis, transcriptional regulation, signaling, oxidative damage, respiration, and mitochondrial biogenesis [30]. The identification of this protein could be related with apoptosis during germination, but its functional relationship with OBs is not clear yet. Manganese superoxide dismutase (Mn-SOD) is involved in the response to oxidative stress. During the early stage of imbibition, the rupture of the testa is accompanied by water infiltration and oxygen diffusion. In addition, many oxidation reactions started during the mobilization of oil which produces active oxygen species [31]. In consequence, many oxidative stress-related proteins are mobilized during germination in order to protect enzymes and other molecules from oxidative stress.

The subcellular localization of some of these identified proteins was previously determined and they were not located in OBs. Increasing evidences support the idea that OBs interact with other organelles probably through protein–protein interactions. For example, animal lipid bodies are often associated with other organelles of the cell as, for example, ER, mitochondria and peroxisomes [32–34]. It is known that plant OBs interact with glyoxysomes, mitochondria and vacuoles [10]. Some studies suggest also that caleosins are involved in the OB-vacuole interaction [11]. Consequently, some proteins involved in these interactions may be purified together with the OB fraction. Interestingly, some proteins identified here as over-expressed during germination are predicted to be located in the mitochondria. These results suggest an interaction between OBs and mitochondria during at least the first steps of germination.

During last years, different authors have demonstrated that animal lipid droplets can serve as transient storage depots for proteins that lack appropriate binding partners in the cell or are temporally in excess, protecting the proteins from degradation, and protecting the cell from detrimental effects of uncontrolled protein activity [35]. Mature plant embryos contain a great amount and diversity of proteins including proteins involved in the degradation of the reserves and in the senescence process that takes place in the organ after the storage reserve mobilization. If plant OBs are also transient storage depots, these may explain why we found proteins associated to OBs that, in principle, may not be located in this organelles.

Acknowledgments

We thank Bertrand Naudin, Annick Schaumann and Sami Irar for their technical assistance. This work was carried out with grants BIO2004-01577 and AGL2009-09151 from Plan Nacional de Investigación Científica y Técnica, by the Centre CONSOLIDER on Agrigenomics and by the Xarxa de Referencia en Biotecnologia of the Generalitat de Catalunya. H.T. was the recipient of a predoctoral I3P (CSIC).

Appendix A. Supplementary data

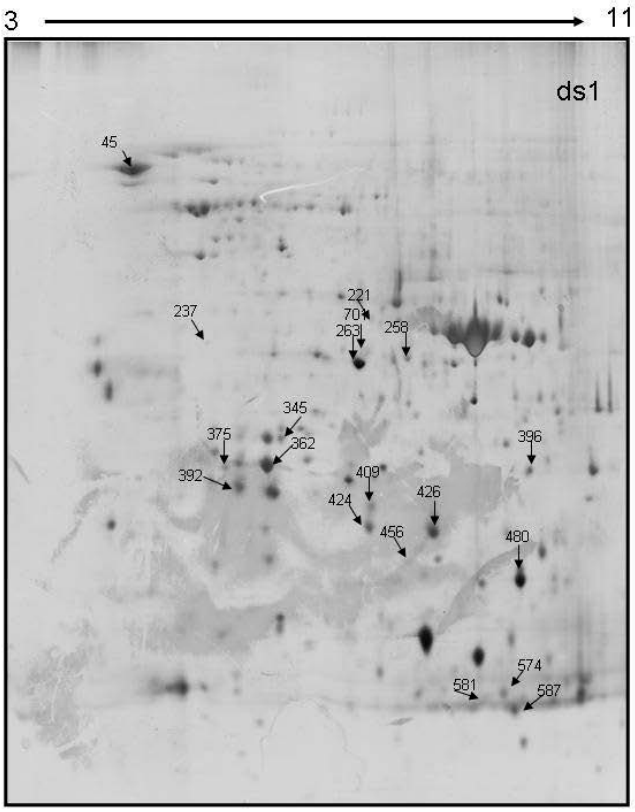
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2012.02.011.

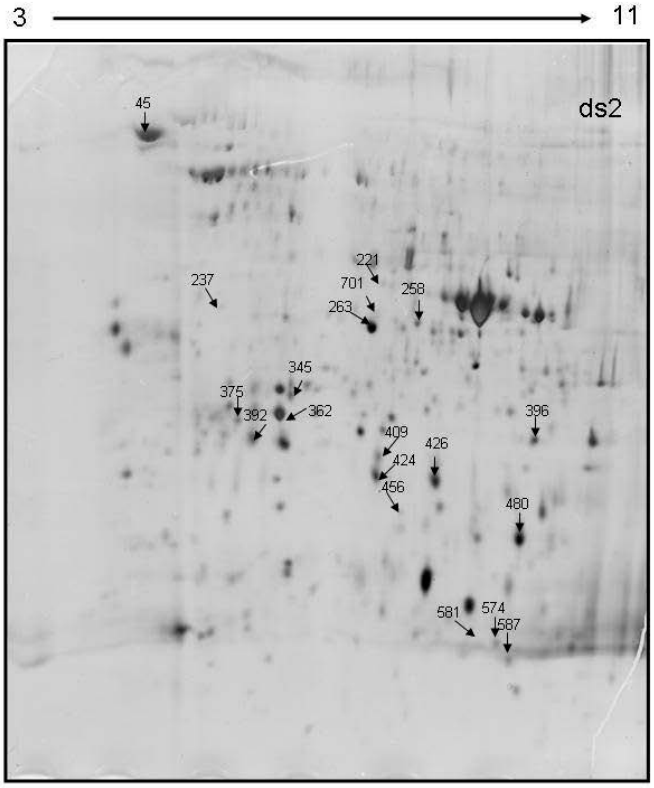
References

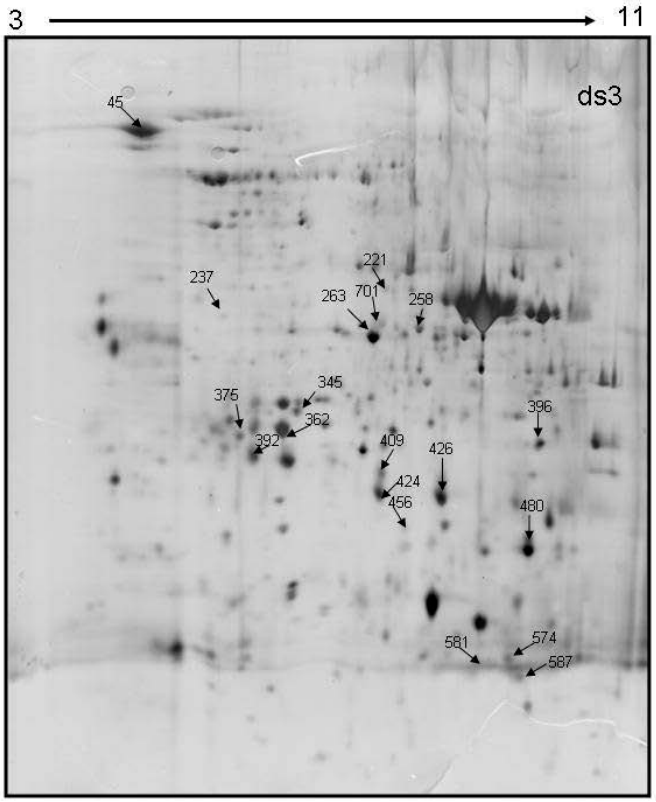
[1] T. Fujimoto, R.G. Parton, Not just fat: the structure and function of the lipid droplet, Cold Spring Harb. Perspect. Biol. 3 (2011) 3.
[2] Z.Z. Purkrtova, P. Jolivet, M. Miquel, T. Chardot, Structure and function of seed lipid-body-associated proteins, C. R. Biol. 331 (2008) 746–754.
[3] J. Pu, C.W. Ha, S. Zhang, J.P. Jung, W.K. Huh, P. Liu, Interactomic study on interaction between lipid droplets and mitochondria, Protein Cell 2 (2011) 487–496.
[4] D.J. Murphy, The biogenesis and functions of lipid bodies in animals, plants and microorganisms, Prog. Lipid Res. 40 (2001) 325–438.
[5] F. Capuano, F. Beaudoin, J.A. Napier, P.R. Shewry, Properties and exploitation of oleosins, Biotechnol. Adv. 25 (2007) 203–206.
[6] Z. Purkrtova, S. d'Andrea, P. Jolivet, P. Lipovova, B. Kralova, M. Kodicek, Y. Chardot, Structural properties of caleosin: a MS and CD study, Arch. Biochem. Biophys. 464 (2007) 335–343.
[7] L.J. Lin, S.S. Tai, C.C. Peng, J.T. Tzen, Steroleosin, a sterol-binding dehydrogenase in seed oil bodies, Plant Physiol. 128 (2002) 1200–1211.
[8] S.C. Bhatla, S. Vandana, V. Kaushik, Recent developments in the localization of oil body-associated signaling molecules during lipolysis in oilseeds, Plant Signal. Behav. 4 (2009) 176–182.
[9] M.F. Yang, Y.J. Liu, Y. Liu, H. Chen, F. Chen, S.H. Shen, Proteomic analysis of oil mobilization in seed germination and postgermination development of *Jatropha curcas*, J. Proteome Res. 8 (2009) 1441–1451.
[10] I.A. Graham, Seed storage oil mobilization, Annu. Rev. Plant Biol. 59 (2008) 115–142.
[11] M. Poxleitner, S.W. Rogers, A.L. Samuels, J. Browse, J.C. Rogers, A role for caleosin in degradation of oil-body storage lipids during seed germination, Plant J. 47 (2006) 917–933.
[12] P. Jolivet, E. Roux, S. D'Andrea, M. Davanture, L. Negroni, M. Zivy, T. Chardot, Protein composition of oil bodies in *Arabidopsis thaliana* ecotype WS, Plant Physiol. Biochem. 42 (2004) 501–509.
[13] V. Katavic, G.K. Agrawal, M. Hajduch, S.L. Harris, J.J. Thelen, Protein and lipid composition analysis of oil bodies from two *Brassica napus* cultivars, Proteomics 6 (2006) 4586–4598.
[14] H. Tnani, I. López, T. Jouenne, C.M. Vicient, Protein composition analysis of oil bodies from maize embryos during germination, J. Plant Physiol. 168 (2011) 510–513.
[15] P. Jolivet, C. Boulard, A. Bellamy, C. Larré, M. Barre, H. Rogniaux, S. d'Andréa, T. Chardot, N. Nesi, Protein composition of oil bodies from mature *Brassica napus* seeds, Proteomics 9 (2009) 3268–3284.
[16] J.T.C. Tzen, C.C. Peng, D.J. Cheng, E.C.F. Chen, J.M.H. Chiu, A new method for seed oil body purification and examination of oil body integrity following germination, J. Biochem. 121 (1997) 762–768.
[17] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] M.W. Murcha, D. Elhafez, A.H. Millar, J. Whelan, The C-terminal region of TIM17 links the outer and inner mitochondrial membranes in *Arabidopsis* and is essential for protein import, J. Biol. Chem. 280 (2005) 16476–16483.
[19] M. Artal-Sanz, N. Tavernarakis, Prohibitin and mitochondrial biology, Trends Endocrinol. Metab. 20 (2009) 394–401.
[20] Y. Wang, A. Ries, K. Wu, A. Yang, N.M. Crawford, The *Arabidopsis* prohibitin gene PHB3 functions in nitric oxide-mediated responses and in hydrogen peroxide-induced nitric oxide accumulation, Plant Cell 22 (2010) 249–259.
[21] C. Jackson, J. Dench, A.L. Moore, B. Halliwell, C.H. Foyer, D.O. Hall, Subcellular localisation and identification of superoxide dismutase in the leaves of higher plants, Eur. J. Biochem. 91 (1978) 339–344.
[22] J.M. Dunwell, A. Purvis, S. Khuri, Cupins: the most functionally diverse protein superfamily, Phytochemistry 65 (2004) 7–17.
[23] D.E. Fernández, R. Qu, A.H. Huang, L.A. Staehelin, Immunogold localization of the L3 protein of maize lipid bodies during germination and seedling growth, Plant Physiol. 86 (1988) 270–274.
[24] H.R. Sadeghipour, S.C. Bhatla, Differential sensitivity of oleosins to proteolysis during oil body mobilization in sunflower seedlings, Plant Cell Physiol. 43 (2002) 1117–1126.
[25] H. Breiteneder, E.N. Mills, Plant food allergens – structural and functional aspects of allergenicity, Biotechnol. Adv. 23 (2005) 395–399.
[26] A.D. Shutov, I.A. Kakhovskaya, Evolution of seed storage globulins and cupin superfamily, Mol. Biol. 45 (2011) 529–535.
[27] S. Martinez-Caballero, S.M. Grigoriev, J.M. Herrmann, M.L. Campo, K.W. Kinnally, Tim17p regulates the twin pore structure and voltage gating of the mitochondrial protein import complex TIM23, J. Biol. Chem. 282 (2007) 3584–3593.
[28] R. Nadimpalli, N. Yalpani, G.S. Johal, C.R. Simmons, Prohibitins, stomatins, and plant disease response genes compose a protein superfamily that controls cell proliferation, ion channel regulation and death, J. Biol. Chem. 275 (2000) 29579–29586.
[29] S. Mishra, L.C. Murphy, L.J. Murphy, The prohibitins: emerging roles in diverse functions, J. Cell. Mol. Med. 10 (2006) 353–363.

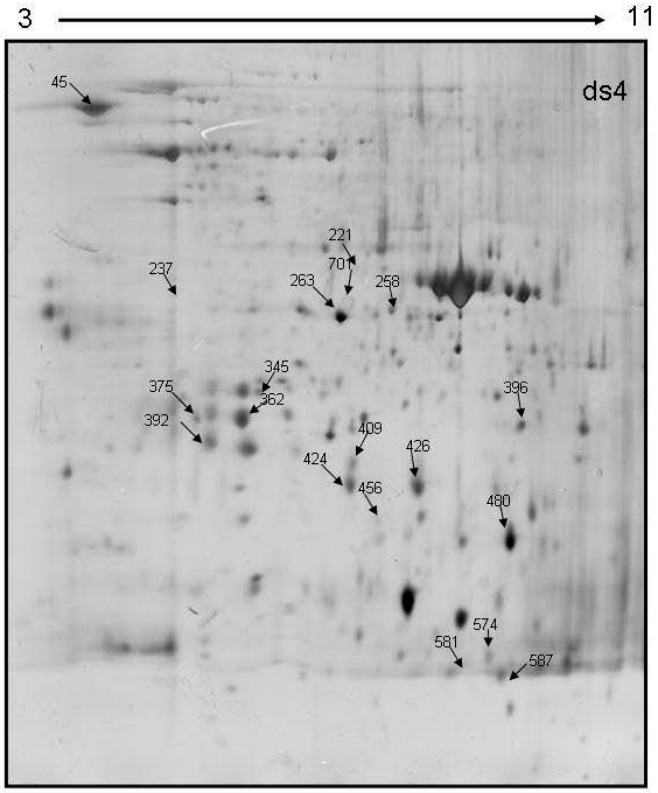
[30] O. Van Aken, J. Whelan, F. Van Breusegem, Prohibitins: mitochondrial partners in development and stress response, Trends Plant Sci. 15 (2010) 275–282.
[31] L. Guan, J.G. Scandalios, Two structurally similar maize cytosolic superoxide dismutase genes, Sod4 and Sod4A, respond differentially to abscisic acid and high osmoticum, Plant Physiol. 117 (1998) 217–224.
[32] T. Levine, Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions, Trends Cell Biol. 14 (2004) 483–490.
[33] T. Fujimoto, Y. Ohsaki, J. Cheng, M. Suzuki, Y. Shinohara, Lipid droplets: a classic organelle with new outfits, Histochem. Cell Biol. 130 (2008) 263–279.
[34] S. Murphy, S. Martin, R.G. Parton, Lipid droplet–organelle interactions; sharing the fats, Biochim. Biophys. Acta 1791 (2009) 441–447.
[35] S. Cermelli, Y. Guo, S.P. Gross, M.A. Welte, The lipid-droplet proteome reveals that droplets are a protein-storage depot, Curr. Biol. 16 (2006) 1783–1795.

Appendix A: Supplementary data of Dry Seed (Ds)

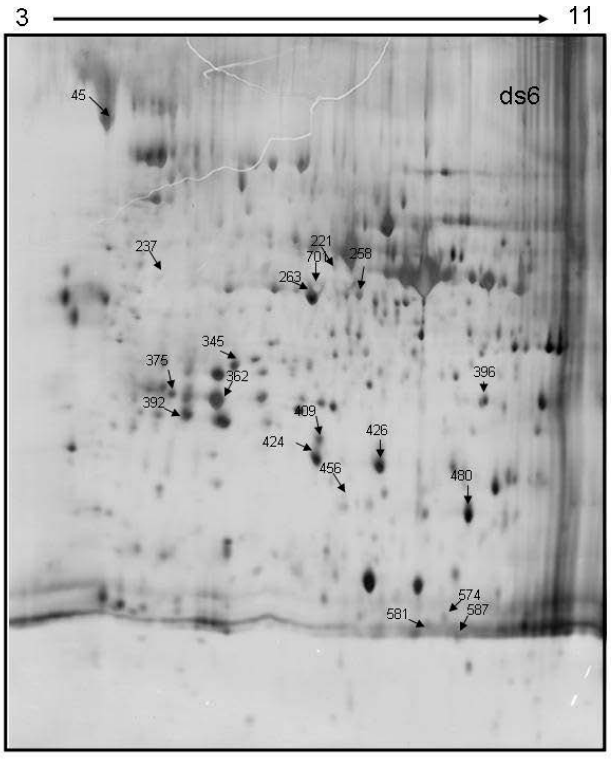




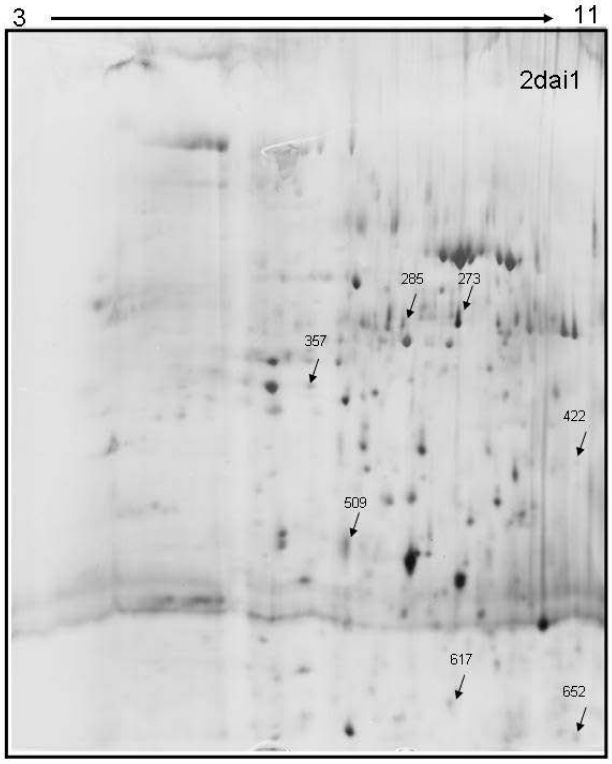

3
11
ds3
45
237
221
263
701
258
345
375
362
392
409
426
424
456
396
480
581
574
587

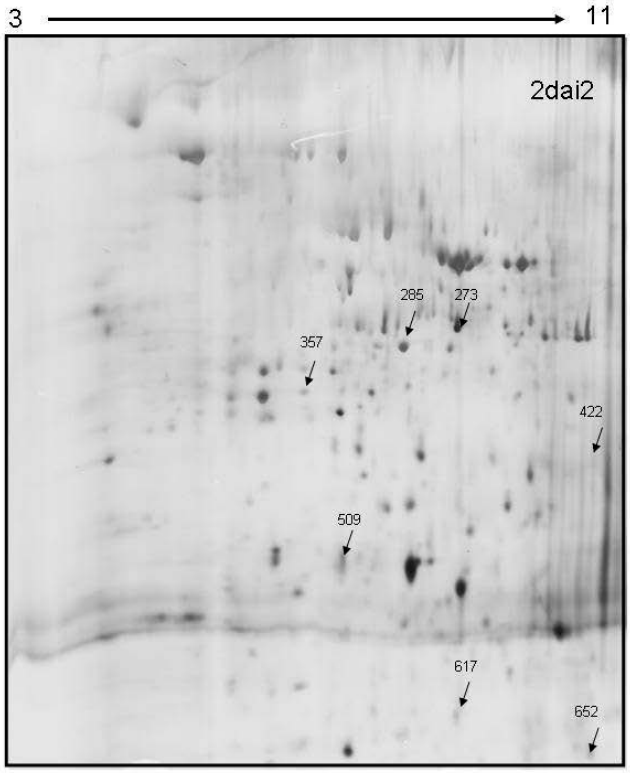

3
11
ds4
45
221
701
237
263
258
345
375
362
392
409
426
424
456
396
480
574
581
587

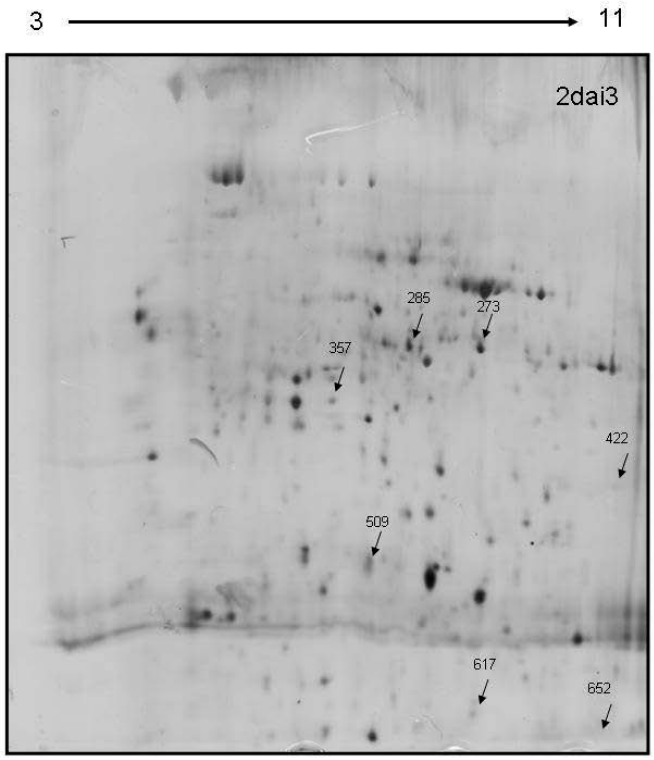

3
11
ds5
45
237
221
701
263
258
345
375
362
396
392
409
424
426
456
480
574
587
581

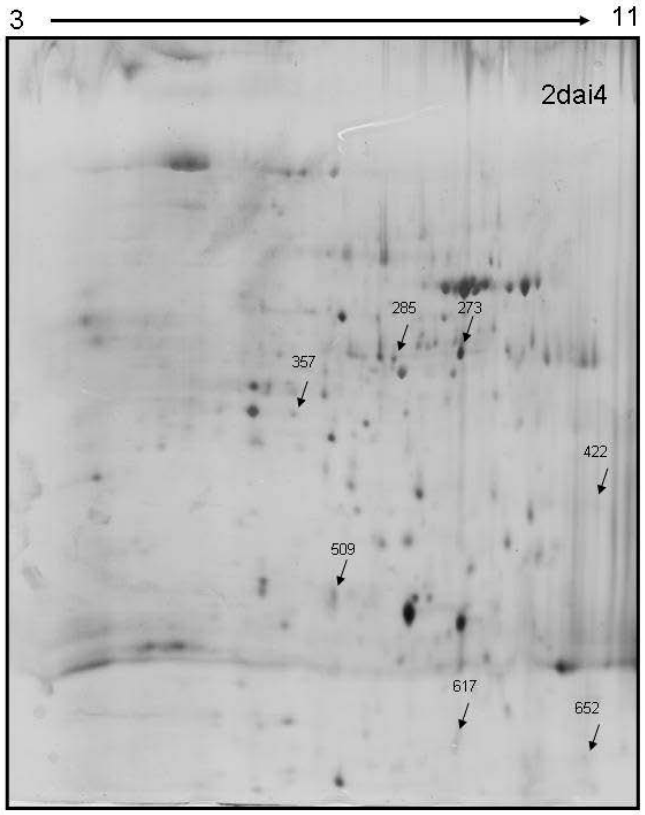

3
11
ds6
45
237
221
701
258
263
345
375
362
396
392
409
424
426
456
480
574
587
581

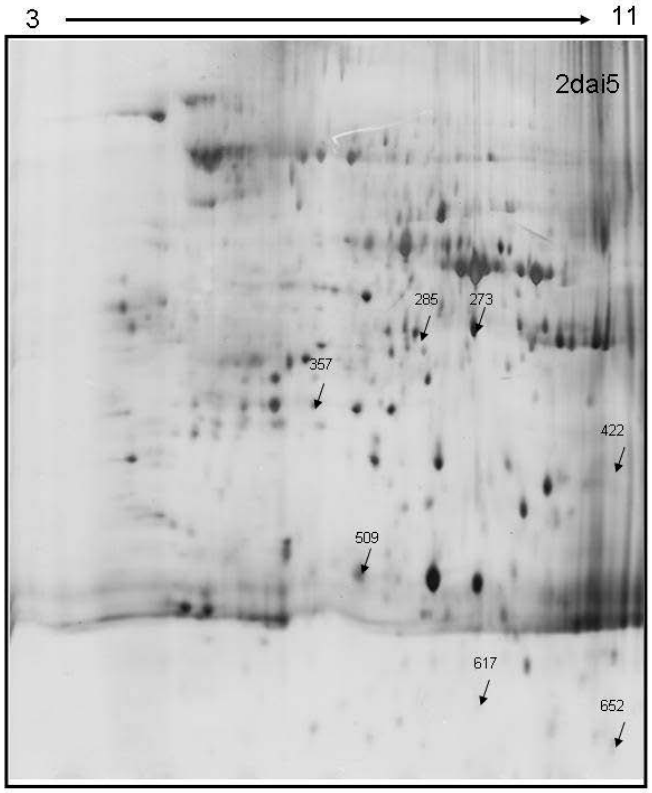
Appendix A: Supplementary data of 2 days after imbibition (2dai)

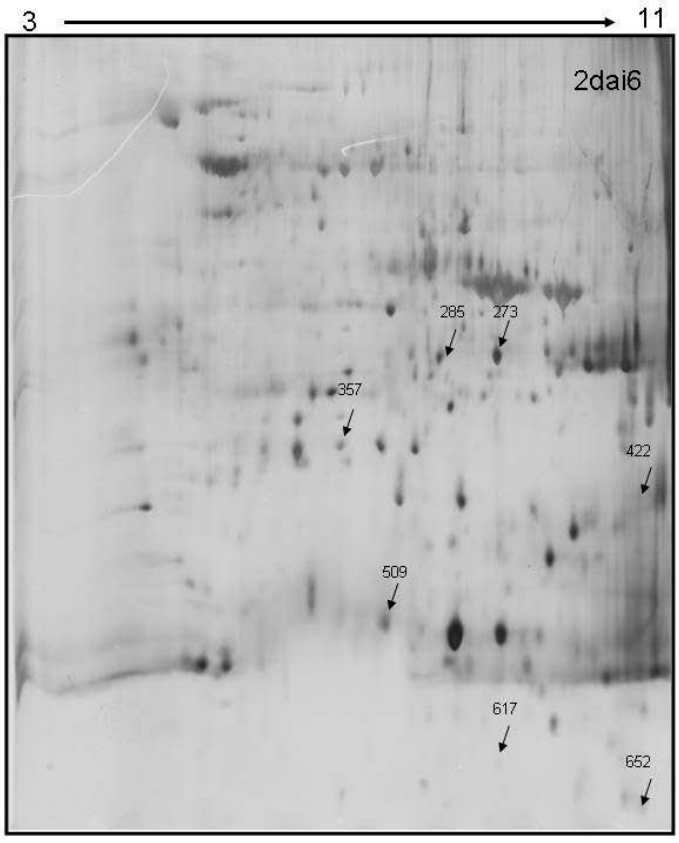





3
11
2dai3
285
273
357
422
509
617
652


3
11
2dai4
285
273
357
422
509
617
652


3
11
2dai5
285
273
357
422
509
617
652


3
11
2dai6
285
273
357
422
509
617
652

4. DISCUSSION

4 DISCUSSION

In this work we have examined different processes that take place in the maize scutellum during germination. Biological processes are often very complex, involving several genes, proteins and mechanisms. In consequence, different approaches are necessary in order to understand them. Proteomic and different transcriptomic approaches provide unvaluable information to understand complex processes.

In the grass caryopsis, the two main parts of the grain are the endosperm and the embryo. The embryo includes the scutellum, considered as a highly modified cotyledon (Higgins and Payne, 1977, 1978a, 1978b, 1981). The scutellum possess a vascular system (which completely develops after imbibition), a parenchyma (involved in the accumulation of storage molecules, mainly lipids), the epidermis (single cell layer in contact with the embryo axis) and the epithelium (single cell layer in contact with the endosperm).

The main role of the scutellum is the accumulation of nutrients but, during germination, the scutellum is also involved in the digestion and transport of the endosperm nutrients towards the embryo axis (Ranki and Sopanen, 1984; Negbi and Sargent, 1986). These roles involve morphological changes (epithelial cell elongation) and changes in gene and protein expression that we have examined here.

4.1 Control of the scutellar epithelial cell elongation during germination in maize (Zea mays)

A series of ultrastructural changes occur in the scutellar epithelium cells during germination. These changes include the partial degradation of the anticlinal walls, resulting in lateral disconnection of individual cells, and their simultaneous elongation that leads to the formation of cylindrical papillae (Swift and O'brien, 1972; Negbi and Sargent, 1986). The length of these cells was about doubled in 10 days after the beginning of the imbibition (Rao and Raju, 1985). These two processes (elongation and separation) produce an important increase in the surface contact area between endosperm and embryo, which allows an increase in the secretion and absorption rates. In some species like oat this adaptation is further developed and the apical region of the

scutellum elongates to form a papillate, finger-like projection that extends through the starchy endosperm towards the distal end of the grain (Negbi and Sargent, 1986).

Many factors are involved in epithelial cell elongation. For example, cell elongation involves wall loosening synthesis of new wall components, which requires energy (Kutschera, 1990). Hormones play a crucial role in regulating elongation process, in special ABA and GAs. ABA and GAs are key regulators of seed germination (Yamaguchi *et al.*, 2007). The germination-inhibiting effect of ABA is counteracted by gibberellins (GAs) and by ethylene. An important feature of GA and ABA metabolism is their interaction. ABA down-regulates GA biosynthesis and up-regulates GA deactivation, thus modulating seed ABA content (Kucera *et al.*, 2005; Holdsworth *et al.*, 2008; Linkies *et al.*, 2009; North *et al.*, 2010). Increase of amino acids uptake following germination was not affected by gibberellic or abscisic acid in barley scutellum but the availability of amino acids in general in the endosperm may regulate the development or the availability of the transport system (Sopanen *et al.*, 1980). This is in agreement with our results. Exogenous GAs stimulate elongation, a reduction in GAs synthesis or perception did not inhibit it despite reducing germination. Thus, although GAs stimulate cell elongation, they are not strictly necessary suggesting that the acceleration of the elongation produced by GAs could be an indirect consequence of the acceleration of germination. Experiments involving embryos of barley, wheat, rice and some subtropical cereals suggested that scutellar enzyme synthesis is largely insensitive to applied GA or GA biosynthetic inhibitors. It is possible either that hydrolase genes are not hormonally controlled in embryonic tissue, or that there are already high endogenous levels of GA. However, using a GA-deficient mutant of barley, it has been shown that high-pI α-amylase gene expression is also induced by GA in the scutellum (Woodger *et al.*, 2010).

ABA inhibits elongation and vp1 mutants (with reduced sensitivity to ABA) fail to undergo normal maturation and germinate (McCarty *et al.*, 1989). In dry seeds and at 1 day, the length/width ratio in the epithelial cells of vp1 was higher than in wt and no significant differences were observed between vp1 epithelial cells in dry seeds and after imbibition. In conclusion, ABA inhibits epithelium cell elongation and in vp1 mutant the elongation has occurred before seed desiccation.

Ethylene has important roles during the late phase of germination and counteracts the ABA inhibition by interfering with ABA signaling, but it does not affect ABA contents (Linkies et *al.*, 2009).

Ethylene has been implicated as a regulator of maize endosperm programmed cell death. A burst of ethylene synthesis is observed prior to the onset of whereas the application of an inhibitor of ethylene synthesis delays the onset of cell death. Moreover, mutations affecting starch biosynthesis such as shrunken2 (sh2) exhibited a premature onset and an accelerated execution of the programmed cell death program during endosperm development which correlates with a significantly higher level of ethylene production in mutant kernels (Young and Gallie, 2000). Ethylene positively influences seed germination (Ogawa *et al.*, 2003) and auxins are involved in embryo development (Chen and Charles An, 2006). However, neither these hormones nor cytokinins, seem to be involved in the regulation of epithelial cell elongation. Furthermore, 1 mM salicylic acid reduces cell elongation. Guan and Scandalios, (1995) reported an increase in catalase activity in maize scutellum in response to salicylic acid (SA), which may be one of the reasons of the decrease in cell elongation. However, SA at the concentration used here did not reduce germination, suggesting an at least partially different regulation of scutellar cell elongation and radicle extrusion. Cumulative evidences suggest a crosstalk between SA and ABA in the regulation of the responses to different stresses and in the regulation of other physiological processes like germination (Xie *et al.*, 2007). SA inhibition of scutellar cell elongation could be an aspect of the SA and ABA crosstalk.

The pH in the endosperm is reduced to 4.9–5.1 during germination due to the release by the aleurone layer of organic and phosphoric acids into the starchy endosperm (Martínez-Camacho *et al.*, 2004). The acidification process provides an optimum pH for several physiological events including phytate solubilization, hydrolytic activity of secreted enzymes, cell wall expansion and activity of nutrient transporters (Rayle and Cleland, 1992). Cell elongation is inhibited in acid environment, but at the pH 5 the elongation is only reduced, not inhibited. This reduction could be due to the increase of amino acids and peptide uptake activity during germination. This reduction of the elongation at pH 3-5 is contradictory with what it has been done in barley where peptide

transport is strongly pH dependent with a pH optimum of 3.8–5.0, which is similar to the pH of the endosperm (Higgins and Payne, 1977).

Scutellar epithelial cell elongation leads to an increase in the contact area between the endosperm and the scutellum and more peptide transport for the growth of the young seedling. Reduction of the elongation inhibits so peptide transport and peptide uptake and thereafter growth of the young plant. A low uptake activity was present in the scutella of ungerminated grains. It began to increase after 6 hours of imbibition and it wasn't affected by gibberellic or abscisic acid (Sopanen *et al.*, 1980).

Moreover, the application of 200 mM of sorbitol reduces cell elongation probably reducing water potential. However, the application of the same concentration of sucrose increased elongation rate. This could be explained by the fact that during the initial maize germination phases, a net flow of sucrose takes place from the scutellum towards the embryo axis and regions that undergo elongation. During this period, sucrose and hexose transporters, become the fundamental proteins that orchestrate the transport of nutrients required for successful germination and active cell elongation to support radicle extension (Sanchez-Linares *et al.*, 2012).

In addition, assimilable carbohydrates probably act as a source of energy and materials for cell wall synthesis. However, the source of carbohydrates should not be a limiting factor because cell elongation is not affected in the mutant Sh2 which contains about 25% of starch respect to wild type (Bhave *et al.*, 1990).

4.2 Expression profile of maize scutellar epithelium during imbibition

Germination represents a rapid transition from dormancy to a high level of metabolic activity. During this period, dramatic physiological changes occur. Lipid and protein reserves are mobilized in the germinating seed to provide energy, carbon and nitrogen for the seedling prior to the initiation of photosynthesis. In Arabidopsis, over 10,000 transcripts were differentially expressed during cold stratification, with equal numbers of up- and down-regulated genes, revealing an active period in preparing seeds for germination, where transcription and RNA degradation both play important roles (Narsai *et al.*, 2011). Very early upon imbibition massive transcriptome changes occur,

which are regulated by temperature, light and plant hormones, among others. ABA and GAs play a major role in regulating seed germination (Weitbrecht *et al.*, 2011).

Transcriptome analysis enables us to take a high-resolution snapshot of the cellular status by providing comprehensive mRNA profiles. These publicly available data are often utilized as a reference in seed biology to make hypotheses, to evaluate physiological results and to identify novel gene functions (Endo *et al.*, 2012).

Changes in the transcriptome following seed imbibition suggest a dynamic relationship between 'stored' RNAs and synthesis of new RNAs related to post-imbibition germinating- or dormant seed states. Transcriptome changes might reflect alteration in dormancy status or enhancement of germination vigor and effects on post-germination functions that relate to seedling growth (Holdsworth *et al.*, 2008). Recent post-genomics approaches suggest that RNA translation and protein post-translation modifications are also major levels of control for germination completion.

cDNA arrays represent a valuable resource to address the question raised above and to gain insight into the orchestration of gene expression in defined tissues. The differential expression profile of maize (*Zea mays* L) scutellar epithelium during imbibition compared to the other parts of the scutellum revealed a series of genes, with a high proportion involved in metabolic processes, the production of energy or in the transport of peptides into the embryo. Similar results have been observed in barley. Genes specifically expressed in the embryo and the scutellum are mainly derived from the functional classes of protein translation, carbohydrate metabolism, nucleotide metabolism, cell cycle and transporters (Potokina *et al.*, 2002; Jiménez-López *et al.*, 2011).

In maize, epithelial scutellar cells elongate and separate ones from the others during imbibition (Negbi and Sargent, 1986), which involves changes in their cell walls. Interestingly, two of the detected genes are involved in cell wall synthesis. The β-N-acetylhexosaminidase catalyzes the hydrolysis of N-acetyl-D-hexosamine and is involved in cell wall modifications. After the initial swelling is completed, all changes in seed size and shape during germination are caused by cell expansion. Expanding plant cells adjust the extensibility of their cell walls by remodelling the major components of the wall, the cellulose microfibrils and/or the pectin/hemicellulose

matrix. Loosening of the wall allows water influx which drives cell expansion and generates cellular turgor pressure (Schopfer, 2006).

This led to the model that embryo growth during germination depends primarily on changes in cell wall extensibility. Holdsworth and collaborators (2008) shown that imbibed after-ripened Arabidopsis seeds show large increases in RNAs encoding proteins associated with cell wall modification in comparison with dormant seeds. Similar changes are also observed at the proteome level (Mak *et al.*, 2009; Rajjou *et al.*, 2011).

One of the selected genes encodes an aspartic proteinase nepenthesin II, which belongs to a proteinase family typical of carnivorous plants (Takahashi *et al.*, 2005). This protein is an extracellular proteinase found in the pitcher fluid of carnivorous plants and is involved in the digestion prey for nitrogen uptake. Almost nothing is known about their role in other plants or organs, but the presence of this enzyme fulfill the major functions of the tissue: production of hydrolytic enzymes, essential for storage protein catabolism in the endosperm. Moreover, consistent with the function of the scutellum in transporting protein degradation products, PTR mRNA from the carnivorous plant *Nepenthes* was detected in phloem cells of the pitcher organs of the plant (Schulze *et al.*, 1999).

Another of the identified genes encodes for the allene oxide synthase which catalyzes the first step in the biosynthesis of Ketol (e.g., KHOD) and cyclopentenone-type oxylipins (e.g., jasmonate) (Vellosillo *et al.*, 2007). Oxylipins were considered to function as signals for triggering plant development or helping the plant respond to abiotic and biotic stresses (Yokoyama *et al.*, 2000; Blée, 2002; Howe and Schilmiller, 2002; Balbi and Devoto, 2008). The possible role of oxylipins in the germinating epithelial cells needs to be addressed.

About 3% of the genes identified in our array analyses have a transport activity. Concordant results have been in detailed transcriptome analysis of barley (*Hordeum vulgare*) grain maturation, desiccation, and germination in two tissue fractions (starchy endosperm/aleurone and embryo/scutellum) using the Affymetrix Barley1 GeneChip where genes for amino acid metabolism, including Asp-derived Met, homo- Ser, and homo-Cys pathways, are activated in the embryo during germination.

Amino acid biosynthesis genes during germination in the embryo/scutellum provides a major nitrogen source for the developing embryo, where large numbers of amino acid, peptide, oligo peptide, and ABC transporters are abundantly expressed in comparison to endosperm/aleurone (Sreenivasulu *et al.*, 2008). These transport processes in the germinating embryo seem to be vital as reported previously for peptide transporters of the barley scutellum (Waterworth *et al.*, 2000). During germination, the nutrients accumulated in the seeds must be transformed into soluble molecules and transported quickly to the embryonic axis to nourish its growth until it is able to perform the photosynthetic function. Enzymatic hydrolysis of starch and storage proteins in the endosperm during germination forms a reservoir of sugars, small peptides and amino acids which are translocated across the scutellum to supply the growing embryo (West *et al.*, 1998).

Finally, the role of many of the genes identified in this study was unknown. Their number will gradually decrease as databases become more complete. Additional information on their tissue specificity and their temporal regulation patterns will assist to elucidate their function.

4.3 ZmPTR1, a maize peptide transporter expressed in the epithelial cells of the scutellum during germination

During cereal grain germination, endosperm protein reserves are hydrolyzed by the concerted action of proteinases and peptidases to form a pool of small peptides and amino acids that are translocated into the embryo across the scutellum, a specialized absorptive tissue which separates the endosperm from the embryo structures.

In this study, a new maize peptide transporter *ZmPTR1* was analyzed. This protein is member of the PTR/NTR1 family. Sequence analysis of the full length mRNA (2295bp) shows that *ZmPTR1* encodes a 587 amino acid protein of predicted molecular mass 64.52 kDa, which displayed 69% identity to the *Arabidopsis thaliana* peptide transporter AtPTR2-B (At2g02040), 59% identity to AtPTR4 (At2g02020) and 62% identity to AtPTR6 (At1g62200). Moreover, ZmPTR1 includes the conserved motif and the transmembrane domains typical of these peptide transporter proteins.

ZmPTR1 is expressed in many plant tissues including scutellum, roots and shoots but highest expression levels are found during germination.

In roots, ZmPTR1 could have the same function as the AtPTR2-A which is expressed in roots and is responsible for absorption of small amounts of organic nitrogen in the form of peptides from the soil (Steiner *et al.*, 1994). Germination and growth studies with ptr1 mutants showed that PTR1 also contributes to dipeptide uptake into roots (Komarova *et al.*, 2008). In the scutellum, the function of ZmPTR1 could be involved in the rapid absorption and thereby transport of endosperm peptides from the hydrolysis of cereal endosperm storage proteins like what has been suggested in the scutellum of germinating barley grain (Higgins and Payne, 1981).

During cereal grain germination, endosperm protein reserves are hydrolyzed by the concerted action of proteinases and peptidases to form a pool of small peptides and amino acids that are translocated into the embryo across the scutellum, a specialized absorptive tissue which separates the endosperm from the embryo structures. Peptide transport plays a vital role in cereal grain germination because peptides, rather than amino acids, form the initial products of endosperm protein breakdown, to provide nutrients for initiation of growth processes (Higgins and Payne, 1977). In germinating cereal grains, peptide transport is solely responsible for the supply of organic nitrogen to the embryo during germination, whereas amino acid transport is essentially a post-germinative event which only becomes significant 48–72 h after the commencement of imbibition (Waterworth *et al.*, 2000).

Like Arabidopsis PTR2, PTR4 and PTR6 (Chiang *et al.*, 2004; Shimaoka *et al.*, 2004; Weichert *et al.*, 2012; Komarova *et al.*, 2012), ZmPTR1 has a tonoplast localization and N-terminal ZmPTR1 contains a peptide similar to the dileucine motif described for tonoplast localization which suggests a role for peptide uptake as well as intracellular peptide transport (Komarova *et al.*, 2012). It is likely that the peptides transported into the vacuole are subsequently degraded to free amino acids by vacuolar endoproteases and exopeptidases. It is also hypothesized that the proteasome and perhaps other, so far unknown, cytosolic proteolytic activities produce peptides from cellular proteins which are readily transported into the vacuole in order to be degraded and recycled to sustain plant metabolism (Ramos *et al.*, 2011).

Experiments showed that ZmPTR1 restores growth of the Arabidopsis plants when cultivated on growth medium where the dipeptide Ala-Ala was the only source of N, implying that ZmPTR1 mediates the uptake of this dipeptide. It has been previously demonstrated that uptake experiments done by Salmenkallio and Sopanen, (1989) showed a more effective uptake of Ala-Ala and Ala-Ala-Ala by maize than by the other cereals (barley, wheat and rice). In addition, when expressed in *Saccharomyces cerevisiae*, AtPTR2-B transports a wide range of dipeptide but has a poor affinity for Gly-Ala and Gly-Gly. It also transports the tripeptide Leu-Leu-Leu. Both AtPTR2-A and B better transport peptides containing one or more hydrophobic amino acid (Ala, Leu and Met), but AtPTR2-B has a lower capacity for toxic peptides fluorophenylalanine and oxalysine than AtPTR2-A (Song *et al.*, 1997; Chiang *et al.*, 2004).

AtPTR5 overexpressing lines promoted better growth of certain dipeptides Ala-Ala, Ala- Asn, and Pro-Ala whereas Ala-Phe and Lys-Asp inhibited growth, indicating that their increased uptake caused a toxic effect either of the peptides or more likely by the amino acids generated (Komarova *et al.*, 2008). Hammes and colaboradors (2010) have shown that the voltage dependence of the apparent affinities differed between Ala-Ala, Ala-Lys, and Ala-Asp and was not conserved between the Arabidopsis transporters AtPTR1 and AtPTR5. Although the substrate range recognized by ZmPTR1 remains to be investigated, we can conclude that it is a functionally active peptide transporter.

4.4 Isolation of proteins associated with oil bodies of maize scutellum

Cereal grains store carbon in the form of starch in the endosperm and oil in the embryo. The kernel of maize is rich in oil. In traditional varieties, the kernel contains 4% oil by weight, this oil being mainly accumulated in the embryo (preferentially in the scutellum) and in the aleurone layer (Barthole *et al.*, 2012). In seed cells, oil is accumulated in subcellular oil bodies (OBs), which consist in a matrix of oil (triacylglycerol) surrounded by a layer of phospholipids embedded with abundant structural proteins, mainly, but not only oleosins. Oleosins contain a unique hydrophobic domain which appears to be inserted into the oil matrix as an α-helical hairpin and stabilize OBs (Capuano *et al.*, 2007).

The expression of the maize 16-KDa and 18-KDa olesosin are coordinated and tissue specific, being expressed only in the embryo (scutellum and axis) and the aleurone layer. OBs are degraded during early stages of germination as a carbon source for the germinating seedling. This lipoxygenase reaction leads to a transient accumulation of ester lipid hydroperoxides in the storage lipids, and the corresponding oxygenated fatty acid moieties are preferentially removed by specific lipases. The free hydroperoxy fatty acids are subsequently reduced to their hydroxy derivatives, which might in turn undergo β-oxidation (Feussner *et al.*, 2001).

In the first paper (Chapter 3.4.1) we examined the protein composition of the OBs during germination in the scutellum of maize seeds 2 days after imbibition. We identified some of the most detected spots. In the second paper (Chapter 3.4.2), we did a quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of maize seeds. Although the two experiments are different and we couldn't compare between them because the spots of the first paper were randomly selected and those of the second one were statistically selected, we could discuss some differences in the results.

Oleosin, the most abundant OB associated protein, have been identified in only one spot in the first study whereas in seven spots in the second study. This is because the predicted pI for oleosin is 9.7 and the pH gradient (IPG) strips used in the first study were from pH 4 to 7 whereas those used in the second study were from pH 3 to 11. Consequently, an important part of the oleosins was lost during first dimension IEF in the first study. In addition, oleosins are especially poor candidates for bidimensional electrophoresis, due to their alkaline pI and to their low solubility in aqueous media (Jolivet *et al.*, 2004).

Caleosins are OB-associated proteins that possess Ca2+-dependent peroxygenase activity and play a role in the degradation of storage lipid in OBs during germination. Its role involves both the normal modification of storage vacuole membrane and the interaction of OBs with vacuoles (Poxleitner et al., 2006). Recent studies have demonstrated that one of the Arabidopsis non-seed caleosins, AtCLO3, is involved in controlling stomatal aperture during the drought response and that the OB-associated Ca2+-binding AtCLO4 protein acts as a negative regulator of ABA responses in Arabidopsis (Kim *et al.*, 2011).

Steroleosin, a third family of OB-associated proteins, are proposed to be regulated by specific sterols to conduct different biological functions possibly related to the formation or degradation of seed OBs (Hsiao and Tzen, 2011), or in signal transduction (Lin *et al.*, 2002).

In *Brassica*, protein and lipid composition analysis of OBs from two *Brassica napus* cultivars have identified oleosin, putative embryo specific protein ATS1, (similar to caleosin), 11-beta-hydroxysteroid dehydrogenase-like protein (steroleosin), and, as we did, several new proteins (Katavic *et al.*, 2006).

A prohibitin has been identified in our comparative study. These proteins are an extensively studied family of proteins that are highly conserved between animals and plants. Prohibitin, a potential tumor suppressor protein, has been shown to inhibit cell proliferation and repress E2F transcriptional activity. Though prohibitin has potent transcriptional functions in the nucleus, a mitochondrial role for prohibitin has also been proposed (Fusaro *et al.*, 2003). Prohibitins serve many functions in such diverse processes as apoptosis and aging, cell cycle progression, tumorigenesis, transcriptional regulation, signaling, oxidative damage, respiration, and mitochondrial biogenesis. The identification of this protein could be related with cell death during germination, but its functional relationship with OBs is not clear yet. It may play a role in OB degradation.

Lipolytic enzymes catalyze the first step of lipid mobilization, with the possibility of subsequently being controlled during and after the germination period (Quettier and Eastmond, 2009; Borgston and Brockman, 1984). The lipase has a very important role in oil degradation and in programmed cell death. The mobilization of the storage oil in the cotyledons of rapeseed seedlings (*Brassica napus* L.) starts about 24 h after imbibition. OBs from cotyledons of rapeseeds started to show autolysis between day 1 and day 2 of germination, with optimum activity at pH 5.0, while optimum activity of the cytoplasmic soluble lipase was at pH 7.0 with native OBs as substrate (Hoppe and Theimer, 1997).

OBs in germinating seeds are not degraded simultaneously. Consumption of triacylglycerols reduced gradually the total amount of OBs in seedlings during germination, whereas no alteration was observed in the integrity of remaining OBs.

It is suggested that glyoxisomes, with the assistance of mitochondria, fuse and digest OBs one, or few, at a time, while the remaining OBs are preserved intact during the whole period of germination (Tzen *et al.*, 1997).

We have not identified peptides belonging to enzymes from the lipid metabolism pathway as lipases as it could be expected. These enzymes could be present in the OBs at low amounts. Although the lipase prepared from 4-day-old rapeseed seedlings hydrolyzes the TAG of the native OBs completely, such lipase was found only with OBs isolated from seedlings at least 2 days old, but not from OBs obtained from dry seeds or l-day-old seedlings. The inhibition of the action of lipase is caused by the half unit membrane surrounding OBs (Hoppe and Theimer, 1997).

5. CONCLUSIONS

5 CONCLUSIONS

- During germination, scutellar epithelial cells suffer an elongation that increases the contact surface between the endosperm and the scutellum and facilitates the transport of the nutrients from the endosperm to the embryo.

- Scutellar cell elongation is inhibited by ABA and salicylic acid, basic and acid pH and high concentrations of sorbitol. Exogenous gibberellins stimulate elongation, but a reduction in gibberellin synthesis or perception does not inhibit it. Elongation is inhibited by sucrose, but not glucose.

- Transcription and translation inhibitors reduce scutellar cell elongation, indicating that transcription and translation are necessary for the elongation process.

- We identified 30 genes showing a significant high expression in scutellar epithelial cells of one day after imbibition. The roles of 43% of these genes remains undetermined, 27% of them are involved in metabolic processes, 13% in protein synthesis or processing and 7% in cell structure.

- ZmPTR1 encode a non-characterized maize peptide transporter protein which has 587 amino acids with a calculated molecular mass of 64.52 kDa. This maize transporter is predominantly expressed in the scutellar epithelium during germination. *ZmPTR1* is also expressed to a less extent in the radicle and the hypocotyl.

- ZmPTR1 is located in the tonoplast and has high sequence similarity with tonoplast di- and tripeptide transporters AtPTR2, AtPTR4 and AtPTR6 from *Arabidopsis thaliana*.

- ZmPTR1 is able to transport at least Ala-Ala dipeptide across the membrane and could have a role in the intracellular transport of di- and tripeptides.

- Protein composition analysis of oil bodies from maize embryos during germination identified, in addition to the previously characterized OB-associated proteins, other proteins of diverse function: an embryonic protein DC-8, a globulin 2, 4 proteins with enzymatic activity (protein disulfide isomerase, xylose isomerase, strictosidine synthase and precursor and ATP synthase beta chain), a protein similar to karyopherin-beta-3 (Kap) and a stress induced membrane pore protein involved in membrane transport.

- Quantitative subproteomic analysis of germinating related changes in the oil bodies of maize scutellum between dry seeds and 2 dai seeds allowed the identification of new proteins interacting with oil bodies in dry seeds or in germinating seeds. In dry seeds: oleosins, cupins, disulfide isomerases, a nucleoside phosphate kinase, a class IV heat shock protein, an embryonic protein DC-8, a 60S acidic ribosomal protein P0 and a rubber elongation factor protein. In germinating seeds: oleosins, mitochondrial protein Tim17, prohibitin-2 and a manganese superoxide dismutase (Mn-SOD).

- Oil bodies interact with other plant cell organelles during germination, as, for example, the mitochondria.

- Plant seed oil bodies may serve as transient storage depots for proteins that lack appropriate binding partners in the cell or are temporally in excess, protecting the proteins from degradation, and protecting the cell from detrimental effects of uncontrolled protein activity.

6. REFERENCES

6 REFERENCES

1. **Aalen R. B.** 1995. The transcripts encoding two oleosin isoforms are both present in the aleurone and in the embryo of barley (Hordeum vulgare L.) seeds. Plant molecular biology **28**:583-8.

2. **Arcalis E., J. Stadlmann, S. Marcel, G. Drakakaki, V. Winter, J. Rodriguez, R. Fischer, F. Altmann, and E. Stoger**. 2010. The changing fate of a secretory glycoprotein in developing maize endosperm. Plant physiology **153**:693-702.

3. **Aubert Y., D. Vile, M. Pervent, D. Aldon, B. Ranty, T. Simonneau, A. Vavasseur, and J.-P. Galaud**. 2010. RD20, a stress-inducible caleosin, participates in stomatal control, transpiration and drought tolerance in Arabidopsis thaliana. Plant & cell physiology **51**:1975-87.

4. **Balbi V., and A. Devoto**. 2008. Jasmonate signalling network in Arabidopsis thaliana: crucial regulatory nodes and new physiological scenarios. The New phytologist **177**:301-18.

5. **Barros M., L. Fleuri, and G. Macedo**. 2010. Seed lipases: sources, applications and properties-a review. Brazilian Journal of Chemical Engineering. SciELO Brasil **27**:15–29.

6. **Barthole G., L. Lepiniec, P. M. Rogowsky, and S. Baud**. 2012. Controlling lipid accumulation in cereal grains. Plant Science. Elsevier Ireland Ltd **185-186**:33-9.

7. **Bethke P. C., S. J. Swanson, S. Hillmer, and R. L. Jones**. 1998. From Storage Compartment to Lytic Organelle: The Metamorphosis of the Aleurone Protein Storage Vacuole. Annals of Botany **82**:399-412.

8. **Bewley J. D.** 1997. Seed Germination and Dormancy. The Plant cell **9**:1055-1066.

9. **Bhatla S. C., V. Kaushik, and M. K. Yadav**. 2010. Use of oil bodies and oleosins in recombinant protein production and other biotechnological applications. Biotechnology advances. Elsevier Inc. **28**:293-300.

10. **Bhave M. R., S. Lawrence, C. Barton, and L. C. Hannah**. 1990. Identification and Molecular Characterization of Shrunken-2 cDNA Clones of Maize. The Plant cell **2**:581-588.

11. **Blée E.** 2002. Impact of phyto-oxylipins in plant defense. Trends in plant science **7**:315-22.

12. **Bogs J., A. Bourbouloux, O. Cagnac, A. Wachter, T. Rausch, and S. Delrot**. 2003. Functional characterization and expression analysis of a glutathione transporter , BjGT1 , from Brassica juncea : evidence for regulation of heavy metal exposure. Plant, Cell and Environment **26**:1703-1711.

13. **Bonsegna S., S. Bettini, R. Pagano, A. Zacheo, V. Vergaro, G. Giovinazzo, G. Caminati, S. Leporatti, L. Valli, and A. Santino**. 2011. Plant oil bodies: novel carriers to deliver lipophilic molecules. Applied biochemistry and biotechnology **163**:792-802.

14. **Briggs D. E.** 1972. Enzyme formation, cellular breakdown and the distribution of gibberellins in the endosperm of barley. Planta. Springer Berlin / Heidelberg **108**:351-358.

15. **Cagnac O., A. Bourbouloux, D. Chakrabarty, Z. Min-Yong, and S. Delrot**. 2004. AtOPT6 transports glutathione derivatives and is induced by primisulfuron. Plant physiology **135**:1378-1387.

16. **Cao J., J. Huang, Y. Yang, and X. Hu**. 2011. Analyses of the oligopeptide transporter gene family in poplar and grape. BMC genomics. BioMed Central Ltd **12**:465.

17. **Capuano F., F. Beaudoin, J. a Napier, and P. R. Shewry**. 2007. Properties and exploitation of oleosins. Biotechnology advances **25**:203-6.

18. **Cejudo F. J., G. Murphy, and D. C. Baulcombe**. 1992. A gibberellin-regulated gene from wheat with sequence homology to cathepsin B of mammalian cells. The Plant Journal **2**:937-948.

19. **Chen J. C., C. C. Tsai, and J. T. Tzen**. 1999. Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. Plant & cell physiology. Jpn Soc Plant Physiol **40**:1079-86.

20. **Chen K., and Y.-Q. Charles An**. 2006. Transcriptional responses to gibberellin and abscisic acid in barley aleurone. Journal of Integrative Plant Biology **48**:591-612.

21. **Chen M. C. M., C.-L. Chyan, T. T. T. Lee, S.-H. Huang, and J. T. C. Tzen**. 2004. Constitution of stable artificial oil bodies with triacylglycerol, phospholipid, and caleosin. Journal of agricultural and food chemistry **52**:3982-7.

22. **Chiang C.-S., G. Stacey, and Y.-F. Tsay**. 2004. Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2. The Journal of biological chemistry **279**:30150-7.

23. **Chávez-Bárcenas A. T., J. J. Valdez-Alarcón, M. Martínez-Trujillo, L. Chen, B. Xoconostle-Cázares, W. J. Lucas, and L. Herrera-Estrella**. 2000. Tissue-Specific and Developmental Pattern of Expression of the Rice sps1 Gene 1. Plant physiology **124**:641-653.

24. **Cruz Pérez A., V. González Hernández, M. Mendoza Castillo, and M. Ortega Delgado**. 2003. Marcadores fisiológicos de la tolerancia al envejecimiento de semilla en maíz. Agrociencia **37**:371-381.

25. **Curie C., Z. Panaviene, C. Loulergue, S. L. Dellaporta, J.-F. Briat, and E. L. Walker**. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature **409**:346-349.

26. **Daussant J., S. Miyata, T. Mitsui, and T. Akazawa**. 1982. Enzymic mechanism of starch breakdown in germinating rice seeds. Plant physiology **71**:88-95.

27. **Dietrich D., U. Hammes, K. Thor, M. Suter-Grotemeyer, R. Flückiger, A. J. Slusarenko, J. M. Ward, and D. Rentsch**. 2004. AtPTR1, a plasma membrane peptide transporter expressed during seed germination and in vascular tissue of Arabidopsis. The Plant journal : for cell and molecular biology **40**:488-99.

28. **Doan N., and G. B. Fincher**. 1988. The A-and B-chains of carboxypeptidase I from germinated barley originate from a single precursor polypeptide. Journal of Biological Chemistry **263**:11106-11110.

29. **El-Metainy A. Y., and A. A. Omar**. 1981. Biochemical genetics of *Zea mays* L. I. Isozymes and the linkage relationship between AP-1 and AP-2 loci. Biochemical Genetics. Springer Netherlands **19**:635-640.

30. **Enari T.-M., and J. Mikola**. 2008. Peptidases in Germinating Barley Grain: Properties, Localization and Possible Functions, pp. 334-352. *In* Ciba Foundation Symposium 50 - Peptide Transport and Hydrolysis. John Wiley & Sons, Ltd.

31. **Endo A., K. Tatematsu, K. Hanada, L. Duermeyer, M. Okamoto, K. Yonekura-Sakakibara, K. Saito, T. Toyoda, N. Kawakami, Y. Kamiya, M. Seki, and E. Nambara**. 2012. Tissue-specific transcriptome analysis reveals cell wall metabolism, flavonol biosynthesis and defense responses are activated in the endosperm of germinating Arabidopsis thaliana seeds. Plant & cell physiology **53**:16-27.

32. **Evers T., and S. Millar**. 2002. Cereal Grain Structure and Development: Some Implications for Quality. Journal of Cereal Science **36**:261-284.

33. **Fath a, P. Bethke, J. Lonsdale, R. Meza-Romero, and R. Jones**. 2000. Programmed cell death in cereal aleurone. Plant molecular biology **44**:255-66.

34. **Feussner I., H. Kühn, and C. Wasternack**. 2001. Lipoxygenase-dependent degradation of storage lipids. Trends in plant science. Elsevier **6**:268–273.

35. **Frandsen G. I., J. Mundy, and J. T. C. Tzen**. 2001. Oil bodies and their associated proteins, oleosin and caleosin. Physiologia Plantarum **112**:301-307.

36. **Frommer W. B., S. Hummel, and D. Rentsch**. 1994. Cloning of an Arabidopsis histidine transporting protein related to nitrate and peptide transporters. FEBS letters **347**:185-9.

37. **Fujimoto T., and R. G. Parton**. 2011. Not Just Fat: The Structure and Function of the Lipid Droplet. Cold Spring Harbor Perspectives in Biology **3**.

38. **Fusaro G., P. Dasgupta, S. Rastogi, B. Joshi, and S. Chellappan**. 2003. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. The Journal of biological chemistry **278**:47853-61.

39. **Gibbons G.** 1981. On the relative role of the scutellum and aleurone in the production of hydrolases during germination of barley. Carlsberg Research Communications **46**:215-225.

40. **Glass A. D. M., D. T. Britto, B. N. Kaiser, J. R. Kinghorn, H. J. Kronzucker, A. Kumar, M. Okamoto, S. Rawat, M. Y. Siddiqi, S. E. Unkles, and J. J. Vidmar**. 2002. The regulation of nitrate and ammonium transport systems in plants. Journal of experimental botany **53**:855-64.

41. **González M., L. Osuna, C. Echevarría, J. Vidal, and F. Cejudo**. 1998. Expression and localization of phosphoenolpyruvate carboxylase in developing and germinating wheat grains. Plant physiology **116**:1249-1258.

42. **Graham I. a**. 2008. Seed storage oil mobilization. Annual review of plant biology **59**:115-42.

43. **Guan L., and J. G. Scandalios**. 1995. Developmentally related responses of maize catalase genes to salicylic acid. Proceedings of the National Academy of Sciences of the United States of America **92**:5930-4.

44. **Gurusamy C., P. Davis, and A. Bal**. 2000. Seasonal changes in perennial nodules of beach pea (Lathyrus maritimus Bigel.) with special reference to oleosomes. International Journal of Plant Sciences **161**:631-638.

45. **Hammes U. Z., S. Meier, D. Dietrich, J. M. Ward, and D. Rentsch**. 2010. Functional properties of the Arabidopsis peptide transporters AtPTR1 and AtPTR5. The Journal of biological chemistry **285**:39710-7.

46. **Hanano A., M. Burcklen, M. Flenet, A. Ivancich, M. Louwagie, J. Garin, and E. Blée**. 2006. Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. The Journal of biological chemistry **281**:33140-51.

47. **Hardy D. J., and J. W. Payne**. 1992. Analysis of the peptide carrier in the scutellum of barley embryos by photoaffinity labelling. Planta. Springer Berlin / Heidelberg **186**:44-51.

48. **Hargin K., W. Morrison, and R. Fulcher**. 1980. Triglyceride deposits in the starchy endosperm of wheat. Cereal Chemistry **57**:320–325.

49. **Heneen W. K., G. Karlsson, K. Brismar, P.-O. Gummeson, S. Marttila, S. Leonova, A. S. Carlsson, M. Bafor, A. Banas, B. Mattsson, H. Debski, and S. Stymne**. 2008. Fusion of oil bodies in endosperm of oat grains. Planta **228**:589-99.

50. **Henikoff S., E. a Greene, S. Pietrokovski, P. Bork, T. K. Attwood, and L. Hood**. 1997. Gene families: the taxonomy of protein paralogs and chimeras. Science (New York, N.Y.) **278**:609-14.

51. **Herman E., and B. Larkins**. 1999. Protein storage bodies and vacuoles. The Plant cell **11**:601-14.

52. **Hernandez-Pinzon I., K. Patel, and D. J. Murphy**. 2001. The Brassica napus calcium-binding protein, caleosin, has distinct endoplasmic reticulum-and lipid body-associated isoforms. Plant Physiology and Biochemistry. Elsevier **39**:615–622.

53. **Higgins C. F., and J. W. Payne**. 1977. Characterization of active dipeptide transport by germinating barley embryos: Effects of pH and metabolic inhibitors. Planta. Springer Berlin / Heidelberg **136**:71-76.

54. **Higgins C. F., and J. W. Payne**. 1977. Peptide transport by germinating barley embryos. Planta. Springer Berlin / Heidelberg **134**:205-206.

55. **Higgins C. F., and J. W. Payne**. 1978. Peptide transport by germinating barley embryos: Uptake of physiological di- and oligopeptides. Planta. Springer Berlin / Heidelberg **138**:211-215.

56. **Higgins C. F., and J. W. Payne**. 1978. Stereospecificity of peptide transport by germinating barley embryos. Planta. Springer Berlin / Heidelberg **142**:299-305.

57. **Higgins C. F., and J. W. Payne**. 1981. The Peptide pools of germinating barley grains: relation to hydrolysis and transport of storage proteins. Plant physiology **67**:785-92.

58. **Hiremath S., C. Patil, and K. Patil**. 2007. Genetic diversity of seed lipid content and fatty acid composition in some species of Sesamum L.(Pedaliaceae). African Journal of Biotechnology **6**:539-543.

59. **Holdsworth M. J., L. Bentsink, and W. J. J. Soppe**. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. The New phytologist **179**:33-54.

60. **Holdsworth M. J., W. E. Finch-Savage, P. Grappin, and D. Job**. 2008. Post-genomics dissection of seed dormancy and germination. Trends in plant science **13**:7-13.

61. **Hoppe A., and R. R. Theimer**. 1997. Degradation of oil bodies isolated from cotyledons during germination of rapeseed seedlings. Journal of Plant Physiology. Gustav Fischer Verlag, Jena **151**:471-478.

62. **Howe G. a, and A. L. Schilmiller**. 2002. Oxylipin metabolism in response to stress. Current opinion in plant biology **5**:230-6.

63. **Hsiao E. S. L., and J. T. C. Tzen**. 2011. Ubiquitination of oleosin-H and caleosin in sesame oil bodies after seed germination. Plant physiology and biochemistry : PPB / Société française de physiologie végétale. Elsevier Masson SAS **49**:77-81.

64. **Huang a H.** 1996. Oleosins and oil bodies in seeds and other organs. Plant physiology **110**:1055-61.

65. **Huang a H. C.** 1992. Oil Bodies and Oleosins in Seeds. Annual Review of Plant Physiology and Plant Molecular Biology **43**:177-200.

66. **Hueros G., E. Gomez, N. Cheikh, J. Edwards, M. Weldon, F. Salamini, and R. Thompson**. 1999. Identification of a promoter sequence from the BETL1 gene cluster able to confer transfer-cell-specific expression in transgenic maize. Plant physiology **121**:1143-52.

67. **Hueros G., S. Varotto, F. Salamini, and R. D. Thompson**. 1995. Molecular characterization of BET1, a gene expressed in the endosperm transfer cells of maize. The Plant cell **7**:747-57.

68. **Jacobsen J. V., R. B. Knox, and N. A. Pyliotis**. 1971. The structure and composition of aleurone grains in the barley aleurone layer. Planta. Springer Berlin / Heidelberg **101**:189-209.

69. **Jerkovic A., A. M. Kriegel, J. R. Bradner, B. J. Atwell, T. H. Roberts, and R. D. Willows**. 2010. Strategic distribution of protective proteins within bran layers of wheat protects the nutrient-rich endosperm. Plant physiology **152**:1459-70.

70. **Jiang P. L., and J. T. C. Tzen**. 2010. Caleosin serves as the major structural protein as efficient as oleosin on the surface of seed oil bodies. Plant signaling & behavior. ACS Publications **5**:447.

71. **Jiang P.-L., G.-Y. Jauh, C.-S. Wang, and J. T. C. Tzen**. 2008. A unique caleosin in oil bodies of lily pollen. Plant & cell physiology **49**:1390-5.

72. **Jiménez-López S., E. Mancera-Martínez, A. Donayre-Torres, C. Rangel, L. Uribe, S. March, G. Jiménez-Sánchez, and E. Sánchez de Jiménez**. 2011. Expression profile of maize (Zea mays L.) embryonic axes during germination: translational regulation of ribosomal protein mRNAs. Plant & cell physiology **52**:1719-33.

73. **Jolivet P., C. Boulard, A. Bellamy, C. Larré, M. Barre, H. Rogniaux, S. d' Andréa, T. Chardot, and N. Nesi**. 2009. Protein composition of oil bodies from mature Brassica napus seeds. PROTEOMICS. WILEY-VCH Verlag **9**:3268-3284.

74. **Jolivet P., E. Roux, S. D'Andrea, M. Davanture, L. Negroni, M. Zivy, and T. Chardot**. 2004. Protein composition of oil bodies in Arabidopsis thaliana ecotype WS. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **42**:501-9.

75. **Jones R. L.** 1969. The fine structure of barley aleurone cells. Planta. Springer Berlin / Heidelberg **85**:359-375.

76. **Kang J., J. Park, H. Choi, B. Burla, T. Kretzschmar, Y. Lee, and E. Martinoia**. 2011. Plant ABC Transporters. The Arabidopsis book / American Society of Plant Biologists **9**:e0153.

77. **Karim S., K.-O. Holmström, A. Mandal, P. Dahl, S. Hohmann, G. Brader, E. T. Palva, and M. Pirhonen**. 2007. AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in Arabidopsis. Planta **225**:1431-45.

78. **Karim S., D. Lundh, K.-O. Holmström, A. Mandal, and M. Pirhonen**. 2005. Structural and functional characterization of AtPTR3, a stress-induced peptide transporter of Arabidopsis. Journal of molecular modeling **11**:226-36.

79. **Karrer E., and R. Rodriguez**. 1992. Metabolic regulation of rice α-amylase and sucrose synthase genes in planta. The Plant Journal **2**:517-523.

80. **Katavic V., G. K. Agrawal, M. Hajduch, S. L. Harris, and J. J. Thelen**. 2006. Protein and lipid composition analysis of oil bodies from two Brassica napus cultivars. Proteomics **6**:4586-98.

81. **Kim Y. Y., K. W. Jung, K. S. Yoo, J. U. Jeung, and J. S. Shin**. 2011. A stress-responsive caleosin-like protein, AtCLO4, acts as a negative regulator of ABA responses in Arabidopsis. Plant & cell physiology **52**:874-84.

82. **Kimura M., and E. Nambara**. 2010. Stored and neosynthesized mRNA in Arabidopsis seeds: effects of cycloheximide and controlled deterioration treatment on the resumption of transcription during imbibition. Plant molecular biology **73**:119-29.

83. **Koh S., A. Wiles, J. Sharp, F. Naider, J. Becker, and G. Stacey**. 2002. An oligopeptide transporter gene family in Arabidopsis. Plant physiology **128**:21-29.

84. **Komarova N. Y., S. Meier, A. Meier, M. S. Grotemeyer, and D. Rentsch**. 2012. Determinants for Arabidopsis Peptide Transporter Targeting to the Tonoplast or Plasma Membrane. Traffic n/a-n/a.

85. **Komarova N. Y., K. Thor, A. Gubler, S. Meier, D. Dietrich, A. Weichert, M. Suter Grotemeyer, M. Tegeder, and D. Rentsch**. 2008. AtPTR1 and AtPTR5 transport dipeptides in planta. Plant physiology **148**:856-69.

86. **Kucera B., M. A. Cohn, and G. Leubner-Metzger**. 2005. Plant hormone interactions during seed dormancy release and germination. Seed Science Research **15**:281-307.

87. **Kumamaru T., M. Ogawa, H. Satoh, and T. Okita**. 2007. Protein Body Biogenesis in Cereal Endosperms Endosperm, pp. 141-158. *In* O.-A. Olsen (ed.), Springer Berlin / Heidelberg.

88. **Kutschera U.** 1990. Cell-wall synthesis and elongation growth in hypocotyls of Helianthus annuus L. Planta. Springer Berlin / Heidelberg **181**:316-323.

89. **Lin L. J., S. S. K. Tai, C. C. Peng, and J. T. C. Tzen**. 2002. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. Plant physiology. Am Soc Plant Biol **128**:1200.

90. **Lin L., S. Tai, and C. Peng**. 2002. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. Plant physiology **128**:1200-1211.

91. **Lin L.-J., P.-C. Liao, H.-H. Yang, and J. T. C. Tzen**. 2005. Determination and analyses of the N-termini of oil-body proteins, steroleosin, caleosin and oleosin. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **43**:770-6.

92. **Lin L.-J., and J. T. C. Tzen**. 2004. Two distinct steroleosins are present in seed oil bodies. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **42**:601-8.

93. **Lin Y. H., L. T. Wimer, and A. H. C. Huang**. 1983. Lipase in the lipid bodies of corn scutella during seedling growth. Plant physiology. Am Soc Plant Biol **73**:460.

94. **Linkies A., K. Müller, K. Morris, V. Turecková, M. Wenk, C. S. C. Cadman, F. Corbineau, M. Strnad, J. R. Lynn, W. E. Finch-Savage, and G. Leubner-Metzger**. 2009. Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using Lepidium sativum and Arabidopsis thaliana. The Plant cell **21**:3803-22.

95. **Liu T., C. Chyan, F. Li, and J. T. C. Tzen**. 2009. Stability of Artificial Oil Bodies Constituted with Recombinant Caleosins. Journal of agricultural and food chemistry. ACS Publications **57**:2308–2313.

96. **Lonsdale J., K. McDonald, and R. Jones**. 1999. High pressure freezing and freeze substitution reveal new aspects of fine structure and maintain protein antigenicity in barley aleurone cells. The Plant Journal **17**:221-229.

97. **Lopes M. a, and B. a Larkins**. 1993. Endosperm origin, development, and function. The Plant cell **5**:1383-99.

98. **Lubkowitz M. a, D. Barnes, M. Breslav, a Burchfield, F. Naider, and J. M. Becker**. 1998. Schizosaccharomyces pombe isp4 encodes a transporter representing a novel family of oligopeptide transporters. Molecular microbiology **28**:729-41.

99. **Lubkowitz M. a, L. Hauser, M. Breslav, F. Naider, and J. M. Becker**. 1997. An oligopeptide transport gene from Candida albicans. Microbiology (Reading, England) **143 (Pt 2**:387-96.

100. **Lubkowitz M.** 2006. The OPT Family Functions in Long-Distance Peptide and Metal Transport in Plants, pp. 35-55. *In* J. Setlow (ed.), Springer US.

101. **Lubkowitz M.** 2011. The Oligopeptide Transporters: A Small Gene Family with a Diverse Group of Substrates and Functions? Molecular plant 1-9.

102. **Madey E., L. Nowack, and J. Thompson**. 2002. Isolation and characterization of lipid in phloem sap of canola. Planta **214**:625-634.

103. **Mak Y., R. Willows, and T. Roberts**. 2009. Germination of wheat: a functional proteomics analysis of the embryo. Cereal Chem. **86**:281-289.

104. **Mak Y., R. D. Willows, T. H. Roberts, C. W. Wrigley, P. J. Sharp, and L. Copeland**. 2009. Germination of Wheat: A Functional Proteomics Analysis of the Embryo. Cereal Chemistry **86**:281-289.

105. **Martinoia E., M. Klein, M. Geisler, L. Bovet, C. Forestier, Ü. Kolukisaoglu, B. Müller-Röber, and B. Schulz**. 2002. Multifunctionality of plant ABC transporters - more than just detoxifiers. Planta **214**:345-355.

106. **Martinoia E., M. Maeshima, and H. E. Neuhaus**. 2007. Vacuolar transporters and their essential role in plant metabolism. Journal of experimental botany **58**:83-102.

107. **Martínez-Camacho J. L., L. González-de la Vara, A. Hamabata, R. Mora-Escobedo, and V. Calderón-Salinas**. 2004. A pH-stating mechanism in isolated wheat (Triticum aestivum) aleurone layers involves malic acid transport. Journal of Plant Physiology **161**:1289-1298.

108. **Maugenest S., I. Martinez, B. Godin, P. Perez, and A.-M. Lescure**. 1999. Structure of two maize phytase genes and their spatio-temporal expression during seedling development. Plant Molecular Biology. Springer Netherlands **39**:503-514.

109. **McCarty D. R., C. B. Carson, P. S. Stinard, and D. S. Robertson**. 1989. Molecular Analysis of viviparous-1: An Abscisic Acid-Insensitive Mutant of Maize. The Plant cell **1**:523-532.

110. **Mikkonen A., I. Porali, M. Cercos, and T.-hua D. Ho**. 1996. A major cysteine proteinase, EPB, in germinating barley seeds: structure of two intronless genes and regulation of expression. Plant Molecular Biology. Springer Netherlands **31**:239-254.

111. **Miranda M., L. Borisjuk, A. Tewes, D. Dietrich, D. Rentsch, H. Weber, and U. Wobus**. 2003. Peptide and amino acid transporters are differentially regulated during seed development and germination in faba bean. Plant Physiology. Am Soc Plant Biol **132**:1950.

112. **Miyata S., K. Okamoto, A. Watanabe, and T. Akazawa**. 1981. Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds. Plant physiology **68**:1314-1318.

113. **Moose S. P., J. W. Dudley, and T. R. Rocheford**. 2004. Maize selection passes the century mark: a unique resource for 21st century genomics. Trends in plant science **9**:358-64.

114. **Murphy D., and I. Hernendez-Pinzon**. 2000. New insights into the mechanisms of lipid-body biogenesis in plants and other organisms. Biochemical Society transactions **28**:710-711.

115. **Murphy D. J.** 2011. The dynamic roles of intracellular lipid droplets: from archaea to mammals. Protoplasma.

116. **Müntz K., M. a Belozersky, Y. E. Dunaevsky, a Schlereth, and J. Tiedemann**. 2001. Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. Journal of experimental botany **52**:1741-52.

117. **Müntz K.** 1998. Deposition of storage proteins. Plant Molecular Biology. Springer Netherlands **38**:77-99.

118. **Naested H., G. I. Frandsen, G. Y. Jauh, I. Hernandez-Pinzon, H. B. Nielsen, D. J. Murphy, J. C. Rogers, and J. Mundy**. 2000. Caleosins: Ca2+-binding proteins associated with lipid bodies. Plant molecular biology **44**:463-76.

119. **Napier J. a, a K. Stobart, and P. R. Shewry**. 1996. The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins. Plant molecular biology **31**:945-56.

120. **Narsai R., S. Law, C. Carrie, and L. Xu**. 2011. In-Depth Temporal Transcriptome Profiling Reveals a Crucial Developmental Switch with Roles for RNA Processing and Organelle Metabolism That Are Essential for. Plant physiology **157**:1342-1362.

121. **Negbi M.** 1984. The structure and function of the scutellum of the Gramineae. Botanical Journal of the Linnean Society. Blackwell Publishing Ltd **88**:205-222.

122. **Negbi M., and J. A. Sargent**. 1986. The scutellum of Avena: a structure to maximize exploitation of endosperm reserves. Botanical Journal of the Linnean Society. Blackwell Publishing Ltd **93**:247-258.

123. **Nomura T., Y. Kono, and T. Akazawa**. 1969. Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds II. Scutellum as the Site of Sucrose Synthesis. Plant physiology **44**:765-9.

124. **Nonogaki H., G. W. Bassel, and J. D. Bewley**. 2010. Germination–Still a mystery. Plant Science. Elsevier **179**:574-581.

125. **North H., S. Baud, I. Debeaujon, C. Dubos, B. Dubreucq, P. Grappin, M. Jullien, L. Lepiniec, A. Marion-Poll, M. Miquel, L. Rajjou, J.-M. Routaboul, and M. Caboche**. 2010. Arabidopsis seed secrets unravelled after a decade of genetic and omics-driven research. The Plant journal : for cell and molecular biology **61**:971-81.

126. **Ogawa M., A. Hanada, Y. Yamauchi, A. Kuwahara, Y. Kamiya, and S. Yamaguchi**. 2003. Gibberellin biosynthesis and response during Arabidopsis seed germination. The Plant Cell Online. Am Soc Plant Biol **15**:1591.

127. **Oikawa A., E. Itoh, A. Ishihara, and H. Iwamura**. 2003. Purification and characterization of β -N-acetylhexosaminidase from maize seedlings. Journal of plant physiology **160**:991-999.

128. **Olsen O.-A.** 2001. ENDOSPERM DEVELOPMENT: Cellularization and Cell Fate Specification. Annual review of plant physiology and plant molecular biology **52**:233-267.

129. **Osawa H., G. Stacey, and W. Gassmann**. 2006. ScOPT1 and AtOPT4 function as proton-coupled oligopeptide transporters with broad but distinct substrate specificities **275**:267-275.

130. **Ouyang J., Z. Cai, K. Xia, Y. Wang, J. Duan, and M. Zhang**. 2010. Identification and analysis of eight peptide transporter homologs in rice. Plant Science. Elsevier Ireland Ltd **179**:374-382.

131. **Partridge M., and D. J. Murphy**. 2009. Roles of a membrane-bound caleosin and putative peroxygenase in biotic and abiotic stress responses in Arabidopsis. Plant physiology and biochemistry : PPB / Société française de physiologie végétale. Elsevier Masson SAS **47**:796-806.

132. **Paungfoo-Lonhienne C., T. G. a Lonhienne, D. Rentsch, N. Robinson, M. Christie, R. I. Webb, H. K. Gamage, B. J. Carroll, P. M. Schenk, and S. Schmidt**. 2008. Plants can use protein as a nitrogen source without assistance from other organisms. Proceedings of the National Academy of Sciences of the United States of America **105**:4524-9.

133. **Paungfoo-Lonhienne C., P. M. Schenk, T. G. a Lonhienne, R. Brackin, S. Meier, D. Rentsch, and S. Schmidt**. 2009. Nitrogen affects cluster root formation and expression of putative peptide transporters. Journal of experimental botany **60**:2665-76.

134. **Perata P., C. Matsukura, P. Vernieri, and J. Yamaguchi**. 1997. Sugar repression of a gibberellin-dependent signaling pathway in barley embryos. The Plant Cell **9**:2197-2208.

135. **Pike S., A. Patel, G. Stacey, and W. Gassmann**. 2009. Arabidopsis OPT6 is an oligopeptide transporter with exceptionally broad substrate specificity. Plant & cell physiology **50**:1923-32.

136. **Pompa A., and A. Vitale**. 2006. Retention of a bean phaseolin/maize gamma-Zein fusion in the endoplasmic reticulum depends on disulfide bond formation. The Plant cell **18**:2608-21.

137. **Potokina E., N. Sreenivasulu, L. Altschmied, W. Michalek, and a Graner**. 2002. Differential gene expression during seed germination in barley (Hordeum vulgare L.). Functional & integrative genomics **2**:28-39.

138. **Poxleitner M., S. W. Rogers, A. Lacey Samuels, and others**. 2006. A role for caleosin in degradation of oil-body storage lipid during seed germination. The Plant Journal. Wiley Online Library **47**:917–933.

139. **Pu J., C. Ha, S. Zhang, J. Jung, W.-K. Huh, and P. Liu**. 2011. Interactomic study on interaction between lipid droplets and mitochondria. Protein & Cell. Higher Education Press, co-published with Springer-Verlag GmbH **2**:487-496.

140. **Purkrtova Z., S. d' Andrea, P. Jolivet, P. Lipovova, B. Kralova, M. Kodicek, and T. Chardot**. 2007. Structural properties of caleosin: a MS and CD study. Archives of biochemistry and biophysics **464**:335-43.

141. **Purkrtova Z., P. Jolivet, M. Miquel, and T. Chardot**. 2008. Structure and function of seed lipid-body-associated proteins. Comptes rendus biologies. Elsevier Masson SAS **331**:746-54.

142. **Rajjou L., M. Duval, K. Gallardo, J. Catusse, J. Bally, C. Job, and D. Job**. 2011. Seed Germination and Vigor. Annual review of plant biology.

143. **Ramos M. S., R. Abele, R. Nagy, M. S. Grotemeyer, R. Tampé, D. Rentsch, and E. Martinoia**. 2011. Characterization of a transport activity for long-chain peptides in barley mesophyll vacuoles. Journal of experimental botany **62**:2403-10.

144. **Randall J. J., D. W. Sutton, S. F. Hanson, and J. D. Kemp**. 2005. BiP and zein binding domains within the delta zein protein. Planta **221**:656-66.

145. **Ranki H., and T. Sopanen**. 1984. Secretion of -Amylase by the Aleurone Layer and the Scutellum of Germinating Barley Grain. Plant Physiology **75**:710-715.

146. **Rao D. V., and M. V. S. Raju**. 1985. Radial elongation of the epidermal cells of scutellum during caryopsis germination of wild oats (Avena fatua). Canadian Journal of Botany. NRC Research Press **63**:1789-1793.

147. **Rayle D. L., and R. E. Cleland**. 1992. The Acid Growth Theory of auxin-induced cell elongation is alive and well. Plant physiology **99**:1271-4.

148. **Rentsch D., M. Laloi, I. Rouhara, E. Schmelzer, S. Delrot, and W. B. Frommer**. 1995. NTR1 encodes a high affinity oligopeptide transporter in Arabidopsis. FEBS letters **370**:264-8.

149. **Rentsch D., S. Schmidt, and M. Tegeder**. 2007. Transporters for uptake and allocation of organic nitrogen compounds in plants. FEBS letters **581**:2281-9.

150. **Rinne P. L., P. M. Kaikuranta, and C. van der Schoot**. 2001. The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. The Plant journal : for cell and molecular biology **26**:249-64.

151. **Saito Y., T. Shigemitsu, K. Tanaka, S. Morita, S. Satoh, and T. Masumura**. 2010. Ultrastructure of Mature Protein Body in the Starchy Endosperm of Dry Cereal Grain. Bioscience, Biotechnology, and Biochemistry **74**:1485-1487.

152. **Salmenkallio M., and T. Sopanen**. 1989. Amino Acid and Peptide uptake in the scutella of germinating grains of barley, wheat, rice, and maize. Plant physiology **89**:1285-91.

153. **Sanchez-Linares L., M. Gavilanes-Ruiz, D. Diaz-Pontones, F. Guzman-Chavez, V. Calzada-Alejo, V. Zurita-Villegas, V. Luna-Loaiza, R. Moreno-Sanchez, I. Bernal-Lugo, and S. Sanchez-Nieto**. 2012. Early carbon mobilization and radicle protrusion in maize germination. Journal of Experimental Botany 1-14.

154. **Sanchez-Linares L., M. Gavilanes-Ruiz, D. Diaz-Pontones, F. Guzman-Chavez, V. Calzada-Alejo, V. Zurita-Villegas, V. Luna-Loaiza, R. Moreno-Sanchez, I. Bernal-Lugo, and S. Sanchez-Nieto**. 2012. Early carbon mobilization and radicle protrusion in maize germination. Journal of Experimental Botany 1-14.

155. **Schaaf G., U. Ludewig, B. Erenoglu, and S. Mori**. 2004. ZmYS1 functions as a proton-coupled symporter for phytosiderophore-and nicotianamine-chelated metals. Journal of Biological **279**:9091-9096.

156. **Schoot C. van der, L. K. Paul, S. B. Paul, and P. L. H. Rinne**. 2011. Plant lipid bodies and cell-cell signaling: a new role for an old organelle? Plant signaling & behavior **6**:1732-8.

157. **Schulze W., W. B. Frommer, and J. M. Ward**. 1999. Transporters for ammonium, amino acids and peptides are expressed in pitchers of the carnivorous plant Nepenthes. The Plant journal : for cell and molecular biology **17**:637-46.

158. **Senna R., V. Simonin, M. a C. Silva-Neto, and E. Fialho**. 2006. Induction of acid phosphatase activity during germination of maize (Zea mays) seeds. Plant physiology and biochemistry **44**:467-73.

159. **Shewry P. R., and N. G. Halford**. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. Journal of experimental botany **53**:947-58.

160. **Shimada T. L., and I. Hara-Nishimura**. 2010. Oil-body-membrane proteins and their physiological functions in plants. Biological & Pharmaceutical Bulletin. J-STAGE **33**:360–363.

161. **Shimada T. L., T. Shimada, H. Takahashi, Y. Fukao, and I. Hara-Nishimura**. 2008. A novel role for oleosins in freezing tolerance of oilseeds in Arabidopsis thaliana. The Plant journal **55**:798-809.

162. **Shimaoka T., M. Ohnishi, T. Sazuka, N. Mitsuhashi, I. Hara-Nishimura, K. I. Shimazaki, M. Maeshima, A. Yokota, K. I. Tomizawa, and T. Mimura**. 2004. Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of Arabidopsis thaliana. Plant and cell physiology. Jpn Soc Plant Physiol **45**:672.

163. **Smritimala G., and S. Swati**. 1993. Effects of ageing on amylase activity and scutellar cell structure during imbibition in wheat seed. Annals of Botany. Annals Botany Co **71**:411.

164. **Song W., S. Koh, M. Czako, L. Marton, E. Drenkard, J. M. Becker, and G. Stacey**. 1997. Antisense expression of the peptide transport gene AtPTR2-B delays flowering and arrests seed development in transgenic Arabidopsis plants. Plant physiology **114**:927-35.

165. **Song W., H. Y. Steiner, L. Zhang, F. Naider, G. Stacey, and J. M. Becker**. 1996. Cloning of a second Arabidopsis peptide transport gene. Plant physiology **110**:171-8.

166. **Sopanen T.** 1979. Development of Peptide Transport Activity in Barley Scutellum during Germination. Plant physiology **64**:570-4.

167. **Sopanen T., M. Uuskallio, and S. Nyman**. 1980. Characteristics and development of leucine transport activity in the scutellum of germinating barley grain. Plant physiology **65**:249-53.

168. **Sopanen T., and E. Väisänen**. 1985. Uptake of glutamine by the scutellum of germinating barley grain. Plant physiology **78**:684-9.

169. **Sreenivasulu N., B. Usadel, A. Winter, V. Radchuk, U. Scholz, N. Stein, W. Weschke, M. Strickert, T. J. Close, M. Stitt, A. Graner, and U. Wobus**. 2008. Barley grain maturation and germination: metabolic pathway and regulatory network

commonalities and differences highlighted by new MapMan/PageMan profiling tools. Plant physiology **146**:1738-58.

170. **Stacey G., S. Koh, C. Granger, and J. M. Becker**. 2002. Peptide transport in plants. Trends in plant science **7**:257-63.

171. **Stacey M., H. Osawa, A. Patel, and W. Gassmann**. 2006. Expression analyses of Arabidopsis oligopeptide transporters during seed germination, vegetative growth and reproduction. Planta **223**:291-305.

172. **Stacey M., A. Patel, W. McClain, and M. Mathieu**. 2008. The Arabidopsis AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. Plant physiology **146**:589-601.

173. **Steiner H. Y., W. Song, L. Zhang, F. Naider, J. M. Becker, and G. Stacey**. 1994. An Arabidopsis peptide transporter is a member of a new class of membrane transport proteins. The Plant cell **6**:1289-99.

174. **Stewart A., H. Nield, and J. N. Lott**. 1988. An investigation of the mineral content of barley grains and seedlings. Plant physiology **86**:93-97.

175. **Stoddart J. L., H. Thomas, and A. Robertson**. 1973. Protein synthesis patterns in barley embryos during germination. Planta. Springer Berlin / Heidelberg **112**:309-321.

176. **Subbarao K. V., R. Datta, and R. Sharma**. 1998. Amylases synthesis in scutellum and aleurone layer of maize seeds. Phytochemistry **49**:657-66.

177. **Swift J.** 1972. The fine structure of the wheat scutellum during germination. Australian Journal of Biological Sciences **25**:469-486.

178. **Swift J., and T. O'Brien**. 1970. Vascularization of the scutellum of wheat. Australian Journal of Botany. CSIRO **18**:45–53.

179. **Swift J., and T. O'brien**. 1972. The fine structure of the wheat scutellum before germination. Australian Journal of Biological Sciences. CSIRO **25**:9–22.

180. **Takahashi K., S. B. P. Athauda, K. Matsumoto, S. Rajapakshe, M. Kuribayashi, M. Kojima, N. Kubomura-Yoshida, A. Iwamatsu, C. Shibata, and H. Inoue**. 2005. Nepenthesin, a Unique Member of a Novel Subfamily of Aspartic Proteinases: Enzymatic and Structural Characteristics. Current Protein and Peptide Science **6**:513-525.

181. **Takahashi S., T. Katagiri, K. Yamaguchi-Shinozaki, and K. Shinozaki**. 2000. An Arabidopsis gene encoding a Ca2+-binding protein is induced by abscisic acid during dehydration. Plant & cell physiology **41**:898-903.

182. **Tegeder M., D. Rentsch, and J. Patrick**. 2011. Organic Carbon and Nitrogen Transporters, pp. 331-352. *In* A.S. Murphy, B. Schulz, and W. Peer (eds.), Springer Berlin / Heidelberg.

183. **Thakur A., J. Kaur, and A. K. Bachhawat**. 2008. Pgt1, a glutathione transporter from the fission yeast Schizosaccharomyces pombe. FEMS yeast research **8**:916-29.

184. **Theodoulou F. L., and P. J. Eastmond**. 2012. Seed storage oil catabolism: a story of give and take. Current opinion in plant biology. Elsevier Ltd **15**:322-328.

185. **Thompson R. D., G. Hueros, H.-a. Becker, and M. Maitz**. 2001. Development and functions of seed transfer cells. Plant science : an international journal of experimental plant biology **160**:775-783.

186. **Tsay Y. F., J. I. Schroeder, K. a Feldmann, and N. M. Crawford**. 1993. The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell **72**:705-13.

187. **Tsay Y.-F., C.-C. Chiu, C.-B. Tsai, C.-H. Ho, and P.-K. Hsu**. 2007. Nitrate transporters and peptide transporters. FEBS letters **581**:2290-300.

188. **Tzen J. T., and a H. Huang**. 1992. Surface structure and properties of plant seed oil bodies. The Journal of cell biology **117**:327-35.

189. **Tzen J. T., Y. K. Lai, K. L. Chan, and a H. Huang**. 1990. Oleosin isoforms of high and low molecular weights are present in the oil bodies of diverse seed species. Plant physiology **94**:1282-9.

190. **Tzen J. T., C. C. Peng, D. J. Cheng, E. C. Chen, and J. M. Chiu**. 1997. A new method for seed oil body purification and examination of oil body integrity following germination. Journal of biochemistry **121**:762-8.

191. **Tzen J. T. C., R. L. C. Chuang, J. C. F. Chen, and L. S. H. Wu**. 1998. Coexistence of both oleosin isoforms on the surface of seed oil bodies and their individual stabilization to the organelles. Journal of biochemistry. Jpn Biochemical Soc **123**:318.

192. **Tzen J., Y. Cao, P. Laurent, C. Ratnayake, and A. Huang**. 1993. Lipids, Proteins, and Structure of Seed Oil Bodies from Diverse Species. Plant physiology **101**:267-276.

193. **Vasconcelos M. W., G. W. Li, M. a. Lubkowitz, and M. a. Grusak**. 2008. Characterization of the PT Clade of Oligopeptide Transporters in Rice. The Plant Genome Journal **1**:77.

194. **Vellosillo T., M. Martínez, M. A. López, J. Vicente, T. Cascón, L. Dolan, M. Hamberg, and C. Castresana**. 2007. Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. The Plant cell **19**:831-46.

195. **Verrier P. J., D. Bird, B. Burla, E. Dassa, C. Forestier, M. Geisler, M. Klein, U. Kolukisaoglu, Y. Lee, E. Martinoia, A. Murphy, P. a Rea, L. Samuels, B. Schulz, E. J. Spalding, K. Yazaki, and F. L. Theodoulou**. 2008. Plant ABC proteins--a unified nomenclature and updated inventory. Trends in plant science **13**:151-9.

196. **Vitale A., and A. Ceriotti**. 2004. Protein quality control mechanisms and protein storage in the endoplasmic reticulum. A conflict of interests? Plant physiology **136**:3420-3426.

197. **Wahlroos T., J. Soukka, A. Denesyuk, R. Wahlroos, T. Korpela, and N. J. Kilby**. 2003. Oleosin expression and trafficking during oil body biogenesis in tobacco leaf cells. Genesis (New York, N.Y. : 2000) **35**:125-32.

198. **Wang H. L., C. E. Offler, and J. W. Patrick**. 1994. Nucellar projection transfer cells in the developing wheat grain. Protoplasma. Springer Wien **182**:39-52.

199. **Wang S., and A. Huang**. 1987. Biosynthesis of lipase in the scutellum of maize kernel. Journal of Biological Chemistry. ASBMB **262**:2270.

200. **Wang S., W. Lue, S. Wu, H. Huang, and J. Chen**. 1997. Characterization of a Maize β-Amylase cDNA Clone and Its Expression during Seed Cermination. Plant physiology **113**:403-409.

201. **Washio K., and K. Ishikawa**. 1994. Organ-specific and hormone-dependent expression of genes for serine carboxypeptidases during development and following germination of rice grains. Plant physiology **105**:1275-1280.

202. **Waterworth W. M., C. E. West, and C. M. Bray**. 2000. The barley scutellar peptide transporter: biochemical characterization and localization to the plasma membrane. Journal of experimental botany **51**:1201-9.

203. **Waterworth W. M., M. K. Ashley, C. E. West, P. a Sunderland, and C. M. Bray**. 2005. A role for phosphorylation in the regulation of the barley scutellar peptide transporter HvPTR1 by amino acids. Journal of experimental botany **56**:1545-52.

204. **Waterworth W. M., and C. M. Bray**. 2006. Enigma variations for peptides and their transporters in higher plants. Annals of botany **98**:1-8.

205. **Weichert A., C. Brinkmann, N. Y. Komarova, D. Dietrich, K. Thor, S. Meier, M. Suter Grotemeyer, and D. Rentsch**. 2012. AtPTR4 and AtPTR6 are differentially expressed, tonoplast-localized members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family. Planta **235**:311-23.

206. **Weitbrecht K., K. Müller, and G. Leubner-Metzger**. 2011. First off the mark: early seed germination. Journal of experimental botany **62**:3289-309.

207. **West C. E., W. M. Waterworth, S. M. Stephens, C. P. Smith, and C. M. Bray**. 1998. Cloning and functional characterisation of a peptide transporter expressed in the scutellum of barley grain during the early stages of germination. The Plant journal : for cell and molecular biology **15**:221-9.

208. **Wiles A., F. Naider, and J. M. Becker**. 2006. Transmembrane domain prediction and consensus sequence identification of the oligopeptide transport family. Research in microbiology **157**:395-406.

209. **Woo Y. M., D. W. Hu, B. a Larkins, and R. Jung**. 2001. Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. The Plant cell **13**:2297-317.

210. **Woodger F., J. V. Jacobsen, and F. Gubler**. 2010. Gibberellin action in germinated cereal grains. Plant Hormones. Springer 221–240.

211. **Xie Z., Z.-L. Zhang, S. Hanzlik, E. Cook, and Q. J. Shen**. 2007. Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. Plant molecular biology **64**:293-303.

212. **Yamagata T., H. Kato, S. Kuroda, S. Abe, and E. Davies**. 2003. Uncleaved legumin in developing maize endosperm: identification, accumulation and putative subcellular localization. Journal of Experimental Botany **54**:913-922.

213. **Yamaguchi S., Y. Kamiya, and E. Nambara**. 2007. Regulation of ABA and GA Levels During Seed Development and Germination in Arabidopsis, pp. 224-247. *In* Annual Plant Reviews Volume 27: Seed Development, Dormancy and Germination. Blackwell Publishing Ltd.

214. **Yang N.-S., and J. G. Scandalios**. 1975. De novo synthesis and developmental control of the multiple gene-controlled malate dehydrogenase isozymes in maize scutella. Biochimica et Biophysica Acta (BBA) - Enzymology **384**:293-306.

215. **Yokoyama M., S. Yamaguchi, S. Inomata, K. Komatsu, and S. Yoshida**. 2000. Stress-Induced Factor Involved in Flower Formation of Lemna is an cr-Ketol Derivative of Linolenic Acid Jasmonic acid **41**:110-113.

216. **Young T. E., and D. R. Gallie**. 1999. Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. Plant molecular biology **39**:915-26.

217. **Young T. E., and D. R. Gallie**. 2000. Programmed cell death during endosperm development. Plant molecular biology **44**:283-301.

218. **Young T. E., and D. R. Gallie**. 2000. Regulation of programmed cell death in maize endosperm by abscisic acid. Plant molecular biology **42**:397-414.

219. **Young T. E., D. R. Gallie, and D. a. DeMason**. 1997. Ethylene-Mediated Programmed Cell Death during Maize Endosperm Development of Wild-Type and shrunken2 Genotypes. Plant physiology **115**:737-751.

220. **Zhang M., A. Bourbouloux, and O. Cagnac**. 2004. A novel family of transporters mediating the transport of glutathione derivatives in plants. Plant **134**:482-491.

221. **Zhao X., J. Huang, H. Yu, L. Wang, and W. Xie**. 2010. Genomic survey, characterization and expression profile analysis of the peptide transporter family in rice (Oryza sativa L.). BMC plant biology **10**:92.

222. **Zheng Y., and Z. Wang**. 2010. Current opinions on endosperm transfer cells in maize. Plant cell reports **29**:935-42.

223. **Zienkiewicz K., A. Zienkiewicz, M. I. Rodriguez-Garcia, and A. J. Castro**. 2011. Characterization of a caleosin expressed during olive (Olea europaea L.) pollen ontogeny. BMC plant biology **11**:122.

224. **Zweytick D., K. Athenstaedt, and G. Daum**. 2000. Intracellular lipid particles of eukaryotic cells. Biochimica et biophysica acta **1469**:101-20.

7. SPANISH SUMMARY

7 SPANISH SUMMARY

7.1 INTRODUCCIÓN

7.1.1 Morfología de la semilla de maíz

La semilla de maíz está formada por tres partes principales: una cubierta el endospermo y el embrión. La cubierta de la semilla se desarrolla a partir delos tegumentos, que originalmente rodean al óvulo. En maíz, esta cubierta está muy endurecida y presenta una cutícula lipídica que, junto con una o varias capas de células protectoras, le confiere un cierto grado de impermeabilidad al agua y los gases, haciendo de esta estructura un elemento muy importante en el control de la germinación.

El endospermo ocupa la mayor parte de la semilla y es donde se encuentran la mayor parte de las reservas de la semilla, principalmente el almidón y las proteínas de reserva. Su capa más externa, la aleurona, posee características diferenciales.

El embrión está formado por el eje embrionario (que incluye la radícula, el hipocotilo y los primordios foliares), y por el escutelo, que es un cotiledón muy modificado.

El escutelo es un órgano típico de los embriones de los cereales y otras Monocotiledóneas y consiste en una estructura en forma de escudo que rodea al eje embrionario. En general se considera que el escutelo es el cotiledón modificado ya que a diferencia de las hojas y del coleoptilo, el escutelo no deriva del meristemo apical y la estructura de los tejidos del escutelo es diferente a la de las hojas verdaderas. El escutelo es un órgano compuesto por tres regiones:

1- El parénquima escutelar

El parénquima escutelar constituye la mayor parte del escutelo y está formado por células de gran tamaño que cumplen funciones de acumulación de reservas, principalmente lípidos, en forma de oleosomas o cuerpos lipídicos. Debido a esta función el contenido de lípidos del escutelo en la semilla madura es elevado. Las células del parénquima escutelar también acumulan proteínas de reserva y fitina.

2- El sistema vascular

El sistema vascular del escutelo se encuentra en forma no diferenciada en el embrión maduro pero se diferencia en floema y xilema a las pocas horas de la germinación (entre 3 y 30 horas). En maíz el sistema vascular del escutelo está constituido por un eje principal que parte del nodo escutelar y que se dirige a la parte coleoptilar del escutelo, donde se ramifica abundantemente a ambos lados y hacia abajo, llegando cerca del epitelio escutelar. En maíz el vaso principal es colateral con xilema en la parte adaxial, pero las ramificaciones son usualmente amfivasales, con el floema en el centro y el xilema alrededor. En las ramas más pequeñas que se aproximan al epitelio el anillo de xilema que rodea el floema o está incompleto o no existe en absoluto. Los vasos juegan un importante papel en las funciones secretoras y absorbentes del escutelo.

3- El epitelio escutelar

El epitelio escutelar o capa glandular es la capa de células más externa del escutelo que está en contacto directo con el endospermo. Está formado por una capa de células en empalizada. Tras la germinación, las células del epitelio se separan unas de otras y sufren un crecimiento en longitud, lo cual incrementa mucho la superficie, permitiendo una más rápida secreción de enzimas hidrolíticas y giberelina, y más eficaz absorción de carbohidratos y péptidos.

El epitelio escutelar presenta además otra particularidad que lo hace muy interesante: en presencia del medio hormonal adecuado el epitelio glandular de embriones inmaduros de maíz es capaz de diferenciarse y formar embriones somáticos. Este aspecto es de gran importancia ya que existen muy pocos tejidos de maíz con totipotencia. Esta propiedad es utilizada para generar callos destinados a la transformación genética.

4- La epidermis escutelar

Es la capa de células que limita el escutelo en la parte más cercana al eje embrionario.

7.1.2 Funciones del escutelo

El escutelo juega un papel importante durante la germinación de las semillas en los cereales no solo por los nutrientes que acumula sino también por su posición intermediaria entre el eje embrionario y el endospermo. Al comienzo de la germinación el escutelo sintetiza giberelinas, que son secretadas al endospermo y llegan a la aleurona, donde inducen la síntesis de enzimas hidrolíticos. La secreción de las giberelinas la realiza el epitelio escutelar. Por otro lado, los nutrientes fruto de la degradación de las reservas del endospermo deben de ser capturados y conducidos hacia el eje por el escutelo, lo cual implica la existencia en este órgano de mecanismos específicos de transporte. Más aún, el propio epitelio escutelar también secreta enzimas que colaboran en la degradación de las reservas del endospermo. Por todo ello muchas veces se habla del escutelo como “órgano digestivo” de la semilla.

Los transportadores son proteínas que abarcan la membrana que selectivamente transportan solutos hidrofílicos de un lado a otro de membranas hidrofóbicas. Están presentes en la membrana plasmática pero también en las membranas de otros orgánulos subcelulares como la vacuola. Estos transportadores pueden transportar compuestos orgánicos como péptidos o azúcares, o bien iones inorgánicos. Los transportadores juegan un papel integral en el metabolismo celular, la homeostasis de iones, osmorregulación, de señalización, y otros procesos. Los transportadores permiten el movimiento de solutos no sólo dentro de las células, sino también entre células, tejidos y órganos de los organismos multicelulares complejos, tales como las plantas superiores. Por lo tanto, ayudan a coordinar los procesos metabólicos, fisiológicos y de desarrollo.

7.1.3 Control de la germinación en la semilla de maíz

Las hormonas de plantas son importantes para la regulación de la dormancia y de la germinación de la semilla. Las giberelinas (GA) y el ácido abscísico (ABA) son los factores principales que regulan la transición del desarrollo de la semilla a la germinación. El ABA induce la dormancia y las GAs promueven la germinación. El equilibrio GA/ABA gobierna la regulación de la maduración y la germinación. La biosíntesis *de novo* de GAs es necesaria durante la imbibición. Las GAs son sintetizadas en el escutelo y son liberadas al endospermo amiláceo. Estas hormonas difunden después hacia la capa de la aleurona. Las células de la aleurona son estimuladas para sintetizar y secretar α-amilasa y otras hidrolasas hacia el endospermo amiláceo. El almidón y otras macromoléculas se degradan hasta pequeñas moléculas. Esos solutos son captados por el escutelo y transportados hacia el embrión en crecimiento. La producción y la secreción de las enzimas hidrolíticas en los tejidos de la aleurona son reguladas por las GAs.

Otras hormonas muy importantes durante la germinación son las auxinas, el ácido indoleacetico (IAA) y las citoquininas. Las auxinas están implicadas en el crecimiento del eje embrionario pero se sabe poco de su influencia sobre la movilización de reservas durante la germinación. El ácido indoleacético (IAA) disminuye durante la imbibición de granos de sorgo, y se ha visto que las auxinas regulan la expresión de la catalasa en el escutelo de maíz durante la germinación.

7.2 OBJETIVOS

El objetivo de la presente tesis es el estudio de los cambios producidos en el escutelo de maíz durante las primeras etapas de germinación en cuanto a aspectos morfológicos, en la transcripción de genes y en la acumulación de proteínas. Para ello hemos dividido nuestro trabajo en 4 partes:

1.- Control de la elongación del epitelio escutelar en maíz durante la germinación.

2.- Identificación de genes que se expresan de manera exclusiva o predominante en el epitelio del escutelo de maíz durante la germinación.

3.- Estudio de un nuevo transportador de péptidos ZmPTR1 en maíz.

4.- Identificación de las proteínas asociadas a cuerpos lipídicos en escutelo de maíz.

7.3 RESULTADOS

7.3.1 Control de la elongación del epitelio escutelar en maíz durante la germinación

Durante la germinación del maíz el escutelo sufre una serie de cambios morfológicos. Aumenta de peso fresco total debido a su hidratación y se hincha e incrementa su tamaño, en especial en su base.

El epitelio escutelar también sufre cambios durante la germinación. En la semilla madura las células del epitelio tienen forma ligeramente elongadas hacia la capa externa y están densamente empaquetadas sin espacios intercelulares. Durante la germinación las células del epitelio pierden la adherencia de unas con otras en sus paredes anticlinales, además, las células del epitelio escutelar sufren una elongación, que en el caso de maíz lleva a un incremento de hasta casi tres veces la longitud inicial, pudiendo llegar a formar protuberancias semejantes a hifas. Por otro lado, el epitelio sufre invaginaciones, creándose estructuras de tipo glandular. Observaciones al microscopio electrónico muestran proyecciones de la superficie de las células del epitelio que se adentran en el endospermo y que aparecen en etapas tardías de la germinación. La separación de las células, el aumento de longitud y la formación de estructuras glandulares contribuyen a un aumento en la superficie de contacto entre endospermo y escutelo, por lo que se cree que facilita los intercambios de materiales entre ambos tejidos y proporcionando así la mayor exposición superficial para la secreción y la absorción, las funciones especializadas de estas células.

A nivel histológico también se observan importantes cambios en las células que componen el escutelo. En la semilla madura, las células del epitelio contienen un granulado fino, uniforme y semitransparente. Inmediatamente después del inicio de la germinación el protoplasma de las células epiteliales se vuelve mucho mas granulado, cosa que se puede asociar a las funciones secretoras. Durante la germinación el número de mitocondrias que se observan en las células del escutelo y, en especial del epitelio, se incrementa.

El objetivo de este estudio fue de determinar los factores hormonales y ambientales que regulan la elongación de las células epiteliales del escutelo en el maíz.

En este trabajo, las semillas de maíz fueron imbibidas en diferentes condiciones y se sometieron a diferentes tratamientos y el alargamiento de las células epiteliales se determinó por microscopía mediante cortes histológicos. No se observaron efectos significativos en el alargamiento o la germinación en las semillas tratadas con el precursor de etileno 1-aminociclopropano-1-carboxílico (ACC), el inhibidor de la síntesis de etileno amino-oxiacético (AOA), o el bloqueador de la acción del etileno, tiosulfato de plata (STS). Tampoco se observaron diferencias frente a tratamientos con tres compuestos de la familia de las auxinas, ácido indol acético [AIA], indol-3-butírico [IBA] y naftalen acético [NAA], ni con el inhibidor de transporte polar auxina el ácido 2,3,5-triyodobenzoico (TIBA). Tampoco se observaron diferencias tras tratamientos con quinetina o 6-bencilaminopurina (BAP). Sin embargo, si se observó una reducción significativa del alargamiento de las células en las semillas tratadas con ácido abscísico (ABA) y con ácido salicílico. En contraste, la aplicación de giberelinas (GA3) produjo un aumento de la elongación, aunque la aplicación de los inhibidores de la síntesis de giberelinas paclobutrazol (PAC) y ancimidol no cambió la tasa de elongación.

Se analizó también el alargamiento de las células epiteliales en los diferentes mutantes de maíz. No se observó un alargamiento significativamente diferente a la del fenotipo salvaje (Wt) en el mutante shrunken2 (mutante deficientes en la acumulación del almidón), en dwarf1 (mutante deficientes en la síntesis de giberelinas) y en dwarf8 (mutante deficientes y en la señalización de la giberelina). Sin embargo, el mutante viviparous1 (vp1), que es deficiente en la señalización del ABA, mostró un alargamiento celular contrastado. No se observaron diferencias significativas entre el tipo salvaje y vp1 cuando se comparan las células a 5 días después de la imbibición. Sin embargo, en semillas secas y a un un día de imbibición la relación longitud / anchura de las células epiteliales de vp1 era mayor. De hecho, no se observaron diferencias significativas en las células epiteliales de vp1 en semillas secas y después de la imbibición. Podemos concluir que en vp1 las células epiteliales ya estaban elongadas en semillas secas y no más cambios se producen después de la imbibición.

Se observó también el efecto de la acidez en el alargamiento de las células epiteliales. El mayor incremento de la longitud se observó con soluciones neutras, mientras que las soluciones de pH ácido y básico producen una reducción tanto en la elongación como en la germinación. Finalmente, la glucosa (200 mM), pero no la sacarosa (200 mM), produce un aumento en el alargamiento. Sin embargo, la incubación en 200 mM de sorbitol redujo significativamente tanto la elongación como la germinación. La incubación en sal o en EDTA no produjo diferencias significativas en la elongación, pero inhibieron totalmente la germinación. La Actinomicina D, un inhibidor de la transcripción, y la cicloheximida, un inhibidor de la traducción, produjeron una reducción significativa en la tasa de elongación y una reducción parcial de la germinación.

7.3.2 Identificación de genes que se expresan de manera exclusiva o predominante en el epitelio del escutelo de maíz durante la germinación

Durante la germinación la región del epitelio del escutelo en contacto con el endospermo sufre importantes cambios morfológicos. Estos cambios van acompañados también de importantes cambios fisiológicos ya que el escutelo actúa como secretor de giberelinas y de enzimas hidrolíticas hacia el endospermo, y, al mismo tiempo, como primer paso del transporte de los nutrientes generados hacia el eje embrionario. Todos estos cambios han de ir necesariamente acompañados de cambios en la regulación y expresión de numerosos genes. El objetivo de esta parte de la tesis ha sido el de identificar estos cambios de expresión mediante transcriptómica. Para ello, nos hemos basado en la construcción de una genoteca de cDNA y su cribado diferencial mediante hibridación de macroarrays. Se identificaron 30 genes que se expresan predominantemente en el epitelio, en comparación con otras partes del escutelo. Una elevada proporción de estos genes está implicada en procesos metabólicos. Otros de los genes identificados están implicados en la síntesis o modificación de las paredes celulares, que puede ser reflejada en los cambios de célula la forma y la composición de la pared celular que se puede observar durante la imbibición. Otro de los genes identificados codifica una proteasa que pertenece a una familia típica de las proteinasas de las plantas carnívoras. Casi nada es sabe acerca de su papel en otras plantas u órganos, pero la presencia en el escutelo puede apuntar a una posible función "digestiva" durante la germinación. Genes que participan en la producción de energía y en el transporte de péptidos fueron también identificados. Uno de estos genes es el ZmPTR1 que hemos estudiado su función, localización y expresión en la tercera parte.

7.3.3 Estudio de un nuevo transportador de péptidos ZmPTR1 en maíz

Las plantas han desarrollado diferentes sistemas de transporte para facilitar la absorción de nitrógeno y la movilización. Además de la absorción de nitrógeno inorgánico (nitrato y amonio), las plantas también absorben y transportan nitrógeno orgánico en forma de aminoácidos, péptidos y proteínas. El transporte de péptidos juega un papel esencial durante la germinación de los cereales. Durante esta etapa, las reservas proteicas del endospermo son hidrolizadas mediante la acción concertada de proteinasas y peptidasas para formar un grupo de pequeños péptidos y aminoácidos que son trasladados al embrión a través del escutelo. Los oligopeptidos, más que los aminoácidos son los productos principales de la degradación de las proteínas del endopsermo. Durante el inicio de la germinación el transporte de péptidos es el único responsable del suministro de nitrógeno orgánico del embrión ya que el transporte de aminoácidos solo llega a ser significativo entre 48 y 72 horas después del comienzo de la imbibición.

Este transporte de péptidos a través de membranas está mediado por tres tipos de transportadores: ATP binding cassette (ABC), los transportadores de oligopéptidos (OPT) y los transportadores de péptidos (PTR/NTR1). Los miembros de la familia del transportador de péptidos PTR/NRT1 transportan una variedad de sustratos tales como nitrato, di-y tripéptidos, auxina y carboxilatos. El elevado número de genes en los genomas vegetales junto con la diversidad de sustratos y las localizaciones celulares sugieren que los transportadores PTR/NTR1 pueden estar implicados en una amplia gama de procesos celulares en las plantas, sin embargo, las funciones fisiológicas de la mayoría de ellos son desconocidas.

Las raíces de las plantas pueden absorber pequeños péptidos desde el suelo y algunos genes que codifican PTR/NTR1 se transcriben en las raíces. También se ha sugerido que la familia PTR es importante para la movilización de nitrógeno durante la germinación y en el transporte de larga distancia de los péptidos a los tejidos en crecimiento.

La secuencia de uno de los cDNAs identificados como de expresión predominante en el epitelio escutelar durante la germinación presenta homología con un transportador de péptidos (PTR). En el presente trabajo hemos aislado y caracterizado este nuevo transportador de péptidos en maíz que hemos llamado ZmPTR1. ZmPTR1 es miembro de la familia del peptide transporter/nitrate transporter 1 (PTR/NRT1) ya que es altamente homólogo a algunos miembros de la caracterizada familia de transportadores de di-y tripéptidos de Arabidopsis (AtPTR2, AtPTR4 y AtPTR6). El análisis bioinformático de la secuencia de aminoácidos ZmPTR1 predice 10 dominios transmembrana conservados (aminoácidos 125-147 y 157-179, 204-226, 233-255, 347-369, 397-418, 432-453, 480-502, 522-544 y 557-579). El patrón de expresión en semilla en germinación fue determinado mediante hibridación *in situ* y demostró que se expresa de manera exclusiva en el epitelio escutelar. Análisis northern muestran que la expresión se inicia tras 1 día de la imbibición y que esta señal es más intensa en embriones de 5 días después de la imbibición. El gen se expresa en el epitelio escutelar y, en menor medida, en la radícula y del hipocotilo. Las plantas tratadas con giberelinas mostraron una expresión más alta del gen.

La región C-terminal de la proteína ZmPTR1 fusionada a la YFP muestra fluorescencia en el tonoplasto. Esta localización ha sido también observada cuando la misma construcción ha sido introducida de forma transitoria en células BY-2 de tabaco.

Las plantas que sobreexpresan ZmPTR1 crecieron mejor que las plantas salvajes de Arabidopsis cuando se cultivaron en un medio con el dipéptido Ala-Ala como única fuente de N. Nuestros estudios sugieren que ZmPTR1 juega un papel importante en el transporte en el embrión de los pequeños péptidos producidos durante la hidrólisis enzimática de las proteínas de reserva del endospermo.

7.3.4 Identificación de las proteínas asociadas a cuerpos lipídicos en maíz

Los lípidos de reserva de las semillas son almacenados en orgánulos subcelulares denominados cuerpos lipídicos. Los lípidos en las semillas de cereales pueden acumularse en el embrión (principalmente el escutelo) o en el endospermo (aleurona). En la mayor parte de las plantas, los lípidos de reserva son triglicéridos que, al asociarse con unas proteínas (principalmente oleosinas), y fosfolípidos, componen los cuerpos lipídicos. Durante la germinación los triglicéridos de los cuerpos lipídicos son catabolizados por la acción de lipasas que hidrolizan los triglicéridos para liberar ácidos grasos. En la mayoría de las semillas, la actividad de la lipasa está ausente antes de la germinación y aparece en la posgerminación previa a la desaparición de los triglicéridos de reserva.

Los cuerpos lipídicos (OB) se componen de un núcleo lipídico neutro rodeado por una monocapa de fosfolípidos incrustado con las proteínas que confieren estabilidad al oleosoma y también pueden estar implicados en las interacciones con otros orgánulos. En las plantas, las más abundantes proteínas descritas asociadas a OB son oleosinas. Los oleosinas poseen un largo dominio hidrófobo central que las ancla en el núcleo de lípidos de los oleosomas, y dos dominios N y C-terminales, hidrofílicos, que están localizados en la superficie del OB. Las oleosinas forman una barrera esférica para evitar la coalescencia de los OB. Otras proteínas descritas asociadas a OB en las plantas son caleosinas y esteroleosinas. Los lípidos degradados durante la imbibición de la semilla proporcionan energía e hidratos de carbono que favorecen el crecimiento de plántulas inmediatamente después de la germinación. Este proceso metabólico se inicia por las lipasas, que catalizan la hidrólisis de triglicéridos (TG) para liberar los ácidos grasos libres y glicerol.

Diferentes estudios proteómicos en semillas de plantas han identificado proteínas asociadas a OB. Además de las proteínas ya conocidas, algunas proteínas menos abundantes asociadas a OB se han identificado incluyendo enzimas, proteínas implicadas en el tráfico de membranas y proteínas de función desconocida.

7.3.4.1 Análisis de la composición proteica de los cuerpos lipídicos de los embriones de maíz durante la germinación

En el primer artículo examinamos la composición proteica de los cuerpos lipídicos durante la germinación. Para ello, el escutelo fue diseccionado de semillas de maíz de 2 días después de la imbibición y las proteínas fueron extraídas de la fracción de los cuerpos lipídicos. El análisis de 2-DE reveló varios *spots* de proteínas asociadas a OBs. 17 fueron cortados del gel, digeridos con tripsina y analizados por LC-MS/MS. Eso nos permitió identificar 11 de las 17 proteínas. Las proteínas identificadas incluyen una oleosina, una caleosina y una esteroleosina. Además de los previamente caracterizadas proteínas asociadas OB hemos identificado otras proteínas de funciones diversas: una proteína embrionaria DC-8, una globulina-2 (ambos son proteínas específicas del embrión con función desconocida), cuatro proteínas con actividades enzimáticas (disulfuro isomerasa, xilosa isomerasa, estrictosidina sintasa y ATP sintasa beta), una proteína similar a carioferina-beta-3 y una proteína de membrana inducida por el estrés.

7.3.4.2 Análisis subproteómica cuantitativa de los cambios relacionados con la germinación en los cuerpos lipídicos del escutelo del maíz

Se realizó un análisis proteómico comparativo utilizando electroforesis bidimensional de proteínas asociadas a OBs extraídos de escutelo de maíz en dos momentos diferentes de desarrollo: semillas secas y 2 días después de la imbibición. La identificación de proteínas por espectrometría de masas nos ha permitido identificar las proteínas asociadas a OB diferencialmente acumuladas en estas dos etapas de desarrollo. El análisis estadístico mostró que 26 proteínas fueron significativamente diferentes entre las dos muestras. Se han encontrado 19 proteínas con una mayor acumulación en las semillas secas y 7 con una mayor acumulación en las semillas 2 dai. Se identificaron proteínas asociadas a OBs que no han sido descritas previamente. De entre las 19 *spots* que presentan mayor acumulación en la semilla seca se identificaron nueve proteínas: cinco *spots* corresponden a dos oleosinas diferentes, cuatro *spots* corresponden a diferentes formas de cupinas (proteínas especificas de la semilla), dos proteínas distintas de disulfuro de isomerasas (triosafosfato isomerasa y nucleósido fosfato quinasa), una proteína de choque térmico de clase IV, una proteína embrionaria DC-8, una proteína ribosomal 60S ácida P0 y un factor de elongación de goma. Los 7 *spots* que presentan una acumulación en el escutelo de 2 dai corresponden a cuatro polipéptidos: dos de ellos oleosinas diferentes, uno corresponde a la proteína mitocondrial Tim17, y dos *spots* corresponden a la prohibitina-2 y una a una manganeso superóxido dismutasa.

7.4 CONCLUSIONES

- Durante la germinación, las células epiteliales del escutelo sufren una elongación que aumenta la superficie de contacto entre el endospermo y el embrión y facilita el transporte de los nutrientes del endospermo al embrión.

- La elongación de las células del escutelo es inhibida por ABA y ácido salicílico, pH básico y ácido y altas concentraciones de sorbitol. Las giberelinas exógenas estimulan la elongación, pero una reducción en la síntesis de giberelinas o percepción no la inhiben. La elongación es inhibida por la sacarosa, pero no la glucosa.

- Los inhibidores de la transcripción y traducción reducen el alargamiento de las células escutelares, lo que indica que la transcripción y la traducción son necesarios para el proceso de alargamiento.

- Se identificaron 30 genes que muestran una expresión diferencial significativa en las células epiteliales del escutelo de un día después de la imbibición. Las funciones de un 43% de estos genes no se conoce, el 27% de ellos están involucrados en los procesos metabólicos, el 13% en la síntesis o la transformación de proteínas y el 7% en la estructura celular.

- ZmPTR1 codifica un transportador de maíz péptido no ha sido previamente caracterizado, se expresa predominantemente en el epitelio escutelar durante la germinación. ZmPTR1 se expresa también en menor medida en la radícula y del hipocotilo.

- La proteína ZmPTR1 se localiza en el tonoplasto y es homóloga a las otras proteínas transportadoras de di-y tripéptidos AtPTR2, AtPTR4 y AtPTR6 de *Arabidopsis thaliana*.

- ZmPTR1 es capaz de transportar al menos el dipéptido Ala-Ala.

- El análisis proteómico de las proteínas asociadas a los cuerpos lipídicos en escutelo de maíz durante la germinación ha permitido la identificación de, además de proteínas previamente caracterizadas asociadas OB, otras proteínas de funciones diversas: una proteína embrionaria DC-8, una globulina-2, cuatro proteínas con actividades enzimáticas, una proteína similar a carioferina-beta-3 y una proteína de membrana inducida por el estrés.

- El Análisis cuantitativo subproteómico de los cambios relacionados con los cuerpos lipídicos en el escutelo de maíz entre semillas secas y semillas 2 dai permitieron la identificación de nuevas proteínas que interactúan con los cuerpos lipídicos en las semillas secas o en la germinación de las semillas. En semillas secas: oleosinas y cupinas, disulfuro isomerasas, un nucleósido fosfato quinasa, una proteína de choque térmico de clase IV y un embrión de la proteína DC-8, una proteína ribosomal 60S ácida P0 y una proteína de factor de elongación. En la germinación de las semillas: oleosinas, proteína mitocondrial Tim17, prohibitina-2 y una superóxido dismutasa de manganeso (Mn-SOD).

- Los cuerpos lipídicos interactúan con otros orgánulos celulares de las plantas durante la germinación, como, por ejemplo, las mitocondrias.

- Los cuerpos lipídicos pueden servir como depósitos de almacenamiento transitorio para algunas proteínas, que puede servir de protección de las proteínas contra la degradación, o para proteger a la célula de los efectos perjudiciales de la actividad incontrolada de la proteína.