Numéro National de Thèse : 2016LYSEN067

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée par
l'Ecole Normale Supérieure de Lyon

Ecole Doctorale N° 340
Biologie Moléculaire, Intégrative et Cellulaire

Discipline : Science de la Vie

Soutenue publiquement le 16/12/2016, par :
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A functional and genetic analysis of novel signaling molecules regulating embryo surface formation in *Arabidopsis thaliana*

Analyse fonctionnelle et génétique de nouvelles molécules de signalisation impliquées dans la régulation de la formation de la surface de l'embryon d'*Arabidopsis thaliana*

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A FUNCTIONAL AND GENETIC ANALYSIS OF NOVEL SIGNALING MOLECULES REGULATING EMBRYO SURFACE FORMATION IN ARABIDOPSIS THALIANA



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This dissertation is submitted for the degree of Doctor of Philosophy

October 2016

“In seed time learn, in harvest teach, in winter enjoy.”

William Blake

ACKNOWLEDGEMENTS / REMERCIEMENTS

Je voudrais d'abord remercier Sébastien Baud, Niko Geldner, Frédéric Domergue and Thierry Gaude pour avoir accepté de faire parti de mon Jury de thèse.

Je voudrais adresser un immense merci à Gwyneth Ingram, ma directrice de thèse. Merci Gwyneth, pour ton énergie à toute épreuve, et pour ton génie, qui a toujours su trouver réponses à mes interrogations. Je suis conscient d'avoir eu un encadrement de très haute qualité, et je t'en serais toujours redevable! Je voudrais aussi remercier Audrey "Deedee" Creff pour son excellent encadrement technique et parce qu'elle a la classe, tout simplement. Un dernier merci à toutes les deux pour votre patience infinie..! Continuez à inspirer ainsi les futurs doctorants qui passeront dans l'équipe SeedDev. A propos, merci pour l'aide sur la fin Nico (et Sof aussi!), et puissent Cerberus&Co combler tes attentes, je te fais confiance pour prendre soin du bébé. Merci Lysiane pour l'initation intense à la Cryo. Enfin, merci à tous les autres membres de l'équipe (Mentions spéciales à Thomas pour les discussions cuticulaires, et à Nat' pour le café).

Le RDP est un endroit peuplé de gens formidables. Je voudrais remercier tous ceux qui ont fait et qui font que ces trois années resteront inoubliables. Tout d'abord Cyril, qui m'a montré le chemin du RDP, et Teva, qui m'en a ouvert la porte. J'ai pu alors rencontrer des gens fantastiques: Marina, Mathilde, Antoine DuveLarrieu, Djo, Jérémy, Carlos, Stan, Laia, Mar, Ursula, Bayle, Tchié, Ben,. Matthieu Pierre Platre, merci pour le sound system des récoltes nocturnes, et pour toutes les autres aventures. Ayant beaucoup eu la bougeotte, merci à tout ceux qui ont eu la charité de m'accueillir dans leurs bureaux. Et bon courage à la nouvelle génération!

Sortons un peu du labo, merci aux collocs du passé et du présent: mon bon Jeanjean, à la passion inspirante; le très grand Vincent Grass, le grand Betsch et la petite Gilles et la Sarah. Et hors des murs du foyer, merci Pierrot, compère de toujours, et Hugo, compagnon de route téméraire depuis toujours aussi. Et puis tous les autres copains et copines parsemés sur la surface du globe à qui je pense toujours sans donner de nouvelles!

Et merci à ma famille: mes deux frères, Léo et Alexi, ces deux zigotos exceptionnels; et mes parents, Hervé et Laurence, toujours aimants et compréhensifs. Merci de m'appeler souvent, mes séjours avec vous sont toujours des plus ressourçants.

Et enfin, le plus grand de tous les merci va à Julie, qui aurait pu être nommée dans presque tous les paragraphes précédents, mais qui a une place toute spéciale ici et ailleurs…

Voilà, merci à tous de m'avoir fait grandir.

ABSTRACT

Seed development is a crucial step in Angiosperms life cycle. The seed is composed of three distinct compartments: (1) The testa, ensuring a protective function, (2) the endosperm, which plays a key nutritive role supporting (3) the embryo, the fate of which is to become the future plant. These three tissues develop concomitantly to form a viable seed. Such developmental coordination necessitates the involvement of communication between the compartments. In this context, I have studied genes involved in the establishment of the embryonic cuticle, a hydrophobic structure that surrounds the embryo, plays an essential post-germination function in regulating water loss and is thus critical for plant survival. At the beginning of my PhD, several proteins were known to be involved in the process of cuticle establishment, some of which were expressed in the endosperm and others in the embryo, hinting at the existence of molecular communication between the two tissues. On the endosperm side, the transcription factor ZOU controls the expression of ALE1, a subtilisin-like serine protease. On the embryo side, two receptors, GSO1 and GSO2, are involved. Genetic interaction between the genes encoding these proteins had confirmed their involvement the same signalling pathway. The molecular identities of these proteins led us to propose the existence of one or more unidentified peptides acting as messengers between the embryo and the endosperm. My research has allowed the characterization of novel proteins involved in the process of embryonic surface formation. The principal subject of my research has been CERBERUS, a peptide produced in the endosperm, the expression of which is controlled by ZOU, and which is necessary both for the formation of an intact embryonic cuticle and the production of a previously uncharacterised structure, the embryo sheath. I have demonstrated novel roles for GSO1 and GSO2 in embryo sheath deposition. Furthermore, I have generated preliminary data suggesting that a protein involved in peptide sulfation, TPST, is involved in the GSO1 GSO2 signalling pathway. Finally, I have shown that another protein involved in posttranslational protein modification, FRIABLE1 is involved in this same pathway. My results have advanced knowledge of the molecular mechanisms controlling embryonic surface formation in *Arabidopsis*.

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List of Abbreviations and Acronyms

ABA	Abscisic Acid
ABC	ATP-BINDING CASSETTE
ACR4	ARABIDOPSIS CRINKLY 4
AFL	AI3, FUS3, LEC1&2
AGL	AGAMOUS-LIKE
AGO1	ARGONAUTE1
AHK	Arabidopsis Histidine Kinase
ALE1	ABNORMAL LEAF SHAPE 1
AP2	APETALA 2
ARF	AUXIN RESPONSE FACTOR
ARR	Arabidopsis Response Regulators
ATHB	Arabidopsis thaliana Homeobox
ATML1	ARABIDOPSIS THALIANA MERISTEM LAYER 1
BAK1	BRI1 ASSOCIATED KINASE
BDG	BODYGUARD
bHLH	basic Helix-Loop-Helix
BR	Brassinosteroids
BIK1	BOTRYTIS-INDUCED KINASE 1
BRI1	BRASSINOSTEROID INSENSITIVE 1
CBF	C-repeat Binding Factor
CK	Cytokinin
CLE	CLAVATA3/ESR-RELATED
CLV	CLAVATA
CRP	Cysteine Rich Peptides
CRS	CERBERUS
ER	ENDOPLASMIC RETICULUM
EREBP	ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN
ESF	EMBRYO SURROUNDING FACTORS
ESR	Embryo Surrounding Region
FDH	FIDDLEHEAD
FER	FERONIA
FIE	FERTILIZATION INDEPENDENT ENDOSPERM
FIS	FERTILIZATION INDEPENDENT SEED
FLC	FLOWERING LOCUS-T
FRB1	FRIABLE 1
GA	Gibberellic acid
GLV	GOLVEN
GRI	GRIM REAPER
GSO	GASSHO
HD-ZIP	Homeodomain leucine zipper
HDG	HOMEODOMAIN GLABROUS

ICE1	INDUCER OF CBF EXPRESSION
IKU2	HAIKU 2
JA	Jasmonic Acid
LEC	LEAFY COTYLEDON
LIP	LOST IN POLLEN TUBE GUIDANCE
LPTG	Glycosylphosphatidylinositol-anchored Lipid Transfer Proteins
LRR	Leucine-Rich Repeat
MAPK	MITOGEN ACTIVATED PROTEIN KINASE
MP	MONOPTEROS
PCR2	Polycomb Repressive Complex 2
PDF2	PROTODERMAL FACTOR 2
PEP	PEPTIDE
PEPR	PEP RECEPTOR
PIN	PIN-formed
PM	Plasma Membrane
PSK	PHYTOSULFOKINE
PT	Pollen Tube
RAM	Root Apical Meristem
RALF	RAPID ALKALINIZATION FACTOR
RGE1	RETARDED GROWTH OF EMBRYO1
RGF	Root Growth Factor
RLCK	Receptor-Like Cytoplasmic Kinase
RLK	Receptor Like Kinase
RPK1	RECEPTOR-LIKE PROTEIN KINASE 1
SAM	Shoot Apical Meristem
SBT	SUBTILISIN
SCRM	SCREAM
SERK	SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE
SP	Signal Peptid
SPTM	Small Post-Translationally Modified
SSP	Specialized Storage Protein
SSP	SHORT SUSPENSOR
STIG	STIGMA SPECIFIC 1
STM	SHOOTMERISTEMLESS
TAG	Triacylglycerol
TF	Transcription Factor
TML1	TMO-Like 1
TMO5	TARGET OF MONOPTEROUS
TOAD2	TOADSTOOL2
TPST	TYROSYLPROTEIN SULFOTRANSFERASE
UTR	Untranslated Region
VLCFA	Very Long Chain Fatty Acids
WOX	WUSCHEL-RELATED HOMEOBOX
WT	Wild Type
WUS	WUSCHEL
ZOU	ZHOUPI

1 General Introduction

1.1 Arabidopsis seed development

1.1.1 Why study seeds?

Seeds are of the utmost importance in human and livestock nutrition. They directly provide the majority of sugars (cereals) and oils (palm oil, rapeseed oil, etc...) consumed by man (www.fao.org/seeds/en). In addition to their agronomic importance, from an evolutionary point of view, the seed can be considered one of the main reasons for the success of the Spermatophytes (Angiosperms & Gymnosperms) as land plants (Linkies et al., 2010). Seeds allow the successful dispersal of the next generation by protecting the future plant, allowing germination to coincide with optimal environmental conditions, and providing nutrition in the first stages of post-embryonic seedling development (Nowack et al., 2010).

Finally, and of particular relevance to the work presented here, the unique structure and genetic composition of the seed make it a powerful system in which to ask fundamental questions about how plant tissues communicate to co-ordinate their development. Structurally, the Angiosperm seed, for which I use *Arabidopsis thaliana* as a genetic model in my thesis, is composed of three genetically distinct tissues. The maternal diploid seed coat, or testa, whose main role is to protect the internal tissues, but which also serves in some cases as an aid to dispersal and/or the regulation of germination, encloses two zygotic tissues: the diploid embryo that will later develop in a functional plant, and the triploid endosperm that is mainly thought to function as a support for the embryo during its development and germination. These three genetically

distinct tissues (the testa, the endosperm and the embryo) must coordinate their development in order to assure the formation of a viable seed. This fact, together with their physical and genetic compartmentalization, makes the developing seed an original model for studies addressing the mechanistic basis for inter-tissue communications events. Despite its advantages, this aspect of the seed system has not, to date, been extensively exploited (Ingram, 2010).

1.1.2 Overview of seed development

Before entering into the details of seed development, it is first necessary to provide a synthetic overview of the spatial and temporal characteristics of this process. Figure 1 (from Le et al., 2010), summarizes the main events occurring within each tissue, as well as providing an approximation of the timescales involved. The developmental stages to which I will later refer in my thesis are also presented in this figure; they are based solely on embryo morphogenetic patterning.

Seed development initiates with double fertilization of the embryo sac and ends with the production of a dormant seed. In Arabidopsis the whole process takes around 20 days to be completed. Two metabolically distinct steps have been defined (Baud et al., 2002). The first step of early seed development, running from the pre-globular stage (Day 1) to the linear torpedo stage (Day 7-8), sees the apparition of the main patterns in the zygotic compartments. The embryo acquires the basic plant body organization and the endosperm first develops into a cœnocyte through free nuclear divisions, then cellularizes by synthesizing cell walls between the majority of the nuclei. In contrast, the basic plan of the seed coat is determined during pre-fertilization ovule development, during which two maternal organs called integuments elongate through cell division to enclose the nucellus, which in turn encloses the female gametophyte. At ovule maturity the outer integument is composed of two juxtaposed epidermal cell layers, while the inner integument, which lies adjacent to the female gametophyte at ovule maturity due to the elimination of much of the nucellus, contains two epidermal cell layers that are separated by an additional third cell layer in more proximal regions. The integuments undergo further limited cell divisions during early post-fertilization development, but the majority of their post-fertilization growth is achieved by cell expansion. The testa reaches its final size at around the point of endosperm cellularization (mid-late heart stage).

The second phase in seed development is termed seed maturation (Baud et al., 2002). During this phase the endosperm breaks down progressively, giving space to the growing embryo. The embryo (and to some extent the endosperm) accumulates nutritional reserves to ensure a successful germination. Once the embryo is fully developed, the seed enters a late maturation phase during which it acquires its tolerance to desiccation, becomes dormant and dries out. Thereafter, the seed leaves the mother through fruit dehiscence or abscission and the development of the embryo reinitiates only once the right environmental conditions are encountered, with the germination of the seed.

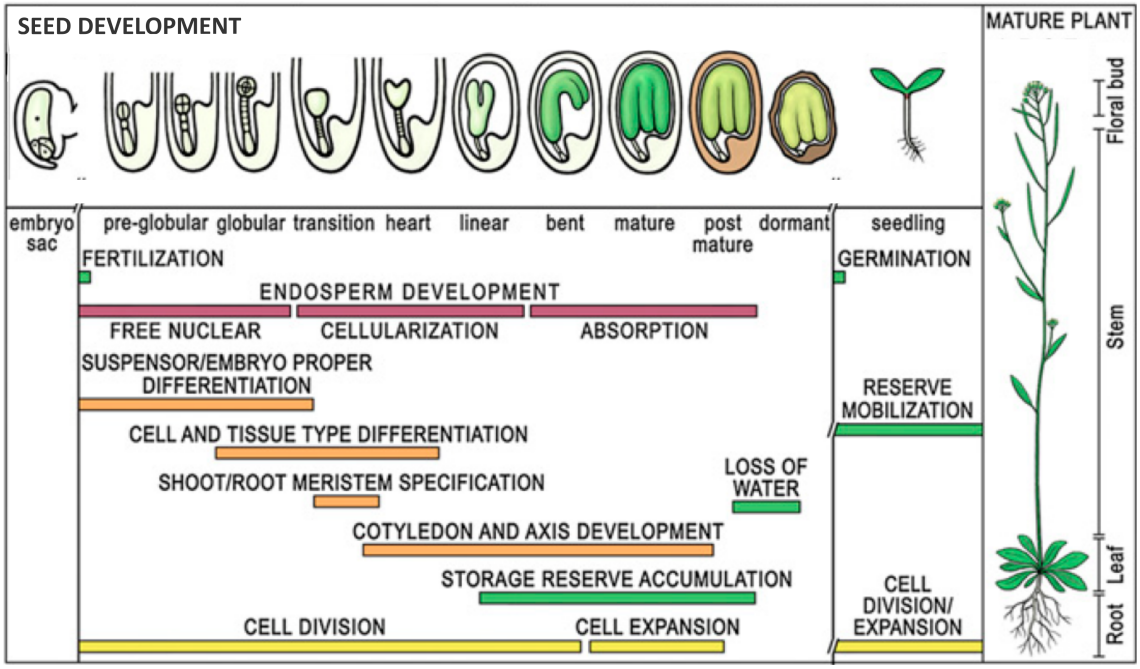

Figure 1. Overview of seed development (adapted from Le et al., 2010).

1.1.3 Where it all begins: gametes and double fertilization in Arabidopsis

The plant sexual life-cycle consists of a succession of haploid and diploid phases, with multicellular haploid (gamete-forming, also known as gametophyte) generations alternating with multicellular diploid (spore-forming, also known as sporophyte) generations. In Angiosperms, the majority of the life cycle is spent as a diploid sporophyte. The post-germination life of the sporophyte can be divided in two main phases of development. The first phase, termed vegetative development is characterized by the production of leaves and roots, ensuring the production of the equipment needed for a sessile lifestyle depending upon photosynthesis and controlled

water/nutrient uptake. The second phase, termed reproductive development, is characterized by the production of sexual organs within the flowers.

Flowering sets the scene for sexual reproduction by enabling the production of both female and male gametophytes. In order to give rise to haploid spores, meiosis occurs within specific organs of the plant, located within the flower (the ovule and the stamens). Spores then undergo limited mitosis to produce haploid gametophytes; the female gametophyte (in Arabidopsis composed of 8 nuclei partitioned into seven cells) enclosed within the ovular tissues, and the male gametophyte (two sperm cells enclosed within a vegetative cell), which is released from the stamen as a pollen grain.

1.1.3.1 Female gametophyte development: Ovule formation

Female gametophytes are located in the female reproductive organ, the gynoecium, more commonly named pistil, in a particular structure called the ovule. In *Arabidopsis*, a functional ovule comprises three distinct tissues: two protective integuments, the nucellus, and the gametophyte proper (Robinson-Beers et al., 1992; Schneitz et al., 1995). Figure 2 summarizes the developmental events leading to the production of a functional ovule. The tissues laid down during ovule development give rise to the seed after double fertilization. The two integuments are destined to become the enclosing seed coat, or testa. They arise on the sides of the ovule primordium and extend as tubular organs during ovule development to enclose a finger of tissue called the nucellus. The point at which the integuments meet over the nucellus is called the micropyle. Within the nucellus a single diploid cell differentiates into the megaspore mother cell (MMC) and undergoes mitosis to generate four haploid megaspores. Three of these megaspores degenerate leaving one viable megaspore that subsequently undergoes 3 rounds of mitosis to give the 8 nuclei of the mature female gametophyte which are partitioned into 7 discrete cells at ovule maturity. 4 distinct cell identities are distinguished in the mature female gametophyte. At the micropylar (distal) pole are the 2 synergids whose main role is in pollen tube attraction and which flank the egg-cell from which is derived the embryo upon fertilization. The central-cell (which in the mature female gametophyte contains two fused haploid nuclei) will also be fertilized to give rise to the triploid endosperm. Finally three antipodal cells with no defined functions are found at the chalazal (proximal) pole of the female gametophyte (Berger and Twell, 2011; Colombo et al., 2008; Endress, 2011).

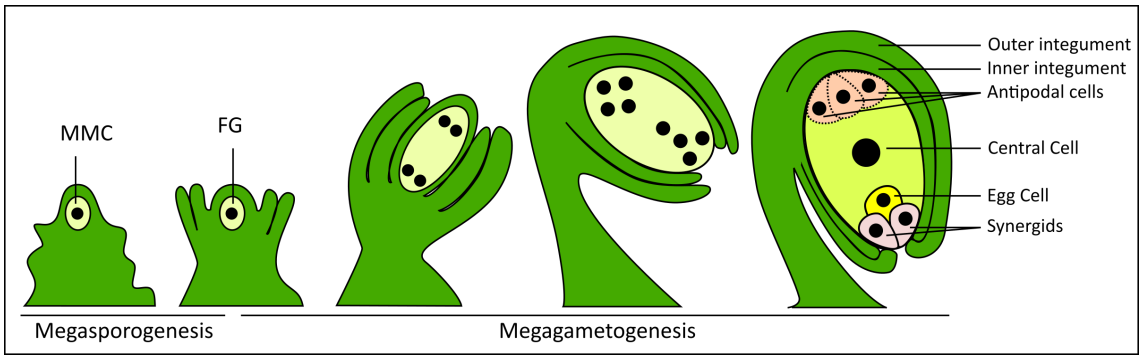

Figure 1. Ovule development. Adapted from Chevalier et al., 2011.

Several cells and tissues are eliminated during ovule development leading to important alterations in the relationships between different tissues. In addition to megaspore elimination, the distal nucellus degenerates prior to ovule maturity meaning that the female gametophyte is left in direct contact with the inner integument (Beeckman et al., 2000; Ingram, 2016).

1.1.3.2 Male gametophyte development: development of the pollen grain

The male gametophyte, known as the pollen grain, is formed in the anthers, which are located at the top of the stamen filament. The young anther is composed of reproductive cells termed microsporocytes enclosed within non-reproductive tissues comprising, from the inside to the outside: the nutritive tapetum, the middle layers, the endothecium and the epidermis. At the start of pollen development, each diploid microsporocyte undergoes microsporogenesis. The two steps of meiosis lead to the formation of a tetrad composed of four haploid cells termed microspores. Each of these microspores then enters the microgametogenesis phase leading to the formation of a functional pollen grain (Figure 3 adapted from Borg et al., 2009). To do so, the microspore undergoes two successive mitoses. The first mitosis is asymmetrical and is preceded by the formation of a rather large vacuole that pushes the nucleus against the side of the cell. The resulting cell division is responsible for the formation of two unequal cells. The first, termed the vegetative cell, is the larger of the two and contains the large vacuole. The smaller cell is termed the generative (or germ) cell. After the first mitosis of the microspore, a unique phenomenon occurs, during which the small generative cell is internalized by the large vegetative cell. There, the generative cell undergoes a second mitosis, producing the two sperm cells required for double fertilization. The sperm cells, enclosed in the protective vegetative cell, form the pollen grain (Berger and Twell, 2011; Borg et al., 2009; Gómez et al., 2015). Pollen bearing anthers enter a dehiscence phase in which the pollen is released and, in Arabidopsis

(which usually self-fertilizes) can be transferred directly onto the neighboring stigmatic papillae.

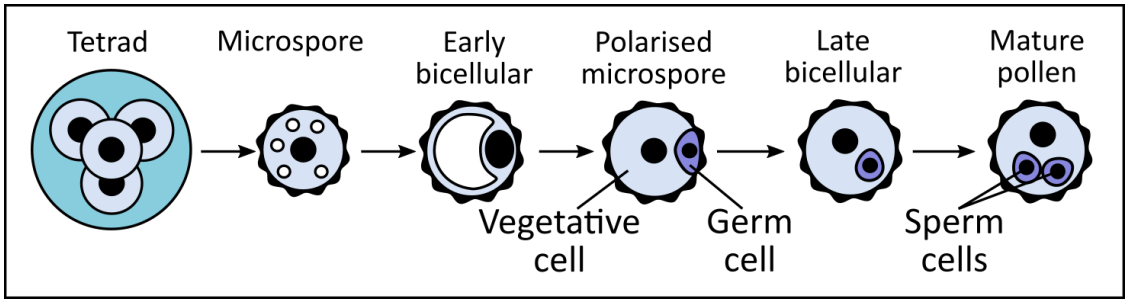

Figure 2. Pollen development after meiosis. Adapted from Borg et al., 2009. The stages of microsporogenesis are described.

1.1.3.3 From the tip of the pistil to the zygote: Mechanisms of double fertilization

In autogamous species such as *Arabidospsis*, (i.e. able to self-fertilize), the pollen grains are generally transferred directly from the dehiscent anther onto the pistil. Allogamous species, require the involvement of physical factors like the wind, or biological ones such as pollinators, to ensure that the pollen reach the pistil (Faegri and Van Der Pijl, 2013). In Arabidopsis, the stigma is termed "dry" as it doesn't produce the characteristic exudates produced by so called "wet" stigmas found in some plants, such as the solanaceae (Dickinson, 1995; Endress and Igersheim, 2000; Heslop-Harrison and Shivanna, 1977). As in most species with dry stigmas, pollen reception in Arabidopsis occurs on specialized cells called stigmatic papillae. The pollen grain interacts with a highly specialized tissue situated at the top of the pistil termed the stigma, adhering to specialized cells, the stigmatic papillae. During a complex

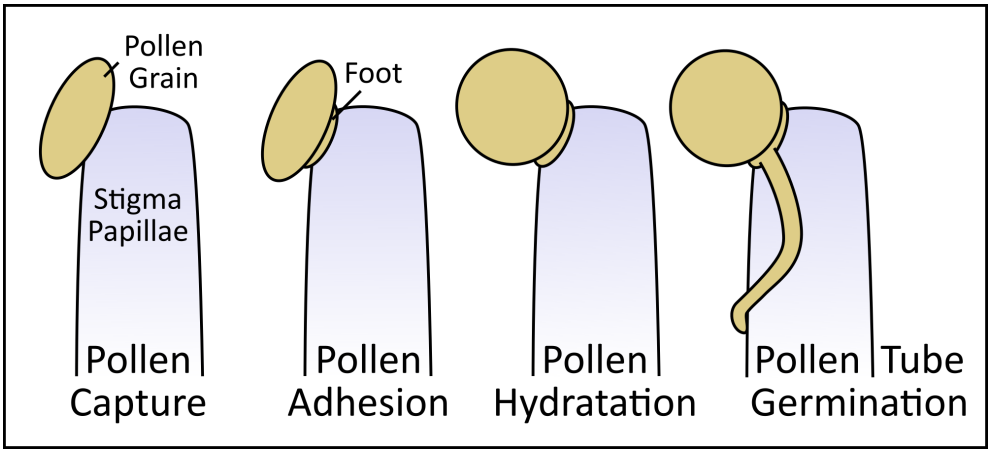

Figure 3 From the dry pollen to the pollen tube: Pollen/Stigma interaction

interaction with the papillae (Figure 4) the dry pollen is rehydrated and germinates, producing a structure called the pollen tube (PT), which contains the vegetative cell and internalized vegetative cells. This rehydratation/germination process is tightly controlled through the mechanism of auto-incompatibility in many species (Chapman and Goring, 2010; Gaude and Dumas, 1987; Takayama and Isogai, 2005), although such mechanism are not present in *Arabidopsis* due to mutations in the loci involved (Nasrallah and Nasrallah, 2014).

After germination of the pollen on the stigma, the pollen tube (PT) starts its journey to the ovule. A depiction of this process is represented in Figure 5, adapted from (Twell, 2006). The PT is a single, rapidly growing cell that navigates through a specialized tissue of the pistil called the transmitting tract, due to its role in conducing the pollen tube to its final destination. Here two different type of guidance can be distinguished: Pre-ovular and ovular guidance. The first ensures that the growing PT enters the transmitting tract and grows toward the ovary, navigating through the tissues of the sporophyte, while the second gives precise positional cues allowing the tip of the PT to grow towards the ovule and ultimately reach the female gametophyte.

Pre-ovular guidance mechanisms appear to act in part through the direct stimulation of pollen tube growth. For example, sporophytic factors such as γ-Aminobutyric acid (GABA) have been shown to activate PT growth (Palanivelu et al., 2003). In species with wet stigmas, exudates have also been shown to play key roles in stimulating PT growth (Chae and Lord, 2011; Dresselhaus and Franklin-Tong, 2013; Huang et al., 2014), and may also provide nutrients to sustain PT growth. Correct ovular guidance of the pollen tube relies heavily on peptide signaling pathways, which will be further discussed in the next part of the introduction. These pathways involve the production and perception of cues informing the PT of the location of the ovule micropyle. The synergid cells play key roles in this process (Higashiyama and Takeuchi, 2015).

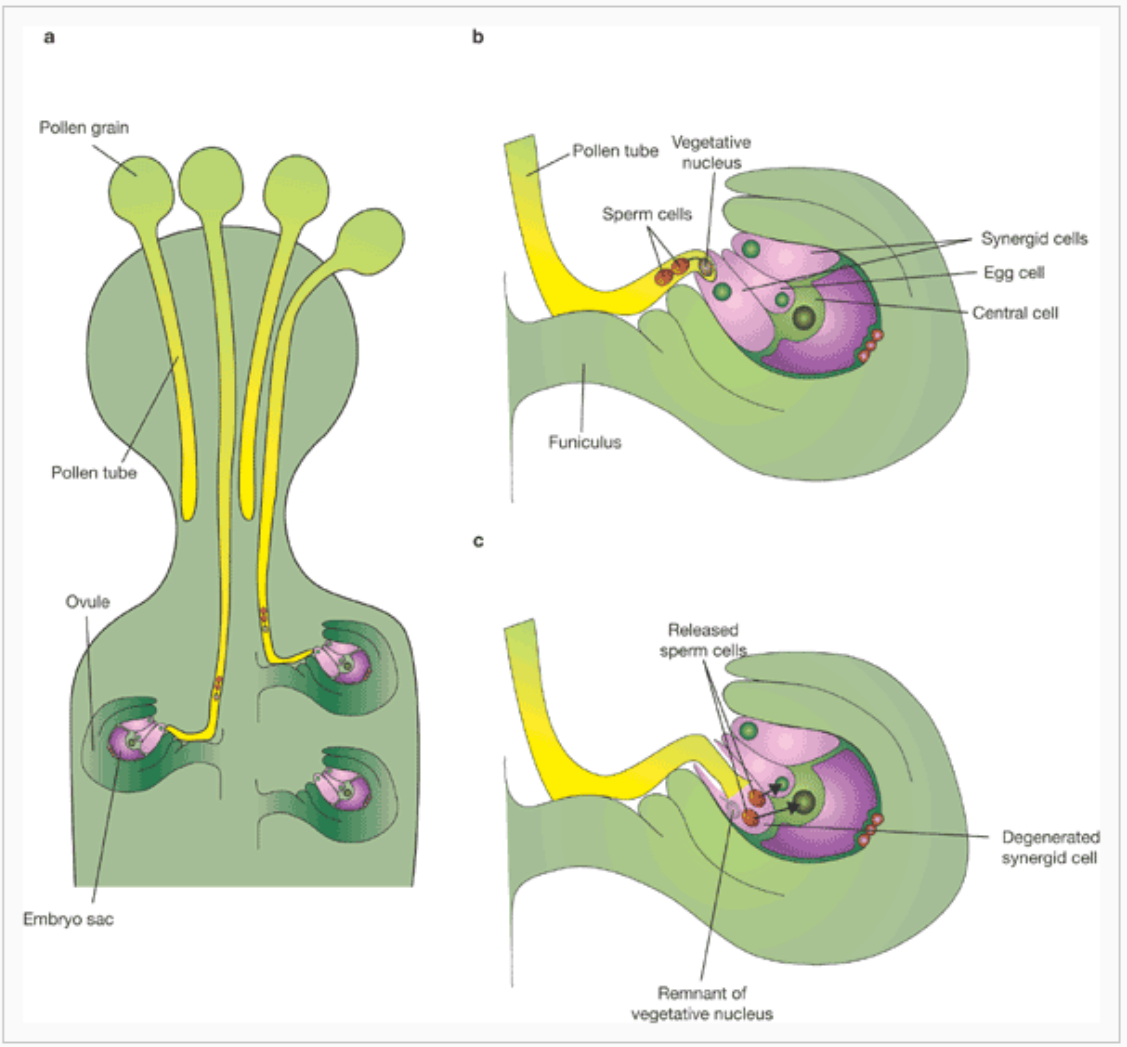

Figure 4 PT growth and double fertilization. Taken from Twell, 2006.

Once the PT arrives at an unfertilized ovule, the PT accesses the embryo sac via the micropyle, enters one of the two synergids and then bursts, releasing both sperm cells into the embryo sac. Both male and female factors regulate the precise timing of PT burst, with some mutants showing a precocious burst and some never bursting (Heydlauff and Gross-Hardt, 2014). Once the PT has burst, the remaining synergid is then eliminated by fusion with the fertilized central-cell (Maruyama et al., 2015). This process is thought to be important because it rapidly dilutes pollen tube attractants and prevents polytubey, the ingress of multiple pollen tubes into a single ovule.

Once the pollen tube has burst, one sperm cell fuses with the egg cell, and the second fuses with the central cell. The fertilized egg cell is known as the diploid zygote after nuclear fusion occurs. This cell is of central importance since it will give rise to the embryo, and thus to the next plant generation. The fertilized central cell, after fusion of its double-haploid nucleus with the second haploid sperm nucleus, results in the triploid

endosperm (Hamamura et al., 2012). It is interesting to note that there is no preference for a specific sperm-cell to fuse with either the egg-cell or the central-cell (Hamamura et al., 2011).

1.1.4 Early seed development

The dramatic events occurring during fertilization kick-start seed development *"sensu stricto"*. The development of the three seed compartments post-fertilization is tightly coordinated by various signaling pathways. They will be discussed in more detail in the next section of the introduction. Here I will describe the structural changes that accompany seed development, concentrating mainly on Arabidopsis, while mentioning examples from other species when relevant.

1.1.4.1 The embryo

The diploid zygote, resulting from the fusion of the haploid egg-cell of the female gametophyte with one of the two haploid sperm-cells from the pollen tube, undergoes successive temporally and spatially controlled cell divisions, accompanied by cell fate specification events, to confer the basic plant body plan on the embryo. The precise timing of these events in Arabidopsis has been recently uncovered through *in vivo* imaging of the developing embryo (Gooh et al., 2015). Embryo development steps (Figure 6) and their control have recently been extensively reviewed by Hove et al., 2015, amongst others.

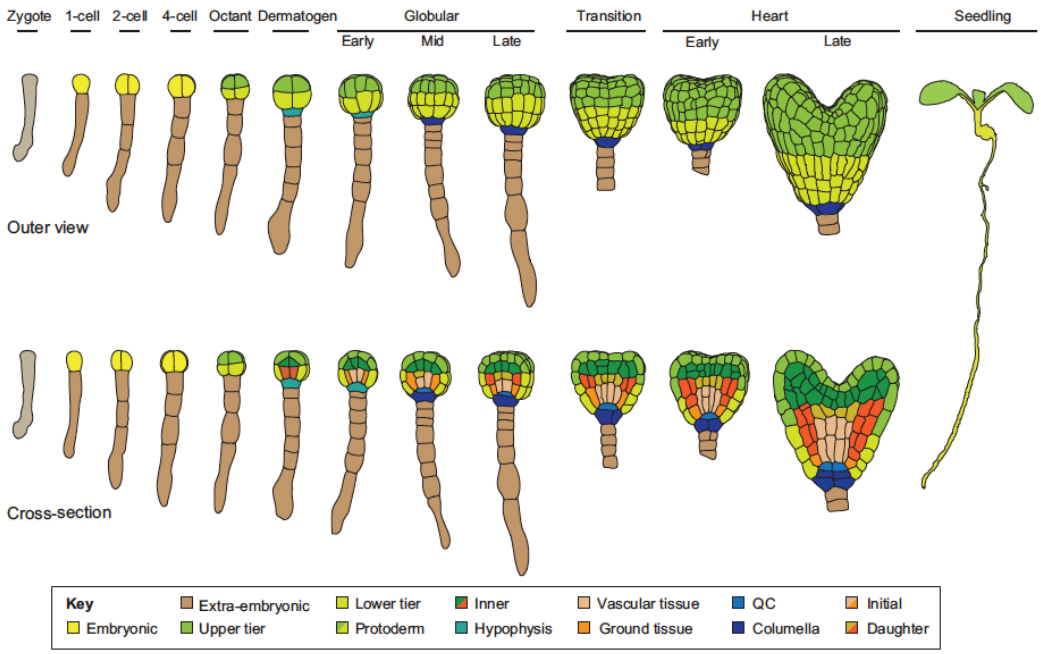

Figure 5 Early embryo development in Arabidopsis Taken from Hove et al., 2015

1.1.4.1.1 Patterning the embryo

The newly formed diploid zygote is a totipotent cell. It will give rise to all the cell types necessary to "make a plant" through cell divisions, cell fate specification and cell-cell communication events. Arabidopsis is one of the few species in which cell division patterns are largely invariant from early on in embryogenesis. Most species having a much more apparently disordered embryonic cell division pattern at first (Mansfield and Briarty, 1991; Pollock and Jensen, 1964). It is unclear to what extent cell division patterns are necessary for cell fate acquisition during early Arabidopsis embryogenesis since several mutants with defective early division planes are capable of establishing relatively normal embryonic body plans (Breuninger et al., 2008; Yoshida et al., 2014). This suggests strongly that positional information, rather than lineage, is of prime importance in embryo patterning.

The very first division of the Arabidopsis zygote occurs roughly 20 min after fertilization. During these first 20 min, the zygote elongates along the apico-basal axis. Subsequently, an asymmetrical division results in the production of two cells: the apical cell that is destined to produce most of the embryo proper, and the basal cell, the larger of the two, which is the precursor of the suspensor and the root quiescent center. The apical cell then undergoes two rounds of longitudinal divisions and one transverse division to give the octant stage embryo composed of 8 cells (Gooh et al., 2015).

1.1.4.1.2 Epidermis specification: Cell fate acquisition of the protoderm.

The first step in the specification of protodermal and inner tissues is the formation of two spatially distinct cell populations by cell division. At the octant stage, the 8 cells of the embryo, which are all in direct contact with the embryo surface, each undergo a tangentially oriented asymmetrical division, producing two distinct concentric cell populations, one internal and one forming the surface of the embryo proper. The resulting structure is called the dermatogen embryo. The outer cells at this stage form the protoderm, which subsequently divides almost exclusively anticlinally, and is entirely responsible for giving rise to the shoot epidermis. The inner cell population is the precursor of most of the non-epidermal shoot tissues, although occasional periclinal divisions in the epidermis can occur during the development of some organs (Furner, 1996; Irish and Jenik, 2001). In terms of the molecular control of these processes, members of the WUSCHEL-RELATED HOMEOBOX (WOX) transcription factor family are expressed in various distinct or overlapping early embryo

regions and play a crucial role in controlling the divisions that give rise to these two distinct layers. Single *wox2* mutants have mild perturbations in early embryonic division patterns (Breuninger et al., 2008; Haecker, 2004). Multiple *wox* mutants show enhanced perturbations, as is the case in *wox1 wox2 wox3* and *wox2 wox8* multiple mutants. Transport, perception and downstream signaling of the key phytohormone auxin is also absolutely crucial for early steps in embryo patterning with perturbations observed in double mutants lacking MONOPTEROS/AUXIN RESPONSE FACTOR5 (MP/ARF5) and WOX2 (Breuninger et al., 2008), and in single and multiple mutants of the PIN family of auxin efflux carriers (Jeong et al., 2016; Smit and Weijers, 2015). Together, WOXs and Auxin therefore set the scene for protodermal differentiation through the formation of a two-layered embryo proper.

Two plant specific transcription factors belonging to the HDZIP-IV family have been shown to play key roles in specifying protodermal identity. The expression of *ARABIDOPSIS THALIANA MERISTEM LAYER 1 (ATML1)* initiates at the two-cell stage of embryogenesis, just after the first division of the zygote, in the apical cell. Expression is maintained in all the cells of the embryo proper until the dermatogen stage, when it is lost from the internal cell population (Lu et al., 1996; Takada and Jurgens, 2007). Subsequently the expression of *ATML1* is restricted to the protoderm. Protodermal identity is very slightly compromised in *atml1* mutants (Abe, 2003). The closest homologue of *ATML1, PROTODERMAL FACTOR 2 (PDF2)* has an almost identical expression pattern to that of *ATML1*, showing ubiquitous expression in the embryo proper at the octant stage, which is then restricted to the protoderm at the dermatogen stage (Abe, 2003). *PDF2* acts largely redundantly with *ATML1* in controlling protodermal identity. Double mutant combinations involving a hypomorphic allele of *ATML1* produce seedlings in which epidermal cells are almost completely replaced by cells with a mesophyll-like identity (Abe, 2003) whereas double combinations of true null alleles arrest at the globular stage of embryo development (San-Bento et al., 2014). Furthermore ectopic expression of *ATML1* leads to the production of cells with epidermal identities in internal cell layers (Javelle et al., 2011; Takada, 2013; Takada et al., 2013). A recent study has shown that the expression of *ATML1* and *PDF2* is positively regulated by the activity of ARABIDOPSIS CRINKLY 4 (ACR4); a Receptor Like Kinase (RLK) specifically expressed in the epidermis. ATML1 and PDF2 negatively act on their own expression and that of *ACR4*, thus

creating a feedback loop necessary for the maintenance of epidermal identity (San-Bento et al., 2014).

During the post-embryonic life of the plant, epidermal identity is continuously maintained and serves as an interface between the plant and its environment, with roles in mediating plant gaseous exchanges, plant pathogens interaction, and nutrient uptake for example (Javelle et al., 2011). Many of the important functions of the epidermis are tightly linked to the cuticle, a layer of lipid-derived polymers secreted by epidermal cells into their outer cell wall. The biogenesis and function of this structure will be discussed in detail in the section III of the introduction, but it is relevant to note now that it is produced de novo on the surface of the developing embryo at an early developmental stage, shortly after epidermal cell fate is acquired in the protoderm (Tanaka et al., 2001 and unpublished results).

1.1.4.1.3 Patterning the inner tissues: radial patterning and stem cell niche formation

At the dermatogen stage, when the future epidermis is specified, the inner tissue is composed of 8 cells. Through longitudinal divisions of the inner cells, the basis for ground and vascular tissues is obtained, leading to the early globular stage of embryogenesis. Little if anything is known about ground tissue specification, although the Auxin Response Factor (ARF) ARF5/MONOPTEROS and downstream factors such as the bHLH transcription factors TARGET OF MONOPTEROS5 (TMO5) and TMO-LIKE1 (TML1) are absolutely necessary for the establishment of the embryonic vasculature, implicating auxin fluxes in this process (Berleth and Jurgens, 1993; Hardtke, 1998; De Rybel et al., 2013). At this stage, new classes of transcription factors start to be expressed in the apical and basal domains of the developing embryo, and are apparently necessary for specifying the “shoot” and “root” domains of the embryo. The HD-ZIP III transcription factors ATHB8, ATHB15, PHABULOSA, PHAVOLUTA and REVOLUTA act redundantly to maintain the Shoot Apical Meristem (SAM) post germination and are expressed in the apical domain of the embryo from the globular stage onwards (Emery et al., 2003). The expression of HD-ZIP III protein encoding genes is prevented in the basal embryo domain by microRNA 165 and 166 (Mallory et al., 2004; Miyashima et al., 2013). The basal domain of the embryo is defined by the expression of the PLETHORA (PLT1, PLT4, PLT3/AIL6 and PLT4/BABYBOOM) (Aida et al., 2004; Galinha et al., 2007). Ectopic expression studies show that the roles of HD-ZIP III and transcription factors and PLT transcription factors in specifying the

ROOT and SHOOT domains of the embryo are largely antagonistic (Smith and Long, 2010).

The transcription factor WUSCHEL (WUS), is required for SAM stem cell maintenance in post-embryonic development (Mayer et al., 1998), and the expression of the *WUS* gene is considered to mark stem cell fate in the SAM. *WUS* expression initiates in the globular embryo, as does the expression of other well-known meristem specifying genes such as *SHOOTMERISTEMLESS (STM)* (Long et al., 1996). However, although several classes of mutants are known to be defective in either the initiation of the expression of these genes, or the maintenance of their expression (reviewed in (Hove et al., 2015)), the exact mechanisms involved in SAM establishment in the embryo remain unclear.

In contrast events leading to Root Apical Meristem (RAM) establishment are better understood. RAM specification initiates at the globular stage with the specification of the hypophysis, which is derived from the apical most cell of a lineage issuing from transverse divisions of the basal cell of the two-cell embryo. The hypophysis expresses the RAM stem cell marker WOX5, and its specification is strongly dependent upon the formation of an auxin maximum by the concerted activity of PIN auxin efflux carriers in the different cells of the early embryo. The capacity to respond to auxin, is naturally also critical in this process, and is again mediated by the ARF5/MP protein (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998). The MP target *TMO7* is also involved, however, this effect is non-cell autonomous since neither *MP* nor *TMO7* is expressed in the hypophysis. Recent work has shown that in fact, TMO7 protein moves into the hypophysis from the vascular precursors in order to specify cell fate (Schlereth et al., 2010). Auxin itself, transported down through the vasculature to the hypophysis is also involved in RAM specification, likely via the activity of ARF9 (Rademacher et al., 2011), and the PLETHORA transcription factors are likely also to be important since mutants severely impact RAM formation (Galinha et al., 2007).

1.1.4.1.4 Acquisition of bilateral symmetry, and cotyledon outgrowth

By the dermatogen stage, the embryo is radially symmetrical and has a well defined apico-basal axis. The out growth of the two cotyledons at the triangular stage confers bilateral symmetry on the embryo proper. The ball-shaped embryo becomes triangular and then heart-shaped due to the appearance of the primordia of the

cotyledons ("embryonic leaves"), which will serve for photosynthetic purposes after germination. The control of cotyledon initiation is determined by the DORNRÖSCHEN (DRN) and DRN-like (DRNL) AP2-type transcription factors. They are specifically expressed at the positions where the cotyledons are initiated (Chandler et al., 2007; Kirch, 2003). *drn drnl* double mutants produce pin-like cotyledon-less embryos. *DRN* is a direct target of ARF5/MP, making it an indirect target of auxin signaling (Cole et al., 2009). Consistent with this, the PIN1 auxin efflux carrier and its regulatory kinase PINOID are necessary for the generation of auxin maxima at the cotyledon tips, and mutants also have defective cotyledon phenotypes (Furutani, 2004; Liu et al., 1993). The CUP-SHAPE COTYLEDONS (CUC) genes are also necessary for proper bilateral symmetry, with corresponding mutants having fused cotyledons. These proteins are necessary for boundary establishment between organs (Aida et al., 1997; Vroemen, 2003). Finally, two receptor kinases, RPK1 and TOADSTOOL2 (TOAD2) are required for embryo patterning, with a role in cotyledon primordial formation (Nodine et al., 2007; Nodine and Tax, 2008). However the precise patterning mechanisms in which these proteins are involved have not yet been elucidated.

1.1.4.2 The suspensor: connecting the embryo to the other tissues

The suspensor is derived entirely from the basal progeny of the basal cell of the two cell embryo, and has a very different fate from than the embryo proper and hypophysis. After the very first division of the zygote giving rise to the embryo proper and the basal cell, the latter elongates and goes through three rounds of transverse divisions to form a filament of 8 cells, reduced to 7 cells when the uppermost cell differentiates into the hypophysis of the embryo. The suspensor is present during most of early seed development, but undergoes developmentally programmed cell death at the torpedo stage, coinciding with the onset of early seed maturation (Bozhkov et al., 2005; Kawashima and Goldberg, 2010).

The main role of the suspensor is to ensure the connectivity between the embryo and its surroundings: the endosperm and the maternal seed coat. The presence of plasmodesmatal structures in the cell wall between suspensor cells and between the suspensor and the embryo proper walls ensures connectivity between these two structures (Zhukova, 2006). Thus, the movement of free cytoplasmic GFP produced specifically in the suspensor into the embryo proper has been reported (Stadler, 2005). In contrast, the suspensor and the embryo are symplastically isolated from surrounding tissues, implying an important role for apoplastic transport in communication with these

tissues. Consistent with this *AtSUC3*, encoding a sucrose transporter, is preferentially expressed in the suspensor, suggesting a role in channeling nutrients to the young growing embryo (Barker et al., 2000; Meyer et al., 2000; Stadler, 2005). Other transporters such as SUC5 and SWEET11/12 have been found in the embryo surrounding endosperm, where they may ensure sucrose loading into the suspensor from the endosperm (Baud et al., 2005; Chen et al., 2015). These observations highlight a connective function of the suspensor ensuring the link between the embryo and the endosperm.

In terms of genetic regulation of the suspensor fate, the WOX8 and WOX9 transcription factors are specifically expressed in this tissue during early embryogenesis. Moreover, while the *wox8* or *wox9* simple mutants do not have any phenotype, the *wox8 wox9* double mutants have a disorganized suspensor and are embryo lethal, suggesting they act redundantly is specifying suspensor fate (Breuninger et al., 2008). Interestingly, WOX8 and WOX9 are also essential for the polarization of the zygote before division, reinforcing their fundamental role in specifying the suspensor lineage (Ueda et al., 2011). Suspensor development also involves a signaling pathway involving the perception of endosperm derived signals (Lukowitz et al., 2004) that will be reviewed in the second part of the introduction.

1.1.4.3 Endosperm development

While the diploid embryo is acquiring its functional pattern, the surrounding triploid endosperm develops. Upon fertilization of the central cell by one of the haploid sperm cells, the development of the triploid endosperm is initiated. During early seed development, the endosperm undergoes several distinct developmental phases. The first step, termed free nuclear endosperm development, is characterized by a rapid expansion of the endosperm domain driven by the rapid expansion of the central vacuole and accompanied by free nuclear divisions without cytokinesis. The second phase, initiated once the final size of the endosperm is acquired, and when the embryo reaches the heart stage of development, involves endosperm cellularization in which the free nuclei are surrounded by cell walls and the central vacuole is fragmented. This phase is followed by endosperm breakdown, which occurs progressively as the embryo expands to fill the endosperm cavity. Ultimately only a single layer of endosperm cells survives in the mature seed, completely surrounding the mature embryo.

1.1.4.3.1 Endosperm coenocytic development

Because of a total lack of cytokinesis, the first stages of endosperm development can be considered to be cœnocytic. Multiple nuclei are generated and a large vacuole is formed, driving the early growth of the seed (Olsen, 2004). The first division of the fertilized central cell triploid nucleus occurs 3 minutes after fertilization as recently revealed by live cell imaging (Gooh et al., 2015). 6 rounds of free nuclear division are necessary to reach a 64-nucleus endosperm. The process is completed around 40 hours after fertilization, which is very rapid compared to embryo development since the embryo has generally just completed its second round of division by this point. By the end of the coenocytic development phase the endosperm is estimated to contain about at 200 nuclei, and the embryo has reached the dermatogen/early globular stage of development (Boisnard-Lorig et al., 2001).

Epigenetic regulation is of the utmost importance in controlling early endosperm development. The activity of the FERTILIZATION INDEPENDENT SEED–Polycomb Repressive Complex 2 (FIS-PCR2) is necessary for the regulation of the key developmental "check point" that acts at fertilization. This complex acts maternally in the diploid central-cell of the young developing endosperm to prevent proliferation by silencing genes necessary for endosperm development. Four proteins compose the complex: FIS2, MEDEA (MEA), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1). FIS2 and MEA are female gametophyte specific. However, FIE and MSI1 are also found in regulatory complexes monitoring other developmental checkpoints in plant development (Derkacheva and Hennig, 2014). Mutations in FIS–PRC2 components leads to a fertilization-independent endosperm development (Chaudhury et al., 1997; Guitton, 2004; Köhler et al., 2003; Ohad et al., 1996), resulting in the initiation of autonomous seeds without embryos. If fertilization occurs, loss of FIS–PRC2 function in the female gametophyte results in the formation of unviable seeds containing an endosperm with an excessively large number of endosperm nuclei, which fails to cellularize. Recent work has shown that one of the key roles of the FIS–PRC2 complex in the central cell is to prevent auxin production, which is necessary for endosperm development. When fertilization occurs, active (non silenced) auxin biosynthetic genes are introduced into the central cell in the sperm cell nucleus, which thus leads to the production of the auxin necessary to initiate endosperm development (Figueiredo et al., 2015).

1.1.4.3.2 Endosperm cellularization and domain specification

After the coenocytic phase, at around the triangular stage of embryo development, the endosperm starts to cellularize in a wave-like manner, from the base of the embryo toward the nucellar/chalazal pole of the seed (Berger et al., 2006; Brown et al., 1999, 2003; Olsen, 2001). This cellularisation process appears to be critical for embryo development past the heart stage, which may explain why mutant seeds maternally lacking the PRCII proteins abort at this stage. It has been proposed that endosperm cellularization is necessary to permit the efficient transport of sugars and other nutrients from the maternal tissues, although this hypothesis remains to be proven (Hehenberger et al., 2012). Cellularisation is a remarkable process involving the progressive formation of cell plates that "grow" into the endosperm from its outer cell wall (Otegui et al., 2001). This process is poorly understood, but is defective in mutants such as *spatzle* (Sorensen, 2002), and when the function of ENDOSPERM DEFECTIVE1 (EDE1), a microtubule binding protein, is impaired (Pignocchi et al., 2009). With cellularization the spatial definition and specification of four major domains in the endosperm is fixed. These are known as the central endosperm, the Embryo surrounding region (ESR), the chalazal endosperm and the aleurone layer. A major repressor of the endosperm cellularization process is the MADS-box transcription factor AGAMOUS-LIKE 62 (AGL62), loss of function of which causes a precocious cellularization of the endosperm, supporting a role in inhibiting the process (Kang et al., 2008). Consistent with this observation, WT seeds show a drop in AGL62 expression prior to cellularization, dependent on the PCR2-FIS complex function (Hehenberger et al., 2012).

Like ovule development, early post fertilization seed development is accompanied by programmed cell death events. The antipodal cells of the female gametophyte are eliminated shortly after fertilization in a process that has not been extensively studied (Kägi et al., 2010; Song et al., 2014). Subsequently, during early endosperm expansion the proximal nucellus partially degenerates, and indeed recent work suggests that the development of the nucellus and the endosperm may be partially antagonistic (Xu et al., 2016). However, the triggers for these cell death events remain uninvestigated.

1.1.4.4 Early seed coat development

As described earlier, the seed coat is composed of two integuments fused together. The outer integument (oi) is made of 2 cell layers with distinct identities. They

were originally termed oi1 (the innermost) and oi2 (the outermost). The inner integument (ii) comprises 3 cell layers, ordered from the inside to the outside: ii1 (commonly referred to as the endothelium), ii1' and ii2 (Figure 7 taken from (Beeckman et al., 2000)). As such, the maternal seed coat is a 5-layered tissue surrounding the endosperm and the embryo. Perhaps unsurprisingly given the death of the intervening nucellar cells during ovule development, the testa and the endosperm are not connected by functional plasmodesmata (Stadler, 2005). Furthermore, it is interesting to note the presence of a cuticle at the interface between the seed coat and the testa, which could potentially gate the apoplastic movement of molecules between the two compartments (Beeckman et al., 2000; De Giorgi et al., 2015). Thus, both apoplastic and symplastic movement might be impeded between the integuments and the embryo sac.

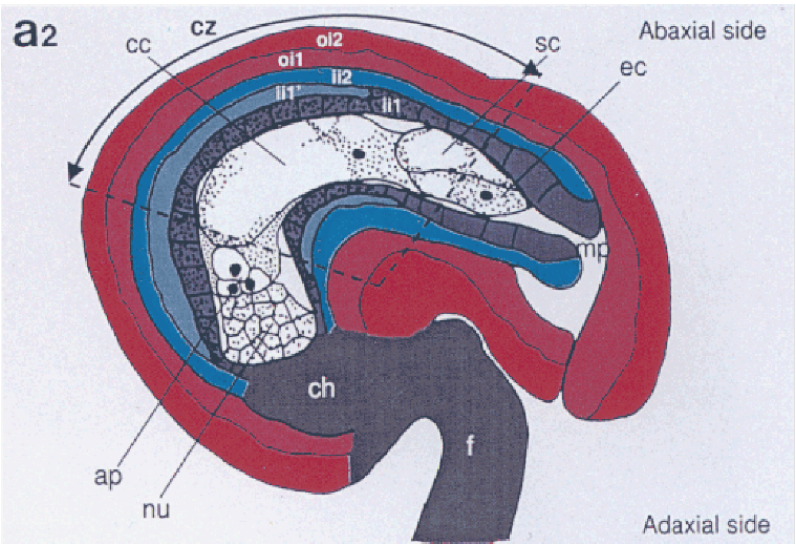

Figure 6 The mature Arabidopsis ovule (Taken from Beeckman et al., 2000). ec = egg cell, sc = synergid cells, nu = proximal nucellus, ap = antipodal cells, ch = chalaza, cc = central cell, cz= central zone, f, funiculus.

After fertilization, the seed coat undergoes a rapid growth phase coordinated with the expansion of the endosperm. Although a few cell divisions occur just after fertilization, most of the growth of the testa is though cell expansion. During this coordinated expansion, the organization of the seed coat remains similar to that of the ovule (Haughn and Chaudhury, 2005), to be later modified during the maturation phase through cell differentiation and further cell elimination events. Notably, at around the torpedo stage of development, ii1' and ii2 undergo a relatively poorly characterized cell death event, leaving the endothelium and the outer integument in close contact

(reviewed in Ingram, 2016). In addition, the outer cell layer of the outer integument (oi2) initiates the differentiation processes that will ultimately give rise to the production of mucilage secretory cells on the seed surface (Francoz et al., 2015). Ultimately, all the cells of the testa will die as the seed desiccates at maturity.

1.1.4.4.1 Seed growth control by the testa

Seed growth in Arabidopsis mainly occurs during early development (fertilization – heart stage) and h

as been shown to be predominantly driven by the zygotic compartment and constrained by the testa. For example, a mutant for the WRKY transcription factor *TRANSPARENT TESTA GLABRA 2 (TTG2)*, which is expressed in the endothelium, has a dramatically reduced seed size, thought to be due to an inability of testa cells to co-ordinate their expansion with that of the endosperm (Garcia, 2005). In contrast seeds in which integument growth is excessive, such as those of *arf2* mutants, plants maternally over expressing the KLUH/ CYP78A5 protein, and seeds lacking the E3 ubiquitin ligase ENHANCER OF DA1 (EOD1)/BIG BROTHER, produce abnormally large seeds post fertilization (Adamski et al., 2009; Schruff, 2005; Xia et al., 2013). Mutants lacking the APETALA2 (AP2) transcription factor produce large misshapen seeds. These mutants have a defect in the specification of outer integument identity, suggesting that this tissue plays a key role in both controlling the expansion of the seed and determining its final shape (Elliott et al., 1996; Jofuku et al., 1994, 2005; Ohto et al., 2005).

1.1.4.4.2 Movement of nutrients to the zygotic compartment

Nutrients arrive in the developing seed from maternal tissues through the funiculus (Figure 7). Phloem contents are actively unloaded in the chalaza of the seed where the vascular tissues terminate (Müller et al., 2015). From there, nutrients can move through a symplastic continuum, which connects the chalazal pole of the seed to the entire outer integument (Stadler, 2005). However, both the inner integument and the endosperm and embryo are symplatically isolated from the chalazal zone of the ovule, implying that the testa must play important and active roles in transporting nutrients from the maternal tissues to zygotic tissues through the expression of proteins capable of exporting nutrients (amino acids and sugars for example) from the maternal symplastic compartment. Studies have shown that such export occurs not only in the chalazal pole of the seed, but throughout the integuments, with a high rate of exporter expression particularly in the micropylar zone of the integuments which immediately

surrounds the embryo-surrounding endosperm (Chen et al., 2015) (Müller et al., 2015). Together, these results support the idea that the testa plays a key role in providing nutrients to the zygotic compartments during seed development.

1.1.5 Maturation of the zygotic compartments in Arabidopsis

1.1.5.1 Embryo growth and reserve accumulation

At the torpedo stage, the maturation phase of seed development begins. The embryo has already acquired the basic features of a plant, including the shoot and root meristems, a functional and self-maintained epidermis, radially patterned inner tissues and two young cotyledons. During maturation, the embryo grows mainly through cell elongation and accumulates storage molecules to ensure a successful germination (Baud et al., 2002, 2008). These reserve compounds are either lipids, mainly triacylglycerols (TAGs), or proteins, referred to as Specialized Storage Proteins (SSPs). Maturation can be divided in three steps. The first involves embryo growth, where the torpedo embryo elongates until it fills the whole seed. The embryo contains large amounts of starch during this phase, and the synthesis of storage compounds initiates. The second phase is characterized by extremely high metabolic activity, with a decrease in starch content and an increase in TAG and SSP production in the embryo. Recent interesting research has also highlighted the fact that storage reserves, also accumulate in the endosperm during seed maturation (Barthole et al., 2014; Troncoso-Ponce et al., 2016). The final step of maturation corresponds to the acquisition of the fully mature seed characteristics: a quiescent metabolism, tolerance to desiccation (González-Morales et al., 2016; Hoekstra et al., 2001) and the acquisition of dormancy (Nonogaki, 2014)

Four major transcription factors, known as the AFL proteins (ABA-INSENSITIVE3 (ABI3), FUSCA3 (FUS3) and LEAFY COTYLEDON 1 & 2 (LEC1 & LEC2)) have been identified as master regulators of the maturation phase (Giraudat et al., 1992; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001) acting in an interconnected regulatory network (Baud et al., 2016; To, 2006). Mutants in each of the AFL-encoding genes exhibit defects in seed maturation, giving similar (but not identical) phenotypes involving a reduction in the expression of seed storage proteins and a reduction in storage lipids. They also show phenotypes associated with defects in other major seed maturation processes, such as a lack of chlorophyll degradation in dry seeds and a reduced sensitivity to acid abscissic acid (ABA) and thus reduced dormancy (in *abi3* mutants), the accumulation of anthocyanins in *fus3* and *lec1*, intolerance to

desiccation in *abi3*, *fus3* and *lec1*, and defects in cotyledon identity in *lec1*, *fus3* and *lec2* (Baumlein et al., 1994; Keith et al., 1994; Kroj et al., 2003; Lotan et al., 1998; Luerssen et al., 1998; Meinke, 1992; Meinke et al., 1994; Nambara et al., 2000; Parcy et al., 1994; Raz et al., 2001; Stone et al., 2001; Vicient et al., 2000; West et al., 1994). MicroRNAs have been shown to control the onset of the activation of these genes (Willmann et al., 2011). Indeed, mutants in ARGONAUTE1 (AGO1), necessary for the maturation of small RNAs, show an early onset of seed maturation, with greening occurring as early as the globular stage, indicating a role for miRNAs in timing the maturation (Tang et al., 2012).

1.1.5.2 Endosperm breakdown

In Arabidopsis, during the early part of maturation, the fully cellularized endosperm degenerates concomitantly with embryo growth. By the end of early maturation, the endosperm is reduced to a single cell layer between the mature embryo and the testa. The process of endosperm breakdown is poorly understood. The bHLH transcription factor ZHOUPI/RETARDED GROWTH OF EMBRYO1 (ZOU/RGE1) is of fundamental importance for endosperm breakdown (Kondou et al., 2008; Yang et al., 2008). Mutants lacking this protein have a fully persistent endosperm and produce embryos of dramatically reduced size. Recently, the bHLH transcription factor INDUCER OF CBF EXPRESSION/ICE1 was shown to form dimers with ZOU, such heterodimers being essential for endosperm breakdown (Denay et al., 2014). ZOU/ICE1 dependent softening of the endosperm cell walls has recently been shown to be important for endosperm breakdown. However, the breakdown of “softened” endosperm only occurs normally if a growing embryo is also present in the seed, suggesting that the role of ZOU/ICE1 complexes may be to predispose the endosperm to physical crushing by the developing embryo, rather than simply to execute the endosperm (Fourquin et al., 2016).

The persistent endosperm of the Arabidopsis seed, despite representing a relatively minor proportion of the mature seed tissue, is of huge physiological importance. Firstly it appears to be a major site in the regulation of dormancy and germination. Both transcriptional studies and elegant “seed bedding” experiments in which the seed coats (in which the endosperm is the only living tissue) and the embryos of mature seeds are separated and recombined with those of other backgrounds, have shown key roles from the endosperm in the hormonal control of germination (Table 2) (Bassel, 2016; Dekkers et al., 2013; Lee et al., 2010). Furthermore, at the purely

physical level, the rupture of the endosperm has been shown to be a key step in regulating the process of germination, although this function is likely tightly regulated by the hormonal pathways controlling dormancy (Iglesias-Fernández et al., 2010, 2013; Linkies et al., 2009; Müller et al., 2013). Finally, as highlighted above, the endosperm of the mature Arabidopsis seed also acts as a storage reserve (Barthole et al., 2014; Troncoso-Ponce et al., 2016).

1.1.6 Concluding remarks

The whole reproduction process in *Arabidopsis thaliana,* and in plants in general, is the result of precisely timed and coordinated events. Seed development demonstrates particularly well this need for coordination, with three genetically distinct tissues necessary for the formation of a functional seed. Coordination demands communication. Therefore, in the next part of the introduction, we review the common principles of cell-cell communication in plants, and their involvement in the communication between the seed coat, endosperm and embryo to make a seed. We focus particularly strongly on the communication events occurring during early seed development as this is the stage addressed by the research carried out in my thesis.

1.2 Inter-compartmental communication during seed development

1.2.1 Overview

1.2.1.1 Means of communication in plant development

The coordination required for proper development is provided by the movement and processing of information between plant cells and tissues. Information can be transmitted in many different forms and via different cellular compartments in order to fulfill this need. Spatially, both short and long distances signals exist. Information must reach every cellular compartment, i.e. both symplastically (in the intracellular space) and apoplastically (in the extracellular space) localized components must be reachable. Temporally, very precisely timed events as well as processes of long duration must be coordinated. The best characterized signalling in plants is of chemical nature, and involves the physical movement of molecules through both simple diffusion and/or active transport. Informative signaling event usually rely on relays and

transduction steps. Three main types of chemical signaling can be defined based on the nature of the messenger involved and their means of transport.

Phytohormones, including the famous auxins, were the first signaling molecules to be described in plants (Darwin, 1881; Tivendale and Cohen, 2015). Phytohormones are small metabolites moving via both active transport and/or passive diffusion, and each one is involved in the control of a wide range of developmental processes. Phytohormones are able to convey information over both short and long distances.

Generally at a small spatial scale, and passing through plasmodesmata, transcription factors (and other proteins) and small RNAs are also able to carry developmental information from one cell to another (Chitwood and Timmermans, 2010; Long et al., 2015). However longer distance movement can be achieved through symplastic movement, when messengers are produced in companion cells and can move through the phloem, a notable example being FLOWERING LOCUS-T (FT) (Turck et al., 2008) and the systemic movement of informative small-RNAs (Yoo, 2004).

More recently, signaling peptides have been uncovered as of a great importance in coordinating developmental processes (Tavormina et al., 2015). These are small apoplastically located molecules involved in short (through cell walls) and long (using the vascular system (xylem)) distance communication.

In addition to chemical communication, plant cells and tissues also respond to mechanical cues triggered by the growth of neighboring cells and tissues. Recent research is increasingly suggesting that such mechanical cues, and their integration with chemical signaling, play critical roles in the coordination of plant development (Hamant, 2013).

In this second part of the introduction, we review the contribution of each type of intercellular communication processes in allowing the coordinated development of the testa, embryo and endosperm in order to make a functional seed.

1.2.1.2 Seed specificities impacting inter-tissue communication

While considering the wide possibilities for inter-compartmental communication during seed development, anatomical specificities/peculiarities of the developing seed must be taken into account. First of all, we must underline the lack of plasmodesmata, and thus the absence of symplastic movement, between the three

compartments (Stadler, 2005). Plasmodesmata are found between most cells in most plant tissues, although their "openness" (defined in terms of the size of molecules which can move from cell to cell via these channels) varies widely between different tissues (Brunkard et al., 2015; Gallagher et al., 2014; Otero et al., 2016; Stahl and Faulkner, 2016). Although plasmodesmata are found between the cells of the female gametophyte, and between the female gametophyte and surrounding tissues, during ovule development, they appear to be absent between both the embryo and endosperm, and the endosperm and testa from shortly after fertilization, leading to the formation of symplastic barriers between these three compartments (Ingram, 2010; Stadler, 2005). The control of this compartmental symplastic isolation in seeds has not been studied in any detail, but it means that symplastic communication, for example through the movement of transcription factors and small RNA, can be disregarded as a means of coordinating the development of the different compartments in seeds. We will therefore consider the three remaining means of communication that make use of the apoplastic space. When considering apoplastic signaling, the potential presence of apoplastic diffusion barriers at both the embryo/endosperm (Tanaka et al., 2001; Xing et al., 2013) and endosperm/testa (De Giorgi et al., 2015) interfaces needs to be noted. Although such barriers could impact apoplastic diffusion between the compartments, their precise properties are, to date unknown. However, despite the presence of these 'barriers", the seed appears to makes wide use of peptide, hormonal and mechanical signaling to ensure developmental coordination.

In the following section I will place particular emphasis on peptide signaling, since this is of the most relevance in the development of my thesis.

1.2.2 Peptide signaling during reproduction and seed development

1.2.2.1 General considerations regarding peptide signaling.

Peptide signaling is a means of carrying information from cell to cell through the apoplastic diffusion of protein-based chemical components, and the perception of these components at the cell surface. Peptides are also referred to as peptide hormones or peptide signals in the literature. Such signals have been extensively studied in animals, with insulin being the very first signaling peptide to have its sequence resolved (Sanger and Tuppy, 1951a, 1951b). In animals, peptide hormones serve many functions, such as developmental patterning (Martí et al., 1995) and neural function (Dodd and

Kelly, 1981). In plants, the number of peptide ligands present in the genome, and their functional importance were long underestimated (De Coninck et al., 2013; Silverstein et al., 2007). However, the last two decades have witnessed the birth and explosive growth of the plant peptide field (Simon and Dresselhaus, 2015). Recent investigations suggest that around 400 different predicted signaling peptides are encoded in the *Arabidopsis* genome (Huang et al., 2015).

Structurally, peptides are small amino-acid chains, composed of 5 to 100 aa Their general mode of action to ensure cellular communication is thought to be the following: They are produced and matured in a given cell, secreted to the apoplast, and finally perceived by another cell. However, variations on this theme are frequent. Their diversity allows them to convey a wide array of developmental information, leading to specific responses in the receiving cells. In plants, peptides have been classified into two main classes: Small PostTranslationally Modified (SPTM) Peptides, and Cysteine Rich Peptides (CRPs) (Tavormina et al., 2015). Peptides are involved in both responses to exogenous cues (abiotic and biotic signals) and developmental regulation. Here I will focus on the peptides involved in development.

Functional, processed SPTM peptides are usually short, ranging from 5 aa to 25 aa in length. Many SPTM peptides have been shown to regulate fundamental developmental processes. A notable example is the 14 amino acid long CLAVATA3 (CLV3) peptide which is crucial in the self-maintenance of the stem cell pool in the shoot apical meristem (Schoof et al., 2000). Other examples of notable SPTM peptides are listed in Table 1.1.

CRPs are longer peptides. As their name suggest they are characterized by the presence of cysteine residues in variable numbers and at conserved positions. Cysteine number and positions define CRPs families. In allowing for the formation of covalent disulfide bonds, cysteines are thought to be crucial for the acquisition of the specific, functional conformation of each peptide in the reducing apoplastic environment. Belonging to the CRP class are the EPF and EPF-Like peptides which regulate stomatal patterning and differentiation in the plant epidermis (Hara et al., 2007; Hunt and Gray, 2009; Lee et al., 2015; Sugano et al., 2010). Other known CRPs involved in development are the LUREs peptides, which act as attractant for the pollen tube (Okuda et al., 2009).

Class	Family	Peptides	Receptors	Modif°	Biological role	References
SMALL POSTTRANSCRIPTIONNALLY MODIFIED PEPTIDES	**CLE** CLAVATA/Endosperm Surrounding Region	CLAVATA 3 (CLV3) CLE40 CLE8 CLE19	CLV1 ACR4? ? ?	Hyp	SAM Maintenance RAM Maintenance Embryo Patterning Embryo/Endosperm Patterning	Fiume and Fletcher, 2012
	CEP C-Terminally Encoded Peptide	CEP1	CEPR1 CEPR2	HyP	-Nitrogen demand regulation	Ohyama et al., 2008 Tabata et al., 2014
	IDA /IDA-Like (**IDL**) INFLORESCENCE DEFICIENT IN ABSCISSION	IDA IDL	HAESA HSL HSL2	HyP	-Flower abscission -LR root emergence	Butenko, 2003 Kumpf et al., 2013 Stenvik et al., 2008
	RGF/GLV Root Growth Factor/ GOLVEN	RGF1 RGF2	RGFR1 RGFR2 RGFR3	TPST SBT6.1 SBT6.2	-Root meristem organization -Root elongation	Fernandez et al., 2013 Ou et al., 2016 Shinohara et al., 2016
	PSY1 PLANT PEPTIDE CONTAINING SULFATED TYROSINE1	PSY1	PSY1R	TPST	-Cell Growth -Cellular proliferation	Amano et al., 2007
	PSK PHYTOSULFOKINE	PSK1 PSK4	PSK1R	TPST SBT1.1	-Cell growth	Matsubayashi, 2006 Srivastava et al., 2008
	AtPEPs	AtPEP1 2,3,4,5,6	PEPR1 PEPR2	HyP	-Immunity	Pearce et al., 2008 Yamaguchi et al., 2006
	SYS SYSTEMINS	SYS HYPSYS	?	HyP	-Immunity Not in brassicacea	Ryan and Pearce, 2003
CYSTEIN-RICH PEPTIDES	**EPF / EPF-Like** EPIDERMAL PATTERNING FACTOR	EPF1 EPF2 EPFL1 EPFL2	ERECTA ERL1	SDD?	-Stomatal patterning -Leaf serration	Lee et al., 2015 von Groll, 2002
	RALF RAPID ALKALINIZATION FACTOR	RALF RALF23	FERONIA	AtS1P	-Cell growth	Haruta et al., 2014 Srivastava et al., 2009
	PDF PLANT DEFENSINS	LURE1	PRK3 PRK6		-Pollen tube local attraction	Okuda et al., 2009 Takeuchi et al., 2016
	ESF EMBRYO SURROUNDING FACTOR	ESF1/ESF2/ ESF3	?		-Embryo patterning	Costa et al., 2014
	STIG1 STIGMA SPECIFIC 1	GRI STIG1 (solanacea)	PRK5	AtMC9	-Cell death propagation -PT penetration	Goldman et al., 1994 Wrzaczek et al., 2015 Huang et al., 2014
sORF	**KOD** KISS OF DEATH	KOD			-Suspensor cell death	Blanvillain et al., 2011

Table 1. Presentation of the different classes and representative families of plant signaling peptides. For each family, notable members are mentioned. Receptors, when identified, are indicated in the adjacent column.

In addition to the CRPs and the SPTMs, there are some signaling peptides that do not fit into the aforementioned groups. Short Open Reading Frames (sORF) peptides are encoded by genomic DNA sequences and targeted to the apoplast, ENOD40 being one of the first identified in plants (Campalans, 2004). More recently, miPEP peptides, originating from the translation of pri-miRNAs, were identified (Lauressergues et al., 2015). Finally, Upstream ORF peptides originate from the translation of the 5'UTR of some genes, and can be crucial in controlling biological processes (von Arnim et al., 2014; Laing et al., 2015).

Fully functional peptide signaling pathways involve other proteins than the peptides themselves (Figure 8). Generally, a secreted peptide starts its journey as a pro-peptide addressed to the secretory pathway. It is thus localized in the Golgi apparatus. There, post-translational modifications may occur such as proline hydroxylation, glycosylation and/or tyrosyl-sulfation (Reviewed in Canut et al., 2016). Such decorations are essential for the function of the mature peptide. Additionally, apoplastically targeted proteases have been shown to be essential in many cases to release the functional peptide by cleaving the propeptide (Rautengarten et al., 2005; Schaller et al., 2012). Whether the cleavage occurs in the apoplast or in the Golgi is still an open question, as is the link (if any) between decoration and maturation by cleavage.

Once the peptide is secreted and matured, it is thought to move through simple diffusion within the cell wall over short distances. It is relevant to note an exception here; recently, long distance movement of CEP peptides from the root to the shoot through the xylem, which is an apoplastic compartment, was shown to be crucial for Nitrogen-demand control (Tabata et al., 2014), giving a first example of long distance apoplastic peptide signalling. How peptide diffusion is affected by decoration and/or the chemical and structural characteristics of specific cell walls is an open question.

The diffusion/movement of peptides allows them to reach other cells, which may be able, if expressing a matching receptor, to perceive the signal and respond accordingly. Receptor-Like Kinases, mostly of the Leucine-Rich Repeat Receptor-Like Kinases (LRR-RLKs) subfamily, are known to specifically bind matching peptide(s) in their extracellular domain, producing a cytoplasmic and/or transcriptional response usually mediated through phosphorylation of target proteins.

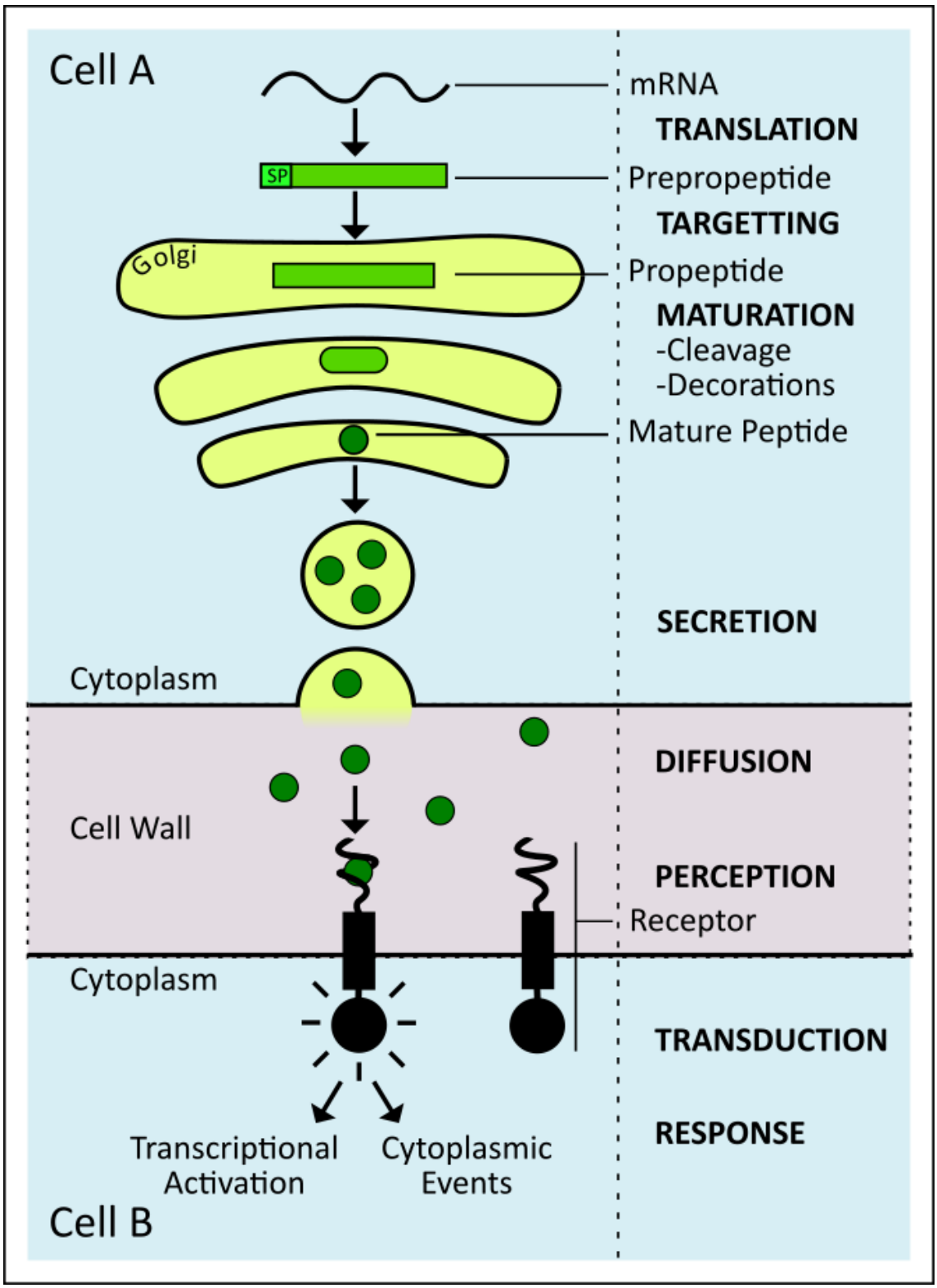

Figure 7. A canonical peptide signalling pathway allowing the transmission of information from one cell to another through the apoplast.

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With a general view on the diversity of peptides and the necessary steps allowing their function as signaling molecules, we can go further with an in depth review of the possible modifications and the mode of perception of these peptides. We will then focus on the peptides and related pathways components known to be involved during reproduction and seed development.

1.2.2.2 Peptide modifiers: Cleavages and Decorations

Secreted peptides almost always undergo post-translational modifications. These produce changes in peptide electrostatic properties, hydrophobicity and spatial conformation. Such properties are essential for correct binding to peptide receptors. Another suspected role, yet still elusive, of such decorations may be to prevent random proteolytic degradation of signaling peptides in the apoplast (Matsubayashi, 2014).

1.2.2.2.1 Posttranslational marks

Common post-translational modifications, occurring on specific residues of the peptide, are hydroxylation and glycosylation. Hydroxylation generally occurs on proline residues. In *Arabidopsis,* Prolyl 4-Hydroxylases (P4Hs) are able to hydroxylate prolines (Hieta and Myllyharju, 2002; Tiainen et al., 2005). Once P4Hs have changed a proline into a hydroxyproline, the resulting decorated residue can be glycosylated. Hydroxyproline O-arabinosyl Transferases (HPATs) are involved in adding arabinose sugars to hydoxyprolines (Ogawa-Ohnishi et al., 2013). The Arabidopsis genome contains 3 HPATs, redundantly involved in the decoration of extracellular proteins including the hydroxyproline-rich extensins such as RSH/EXT3 (Hall, 2002). Biochemical evidence shows that HPAT3 is also involved in the glycosylation of CLE peptides (Ogawa-Ohnishi et al., 2013). Additional arabinoses can be added through the action of XEG113 (Gille et al., 2009), RRA1, RRA2 (Egelund et al., 2007) and RRA3 (Velasquez et al., 2011). To date however, no role for such enzymes has been demonstrated during seed development, although RRA1 for example is very strongly expressed in the developing endosperm (Le et al., 2010). Another type of

posttranslational modification involves the in addition of sulfate groups to tyrosine residues, and is mediated by the TYROSYLPROTEIN SULFOTRANSFERASE (TPST) protein (Komori et al., 2009). The discovery of this protein was long awaited because PHYTOSULFOKINE (PSK), one of the earliest discovered plant peptides (Matsubayashi and Sakagami, 1996), is sulfated. Other peptides involved in root growth, the Root Growth Factor (RGF) / GOLVEN (GLV) peptides have also been shown to be sulfated by TPST (Zhou et al., 2010).

1.2.2.2.2 Peptide cleavage

Small signalling peptides are secreted molecules and therefore often contain a canonical N-terminal signal peptide (SP) addressing them to the secretory pathway. SPs are known to be removed by the action of signal peptidases (Tuteja, 2005) . In addition, signaling peptides often undergo endoproteolytic cleavage. Subtilisin-like serine proteases have been shown to be involved in peptide cleavage in *Arabidopsis*. For example, SBT1.1 is involved in the maturation of PSK4 (Srivastava et al., 2008). Recently, SBT6.1 and SBT6.2 were both shown to cleave GOLVEN1 (GLV1), with such a cleavage necessary root cell elongation mediated by the peptide (Ghorbani et al., 2016). Another class of plant proteases, Metacaspases (MCs), also play roles in maturing peptides, a recent example being ATMC9, which processes a cell-death inducing peptide from the GRIM REAPER (GRI) protein (Wrzaczek et al., 2015).

1.2.2.3 Peptide perception: RLKs and downstream events

1.2.2.3.1 Receptors physically interact with peptides

Once peptides are matured and secreted into the apoplast, receiving cells recognize specific peptides and give an appropriate response. Such recognition is generally mediated through transmembrane proteins known as Receptor-Like Kinases (RLKs). RLKs are composed of three main domains: (1) An N-terminal ectodomain located in the apoplast, (2) a transmembrane domain allowing the RLK to be integrated in the membrane, and (3) a cytoplasmic C-terminal kinase domain able to initiate a phosphorylation cascade generating either cytoplasmic and/or nuclear (transcriptional) responses. Peptide ligands involved in development usually bind physically to the ectodomain of RLKs, triggering either conformational changes and/or co-receptor binding and ultimately leading to the activation of the intracellular kinase domain. The *Arabidopsis* genome comprises more than 610 RLKs (Shiu and Bleecker, 2003). Considering their number, our relative knowledge regarding their functions remains

poor. Leucine Rich Repeat RLKS (LRR-RLKs) compose the largest RLK family with 235 members (Hove et al., 2011). They have a corkscrew-shaped ectodomain composed of Leucine Rich Repeats, hence the name. This ectodomain varies in sequence, length and number of repeat between the different LRR-RLKs (Shiu and Bleecker, 2003). Perhaps unsurprisingly given its size, this is the family for which the greatest numbers of functional analyses have been carried out. LRR-RLKs can be subdivided into 14 subfamilies (Shiu and Bleecker, 2003). Of these, family LRR-XI contains a remarkably large number of RLKs involved in developmental processes. Examples include CLAVATA1 (part of the receptor complex binding the CLV3 peptide) and close homologues, which are involved in shoot apical meristem maintenance (Clark et al., 1993, 1995; Hazak and Hardtke, 2016; Ogawa et al., 2008). Belonging to the same subgroup, HAESA, HSL1 and HSL2 regulate processes like organ abscission and lateral root emergence (Jinn et al., 2000; Kumpf et al., 2013), while HAIKU2 (IKU2) plays a key role in seed development (Garcia, 2003; Luo et al., 2005). Plant defence responses are also controlled by LRR-XI members with the PEPR1 and PEPR2 RLKs binding the endogenous AtPEP peptides produced during pathogen attack and acting to enhance/modulate defence responses (Krol et al., 2010; Yamaguchi et al., 2010).

1.2.2.3.2 Co-receptors are receptor partners mediating peptide perception

Peptide perception sometimes involves the recruitment of a co-receptor by an RLK. Co-receptors are often other receptor kinases, frequently with small ectodomains, that can heterodimerize with receptors and induce subsequent trans-phosphorylation events of their kinase domains. The LRR-RLK II family is comprised of various RLKs known to act as co-receptors (Shiu and Bleecker, 2003). Included in this family are the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs), amongst which the SERK3/BRI1 ASSOCIATED KINASE (BAK1) protein (Brandt and Hothorn, 2016; Li et al., 2002) is a notable example. SERKs are co-receptors that are able to dimerize with multiple receptors involved in the perception of distinct signals. Such receptor include the PEPRs (Yamada et al., 2016), HAESA (Meng et al., 2016; Santiago et al., 2016) and FLS2 (Chinchilla et al., 2007; Heese et al., 2007). In these cases, the receptor/co-receptor dimerization is ligand dependent; the peptide serving as a "molecular glue" between the two RLKs (Santiago et al., 2016).

Receptor-Like Proteins (RLPs) also act as partners for RLKs. They are very similar in structure to RLKs, except that they lack the canonical kinase domain. They are able to physically interact with LRR-RLKs, yet are not themselves capable of

transphosphorylation, and thus require other partners. In the meristem, CLAVATA2 (CLV2) (an RLP) has been shown to interact with CORYNE (an RLK with a transmembrane domain but a highly truncated extracellular domain) (Bleckmann et al., 2010; Müller et al., 2008; Pallakies and Simon, 2014; Zhu et al., 2010). This heterodimerization localizes CLV2 to the membrane and effectively reconstitutes a functional RLK capable of perceiving CLV3 peptide and other CLE peptides. Another RLP required for peptide perception is PRK5, which is known to bind the cell death related peptide GRIp and to convey its information. In the case of PRK5 a kinase domain is present but lacks key residues necessary for enzymatic activity. It seems likely that PRK5 could heterodimerize with another kinase, but this putative partner has not yet been identified. (Wrzaczek et al., 2015)

A third family of protein is involved in peptide signal perception at the plasma membrane. Some signaling pathways require the action of Receptor-Like Cytoplasmic Kinases (RLCKs). These are membrane associated kinases that can be recruited by specific RLKs in presence of the peptide, and mediate phosphorylation events necessary for the transduction of the signal. A prime example of RLCK is the BOTRYTIS-INDUCED KINASE 1 (BIK1) protein. BIK1 has been shown to be involved in FLS2 (Lu et al., 2010), BRI1 (Lin et al., 2013), and PEPR (Liu et al., 2013) mediated signaling. Structurally, BIK1 and BAK1 form a complex and their phosphorylation has been shown to be a necessary step in mediating immunity related signals (Lin et al., 2014). More recently the SCHENGEN 1 (SGN1) RLCK has been shown to play a key role in RLK-mediated signaling necessary for the formation of an intact Casparian strip in the root endodermis (Alassimone et al., 2016). SGN1 and an RLK, SGN3/GASSHO1 (GSO1) are both necessary for Casparian strip formation, but show subcellular localisations with only a very small overlap. Regulation of the subcellular localization of receptor complex components could thus play a key role in regulating their ability to perceive apoplastic signals.

1.2.2.3.3 Downstream signaling events

Peptide perception by its LRR-RLK initiates downstream signaling events leading to a proper cellular response. Both transcriptional activation and cytoplasmic responses have been reported to date. Transcription factors, such as the WRKY-type transcription factors can be activated by LRR-RLKs, giving a transcriptional output to a given apoplastic input. An excellent example is WRKY33, a key coordinator of stress responses, whose activity is regulated by interaction with MAP-KINASE

SUBSTRATE1 (MKS1), a VQ motif containing protein (Qiu et al., 2008). This interaction is in turn regulated by the phosphorylation of MKS1 by MITOGEN ACTIVATED PROTEIN KINASES (MAPKs), activated (via an as yet unidentified intermediary step) in cascades of phosphorylation events downstream of the perception of Pathogen Associated Molecular Patterns (PAMPs) by RLK-RLKs. Canonical MAPK signaling involves MAPKKKs, MAPKKs and finally MAPKs to regulate transcription factor activity (Reviewed in (Cristina et al., 2010). MKS1 is a substrate of MPK4 (Qiu et al., 2008), but the regulation of WRKY transcription factor through the MPK-mediated phosphorylation of interacting VQ-domain containing proteins may well be a much more globally used mechanism in plants than previously reported (Pecher et al., 2014). A similar mechanism has, for example, recently been shown to regulate pollen development (Guan et al., 2014). Among others, MPK3 and MPK6 have also been shown to be involved in developmental processes such as stomatal patterning (Bergmann, 2004; Wang et al., 2007). Thus, peptides can readily act on cells by changing their transcriptional landscape.

The other kind of response achievable downstream of RLK-mediated peptide perception is a so-called cytoplasmic response. Pathogen attacks, for example, induce not only transcriptional regulation but also rapid cellular responses such as the production of apoplastic Reactive Oxygen Species (ROS) and the influx of calcium. These responses are achieved downstream of RLK signaling, for example through the regulation of NADPH Oxidase activity (Kadota et al., 2015; Steinhorst and Kudla, 2013). Another, possibility is that signaling can regulate the secretion of apoplastic components (including cell wall components, but potentially also peptide signals). A good example of this is the rapid and highly localized deposition of callose observed upon hyphal penetration into epidermal cells (Ellinger and Voigt, 2014; Voigt, 2014). Interestingly, a very recent study has shown that ERECTA family receptors are able to locally reduce Endoplasmic Reticulum / Plasma Membrane distance, and thus potentially regulate secretion, through their interaction with VAP-RELATED SUPPRESSOR OF TMM (VST) proteins (Ho et al., 2016).

1.2.2.4 Peptide signalling during seed development

1.2.2.4.1 Peptides involved in inter-compartmental communication

Once fertilization has occurred, a signaling pathway involving central-cell derived ESF peptides is necessary for proper embryo development (Costa et al., 2014).

ESF peptides belong to the CRP class. The expression of *ESF1, ESF2* and *ESF3* initiates before ovule fertilization and continues during early seed development. Their expression is central-cell (CC) specific and, after fertilization when the CC becomes the endosperm, it remains endosperm specific. The ESF peptides are necessary for patterning of both the suspensor and the embryo proper, as mutants have division defects in both tissues. Genetic interaction investigation has shown they act in the same pathway as SHORT SUSPENSOR (SSP) (a cytoplasmic interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase) and YODA (MAPKKK1)/MPK3/6 signalling (Bayer et al., 2009; Costa et al., 2014; Lukowitz et al., 2004). However the RLK involved in this signaling pathway has not yet been identified. Interestingly, the Arabidopsis ESF peptides are the closest orthologues of the maize MATERNALLY EXPRESSED GENE1 (MEG1) peptide which plays a key role in specifying the BETL layer, a specialized zone equivalent to the chalazal endosperm in the developing maize kernel (Gutiérrez-Marcos et al., 2004). Adding to this structural similarity, it is interesting to note that the expression of both MEG1 and the EPF peptides occurs specifically from the maternal genome in both cases. In contrast the expression of part of the likely ESF signaling pathway, the SSP protein, occurs uniquely from the paternal genome (Bayer et al., 2009), hinting at parental conflict over processes controlled by peptide signaling in seeds (Ingram and Gutierrez-Marcos, 2015)

CLAVATA/Endosperm Surrounding Region peptides, commonly referred to as CLE peptides, may also be involved in early embryo patterning at the embryo/endosperm interface. One of the founding members of this family was first identified in the endosperm-surrounding endosperm of maize, although its function in this tissue remains a mystery (Bonello et al., 2002, 2000; Opsahl-Ferstad et al., 1997). The endosperm- and embryo-expressed CLE8 peptide regulates both embryo and endosperm development in *Arabidopsis* (Fiume and Fletcher, 2012). The regulation of WOX8 expression in the basal part of embryo is impacted by this peptide. Consistent with this, *cle8* mutants have larger suspensors than wild-type, and problems in hypophysis specification. However they also show a reduction in the number of cell divisions in the coenocytic endosperm, leading to the formation of small seeds. Another CLE peptide, the embryo cotyledon specific CLE19, regulates both embryo and endosperm development (Xu et al., 2015). Mutant seeds have defects in embryo cotyledon expansion and delayed endosperm cellularization. Complementation of the mutant using an endosperm specific promoter rescues only the endosperm phenotype

while the embryo still has short cotyledons. These results appear to indicate the existence of a communication pathway in which CLE19 acts cell-autonomously in embryo development and non-cell autonomously in endosperm cellularization.

Finally, a unique peptide from the sORF peptide class, KISS OF DEATH (KOD), is expressed in the embryo and has been shown to be necessary for suspensor cell death (Blanvillain et al., 2011). Consistent with this, components activated downstream of KOD signaling appear to include regulators of programmed cell-death pathways. However, the receptor involved in the KOD signaling pathway remains unclear. Interestingly, KOD subcellular localization is cytosolic, indicating either the need for cell death to propagate the signal, or an intracellular localization of the receptor(s).

1.2.2.4.2 Modifying enzymes

Globally, genome wide expression studies have underlined a notable prominence of CRP peptide expression in the various seed compartments during both fertilization and seed development. The abundance of CRPs in the reproductive steps render them attractive as candidates in mediating developmental coordination processes during seed development (Huang et al., 2015; Ingram and Gutierrez-Marcos, 2015; Bircheneder and Dresselhaus, 2016). CRP peptides are not thought to be extensively post-translationally modified, and this may explain why information regarding roles for modifying enzymes such as proteases, or decorating enzymes involved during seed development is relatively scarce. Nonetheless, several subtilisin-like serine proteases have been shown to regulate seed development coordination. The endosperm ESR specific ABNORMAL LEAF SHAPE 1 (ALE1) regulates embryonic cuticle formation in a non-cell autonomous fashion (Tanaka et al., 2001, 2004). ALE1 has been shown to act in the same pathway as the two embryo-expressed LRR-RLKs, GASSHO1 (GSO1) and GSO2 (Xing et al., 2013). However, no substrate for ALE1, nor ligand for GSO1 GSO2, has yet been identified. In *Medicago trunculata* and *Pisum sativum*, the endosperm-specific subtilisin SBT1.1 is a positive regulator of seed size with a possible action in determining cotyledon cell number, probably through inter-tissue sigalling (D'Erfurth et al., 2012). In *Arabidopsis,* yet another subtilisin, ATSBT1.7 is expressed specifically in the seed coat, where it acts in a cell autonomous manner during the regulation of mucilage deposition in the outer integument (Rautengarten et al., 2008). However, although these subtilisins are clearly involved in seed development, and it is tempting to speculate that they could be involved in signaling peptide processing,

subtilisins have also been implicated in the processing of other apoplastically located proteins (Senechal et al., 2014), and therefore their phenotypes cannot be unambiguously taken as proof of functions for processed signaling peptides in the developing seed. Identification of substrates will be an important next step.

Interestingly, none of the enzymes known to mediate the addition of post-translational decorations to signaling peptides have been shown to be involved in seed development to date. However, this could well be due to oversight, or alternatively due to the fact that loss of function of some of these enzymes, such as TPST, lead to severe pleiotropic phenotypes which could affect fertility.

1.2.2.4.3 Receptors and downstream components involved in seed development.

Several RLKs have been implicated in embryo development, and could potentially play important roles in the communication of the developing embryo with its surrounding tissues. Examples include RLKs necessary for the formation of the embryonic epidermis such as the redundantly acting PRK1 and TOADSTOOL2 RLKs, the ARABIDOPSIS CRINKLY4 protein, and the ABNORMAL LEAF-SHAPE2 protein (Nodine et al., 2007; Watanabe et al., 2004), In addition two redundantly acting LRR-RLK-XI proteins, GASSHO1 and GASSHO2 (GSO1 & GSO2) have been shown to control embryonic cuticle integrity (Tanaka et al., 2007; Tsuwamoto et al., 2008) in an inter-compartmental interplay with the endosperm-specific ZOU transcription factor and ALE1 subtilisin-like serine protease (Tanaka et al., 2001; Xing et al., 2013). Relatively few other functional analyses have reported important roles for LRR-RLKs in seed development. However, the endosperm-specific HAIKU2 (IKU2) protein, another member of the LRR-RLK-XI family, is essential seed size control (Garcia, 2003; Luo et al., 2005). Interestingly a WRKY transcription factor, MINISEED3 (MINI3)/WRKY10 and a VQ-motif protein, HAIKU1 also act in the IKU2 signalling pathway, although the molecular mechanisms involved have yet to be explored. To date, no ligands have been identified for IKU2. Finally, as discussed above, suspensor development is also a likely scene of peptide signaling due to the implication of the SHORT SUSPENSOR SSP protein, an RLCKII acting in controlling suspensor length (Bayer et al., 2009; Kawashima and Goldberg, 2010). Although the ESF1 peptides have been recently showed to act in the same pathway as SSP, no LRR-RLK bridging physically these components has been discovered to date (Costa et al., 2014). However,

tantalizingly, both MAP Kinase signaling and a WRKY transcription factor (WRKY2) have also been implicated in this process (Lukowitz et al., 2004; Ueda et al., 2011).

Although peptide mediated signaling is clearly a potentially important factor in regulating seed development, and is the main subject of this thesis, other means of communication cannot be ignored. For completeness I will therefore briefly consider mechanical and hormonal communication in this context.

1.2.3 Mechanical signaling

1.2.3.1 General considerations

Since the early onset of biochemistry and the revolution of molecular biology in the 60s, biologists have tended to explain biological phenomena in the light of molecular interactions. Indeed, the peptides I have considered until now are of chemical nature. Yet, organisms, evolving in the physical world, are also subject to its fundamental laws. For this reason, mechanical cues are likely to be informative signals of great importance for plant development. The wind sculpting the shape of tree (known as anemomorphosis) and the root making its way through the soil, are well known examples of developmental processes strongly influenced by exogenous mechanical cues (Bengough et al., 2011; Telewski, 1995), although obviously other non-mechanical signals are also at play during these processes. The integration of exogenous mechanical cues with other environmental signals is thus critical in allowing the plant to adapt to its environment. Recent research in developmental biology has taken this idea to its logical conclusion, and is rapidly uncovering evidence to suggest that plants also make also use of endogenous physical cues, generated by growth, to co-ordinate their development (Hamant, 2013). In the developing plant, coordination through mechanical cues can be found at all biological levels: Organs (Couturier et al., 2011), tissues (Lucas et al., 2013), cells (Hamant et al., 2008) and molecules (Shih et al., 2014). Here we will review the potential functional significance of mechanical cues for seed development.

1.2.3.2 Importance of mechanical stresses in seed development

Physical interactions between different tissues composing an organ can provide key developmental cues. In the developing *Arabidopsis* seed mechanical interactions between the three seed tissues, which are organized in a Russian doll-like manner and thus unavoidably physically linked, have recently been shown to be involved in developmental co-ordination. It has long been assumed that the expansion of the early

coenocytic endosperm drives the expansion of the early seed, allowing it to acquire its proper size. This supposition is supported by genetic data showing that growth is driven by the early zygotic compartment (Garcia, 2003, 2005; Ingram, 2010). Since plant growth is entirely driven by cellular turgor pressure, this suggests that the turgor of the coenocytic endosperm could be a critical factor in driving seed growth. A recent study has focused on measuring the turgor of the developing endosperm and shown that, consistent with the idea that endosperm turgor drives seed growth, it is relatively elevated during early seed expansion, decreases as seed growth slows and stops with the cellularization of the endosperm (Beauzamy et al., 2016). High turgor pressure in early seed development could therefore generate a mechanical heterogeneity between the endosperm and the seed coat, thus inducing seed expansion. How this turgor is generated, and, more importantly, regulated remains an open question, but one which could ultimately link seed development to basic seed physiology, since it seems likely that basic metabolites such as sugars and amino acids, which are selectively imported into the symplastically isolated zygotic seed compartment, could play key roles in turgor generation (Beauzamy et al., 2016).

In addition to the idea that the endosperm acts to drive seed growth, genetic studies in several species have also shown an important role for the maternal tissues of the seed (the seed coat) in restricting seed growth (Garcia, 2005; Jiang et al., 2013). A recent study has shown that seed expansion is in fact subject to a kind of mechanically mediated retro-control, during which a mechanosensitive layer of the seed coat (oi-1) produces a thickened cell wall upon endosperm expansion, therefore increasing the resistance of the seed coat to endosperm derived tension (Creff et al., 2015). Interestingly, expression of the *ELA1* gene, which is a known regulator of seed growth (Zhang et al., 2011) is induced upon mechanical stress specifically in the oi1. ELA1 is known control growth in other tissues via the breakdown of active Gibberellins (GAs) (Zhang et al., 2011), suggesting that GA-mediated growth control could be controlled by mechanical signals in the testa (Creff et al., 2015).

Finally, a potential role for mechanical cues in the regulation of the breakdown of the cellularised endosperm has also recently been suggested. In Arabidopsis this breakdown occurs concomitantly with embryo growth, until the endosperm is reduced to a single cell layer, the aleurone. A coupling between the pressure generated by the growing embryo, and the genetically controlled softening of the endosperm cell walls, has been recently proposed to be responsible for endosperm elimination (Fourquin et

al., 2016). These seed-derived examples illustrate the ability of mechanical cues to convey information between distinct tissues.

1.2.4 Phytohormones in seed development

1.2.4.1 Phytohormones overview

Phytohormones are involved in governing nearly every developmental process throughout the plant life cycle. They are small diffusible chemicals, and the production and perception of such molecules by groups of cells or tissues can provide both positional information allowing pattern formation, and more general information regarding the physiological status of the plant. When considering hormone signalling, three key steps need to be taken into account: (1) Biosynthesis and degradation/inactivation, which together determine the quantity of hormones provided by given cells and tissues and thus available for transport/signalling. (2) Diffusion/transport, which can be either active or passive, and is able to generate spatially precise concentration gradients. (3) The expression and activity of the multiple signalling pathway components for each phytohormone, as these components are ultimately responsible for the transduction of the signal contributing to an appropriate transcriptional or cytoplasmic response. In an added level of complexity, the fact that any given cell may be able to perceive and respond to several phytohormones simultaneously also needs to be taken into account.

Phytohormone	Function (in early seed development)	Biosynthesis/ transporters	Receptor/ signalling system	Effectors / Intermediates
Auxins *(Enders and Strader, 2015; Kasahara, 2016; Robert et al., 2015; Smit and Weijers, 2015; Strader and Zhao, 2016)*	Vascular differentiation, cellular elongation (tropism), prevention of abscission, apical dominance, ethylene release, fruit development, embryo patterning, endosperm development.	WEI2, WEI7, WEI8, TAA1 TARs PINS, ABCB/PGPs AUX1/LAX	TIR1, AFB1-5 (F-box) SCF-mediated protein degradation	ARFs AUX/IAAs
Jasmonates *(Chini et al., 2016; Yuan and Zhang, 2015)*	Response to stresses, control of seed germination, male fertility.	AOC, OPR3 ACX1, JAR1 NPFs *	COI1 (F-box) SCF-mediated protein degradation	MYC2 (+ other bHLH TFs) JAZs
Gibberellins *(Davière and Achard, 2016; Lor and Olszewski, 2015)*	Stem elongation, mobilisation of storage materials during germination, vegetative-floral transition, pollen tube growth, control of seed size.	GA20ox, GA3ox NPFs *	GID1a-GID1c (Lipase) SCF-mediated protein degradation	PIFs (+ other bHLH TFs) SLY, SNE, DELLAs
Strigolactones *(Koltai, 2015; Seto and Yamaguchi, 2014)*	Inhibit shoot branching.	CCD7 (MAX3) CCD8 (MAX4) MAX1 PDR1/ABCGs*	DWARF 14 (Hydroxylase) SCF-mediated protein degradation	BES1 DWARF 55
Salicylic Acid *(Liu et al., 2016; Yan and Dong, 2014)*	Regulation of plant defence related transcription.	ICS1 ?	NPR1, NPR3 and NPR4 ? (BTB-POZ domain) Protein degradation	TGA (+ other bZIP TFs) NPR1
Abscisic Acid *(Dong et al., 2015; Merilo et al., 2015)*	Maintains seed dormancy, maintain bud dormancy, stimulates stomatal closure, regulates senescence.	ZEP, NCED,SDR, AOO, ABCG25, ABCG40, ..*	PYR1 – PYR14 (START-domain) Phosphorylation	ABI3, ABI5 + other bZIP TFs SnRK2.2,.3 & .6, PP2Cs
Ethylene *(Ju and Chang, 2015; Poel et al., 2015)*	Fruit ripening, promotes abscission, formation of aerenchyma, sex determination (cucurbits), control of dormancy and germination.	ACSs ACO ?(LHT1 transports the ethylene precursor ACC)	ETR1, ETR2, EIN4, ERS1, ERS2 (Histidine Kinase) SCF-mediated protein degradation and phosphorylation	EIN3, EILs CTR1, EIN2
Cytokinins *(Zürcher and Müller, 2016)*	Affect cell division, delay senescence, activate dormant buds, vascular development, female gametophyte development,embryo patterning.	IPTs, Cyp753A LOGs ABCG14,..* (Ko et al., 2014; Zhang et al., 2014) PUP14 (Zurcher et al., 2016)	AHK2-AHK-4 (Histidine kinase) Phosphorylation	A and B-type ARRs AHP1-AHP6 CKI1
Brassinolides *(Belkhadir and Jaillais, 2015; Singh and Savaldi-Goldstein, 2015)*	Promote elongation,stimulate flowering, promote cell division, control of seed size.	DET2, DWF4 and others ?	BRI1, BRL1 and BRL3 (Serine/threonine protein kinase) Phosphorylation	BZR1, BES1 BAK1, BKI1, BSK1, BSU1, BIN2

Table 1. In this table relevant reviews are cited under hormone names. Where reviews do not cover specific aspects, research articles are cited. * ABCG transporters and NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER (NPF) proteins are increasingly being uncovered as transporters of multiple plant hormones (most recently reviewed in Lacombe and Achard, 2016).

Nine types of phytohormones and their respective transduction pathways have been uncovered in plants to date: Auxins, Cytokinins (CKs), Gibberellins (GAs) Abcissic Acid (ABA), Brassinosteroids (BRs), Ethylene (ET), Strigolactones (SLs), Jasmonic Acid (JA) and Salicylic Acid (SA) (Larrieu and Vernoux, 2015). Many of these hormones have no described roles in early developing seeds, and as a result I have chosen simply to present their main functions, and relevant recent reviews in tabular form (Table 1.2). I have highlighted processes of importance to early seed development in red in this table and I will elaborate briefly on these processes in the following few paragraphs.

1.2.4.2 Hormones in seed developmental coordination

As can be seen in Table 1.2, several phytohormones have known roles in early seed development. Globally, these roles may appear relatively minor compared to functions in other tissues. It should, however, be borne in mind that gamete-specific sterility and/or strong pleiotropic phenotypes are associated with a strong lack of production/signalling/perception of various hormones including jasmonic acid, brassinosteroids, gibberellins and auxins, and as a result it is often difficult to observe and/or interpret seed/embryo phenotypes in these backgrounds. The roles of some of these hormones in signalling during seed development may therefore have been underestimated.

1.2.4.2.1 Auxins

Historically the major role for auxin in the developing seed has been associated with embryo patterning. During the very earliest stages of embryo development local auxin production in the suspensor (Robert et al., 2013) and polar auxin transport mediated by PIN7 (Friml et al., 2003) lead to the production of an auxin response maximum in the embryo proper, which contributes to its specification (Lokerse and Weijers, 2009). Subsequently auxin biosynthesis initiates in the embryo proper at the globular stage. This leads to the polarised localisation of another auxin transporter, PIN1 towards the basal pole of the embryo proper resulting in the formation of an auxin response maximum in the hypophysis, which is implicated in root pole specification (Friml et al., 2003). Consistent with the important role of auxin in embryo patterning, auxin biosynthetic mutant show serious patterning defects from the globular stage onwards (Cheng et al., 2007; Robert et al., 2013). Interestingly the expression of the key *YUCCA1 (YUC1), YUC4* and *YUC10* auxin biosynthesis genes is controlled by the seed

development orchestrating transcription factor LEC2, with YUC4 being a direct target (Stone et al., 2008; Wójcikowska et al., 2013). The auxin response maxima observed during early embryogenesis are dependent upon the presence of appropriate effectors within the cells of the early embryo. Consistent with this, a comprehensive study has shown dynamic complementary and overlapping expression patterns for a large number of AUXIN RESPONSE FACTOR (ARF)-encoding genes in the early embryo (Rademacher et al., 2011). These transcription factors, whose activity is determined by the presence of auxin are also very important in embryo development. For example, as mentioned in the first part of the introduction, MP/ARF5 is essential for correct embryo patterning (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998).

With the exception of the embryo, relatively little has been published about the role of auxin in the other compartments of the developing seed. Nonetheless, recent work has started to show key roles for auxin in the development of the endosperm. Just after fertilization, there is a dramatic increase in auxin levels in the young endosperm, and this auxin is sufficient to trigger endosperm development (Figueiredo et al., 2015). This dynamic change in auxin levels has been shown to be due to the fact that the auxin biosynthesis genes *TAR1, TAR2* and *WEI8* are maternally repressed in the central-cell before fertilization. The arrival of non-repressed paternal copies of these genes during fertilization permits a wave of auxin production, and thus triggers endosperm development (Figueiredo et al., 2015). Additionally, the authors of this insightful study have shown that auxin mediated endosperm development requires the function of AGL62 (an endosperm specific MADS-box transcription factor), which in another study from the same group has also been shown to be necessary for the initiation of seed coat development upon fertilization (Roszak and Kohler, 2011), raising the exciting possibility that auxin from the endosperm could co-ordinate the development of the maternal and zygotic tissues during post-fertilization seed development. Although no ARFs have been reported to date to have a function in controlling endosperm development, this could be due to redundancy since several members of this transcription factor family are expressed in the endosperm (Rademacher et al., 2011).

In contrast, the potential importance of auxin on the testa is underlined by important roles of ARFs in testa development. ARF2, which is expressed in the integuments, is a negative regulator of seed size. Mutants have a larger integument due to an increased number of cells generated prior to fertilization (Adamski et al., 2009; Schruff, 2005; Xia et al., 2013). In addition, ARF3/ETTIN(ETT) also controls the

development of the integuments. In *arf3*/*ett* mutants, the outer and inner integuments are not well defined, resulting in the production of seeds with a rounded shape and variable size (Kelley et al., 2012). However, ARF3/ETTIN(ETT) is likely to act in an auxin independent fashion (Vernoux et al., 2014).

1.2.4.2.2 Cytokinins (CKs)

Genes encoding enzymes necessary for regulating CK levels are necessary during the first steps of seed development. They have effects on both the endosperm and the seed coat, and could therefore also be involved in the proposed cross-talk between these two compartments (Garcia, 2005). Cytokinin oxidases (CKXs), involved in CK degradation are necessary for the control of seed size, with *ckx1* and *ckx3* mutants showing abnormally larger seeds with large embryos (Werner, 2003). Seed size has also been reported to be increased in mutants lacking the AHK2, AHK3 and AHK4 receptors (Riefler, 2006). Another CK degrading gene, *CKX2*, is a target of the IKU signalling pathway which plays an important role in the control of endosperm expansion and cellularization, as well as seed size (Li et al., 2013). Furthermore, *CKX2* appears to be maternally repressed through the action of PRCII (Li et al., 2013). Cytokinins have also been shown to be necessary for the actual specification of the central-cell during gametophyte formation, through the action of the CYTOKININ INDEPENDENT 1 (CKI1) histidine kinase, a kinase involved in the phosphorylation cascade induced by cytokinin perception. *CKI1* expression is restricted to the forming central-cell and ectopic expression in the egg-cell induces the formation of non viable ovules with two central cells, making CKI1 a key actor of central-cell specification (Yuan et al., 2016)

Analogous to ARFs for auxins, the Arabidopsis Response Regulators (ARRs) are the transcription factor effectors of CK signalling. Although the endosperm is the scene of the preferential expression of *ARR9, ARR18, ARR19* and *ARR21* (Day et al., 2008), no very convincing seed phenotypes have been reported in mutants lacking ARR proteins. Interestingly however, cytokinin production appears to play an important role downstream of auxin signalling during the patterning of the embryonic vasculature (Rybel et al., 2014).

1.2.4.2.3 Brassinosteroids

The plant steroid hormones, called Brassinosteroids, have also recently been shown to be involved in seed development. They regulate seed shape and size, and also interact with other hormonal signalling pathways in the seed. In *Arabidopsis,* the

BRASSINAZOLE RESISTANT1 (BZR1) protein plays a central role as an integrator of many seed development processes by controlling the expression of key seed development genes (Jiang et al., 2013). In particular, BZR1 activates expression of the *IKU1 and IKU2* genes in the endosperm, and down regulates the transcription factor encoding *AP2* and *ARF2* genes in the seed coat. Another gene, *BIN2,* has been shown to integrate auxin signalling and BR signalling through the control of *ARF2* (Vert et al., 2008). However the sites of BR biosynthesis and signalling in the developing seed remain uninvestigated.

1.2.4.2.4 Abscissic acid & Gibberellins

It is interesting to note that as the literature stands, two distinct sets of phytohormones appear to regulate the early and late phases of seed development. On one side, early seed development involves Auxins, Cytokinins and Brassinosteroids, which both promote the development of single compartments, and also, potentially mediate intercompartmental interactions. During later seed development Abscisic Acid and Gibberellins are generally cited as being involved in the acquisition and maintenance of dormancy as the seed matures (Bassel, 2016). These processes will not be addressed in detail here, although it is interesting to note that the regulation of embryo dormancy appears, based on seed bedding assays, to be dependent upon the residual endosperm, suggesting an important role for inter-organismal signalling in the regulation of germination (Lee et al., 2010). Interestingly Gibberellins at least, increasingly appear to play roles in earlier seed development. Notably, a study has shown that the cytochrome P450 monooxygenases ELA1 and ELA2, which have been shown to be involved in degrading active Gibberellins, are required to restrict seed expansion (Zhang et al., 2011), consistent with the role of Gibberellins in promoting cell elongation. As mentioned previously, more recently the *ELA1* gene has been found to be positively regulated by mechanical tension in the testa during seed expansion, suggesting that alteration of GA levels in this tissue could be a means of restricting endosperm-mediated seed expansion during early seed development (Creff et al., 2015). Where the GA in the seed originates is unclear, although two redundantly acting Ent-kaurenoic acid oxidase (KAO) proteins, members of the CYP88A class of cytochrome P450 monooxygenases, which play critical roles in GA biosynthesis (Regnault et al., 2014), show very strong and specific endosperm-specific expression in *in silico* data from early in seed development. This raised the exciting possibility that GA from the endosperm moves to the testa, where levels are actively controlled.

With this overview of inter-compartmental signalming in the seed complete, I will now address the final section of the introduction, which considers a particular structure developing within the seed, the embryonic cuticle

1.3 Formation of the Embryonic Cuticle: *De novo* formation of an apoplastic barrier

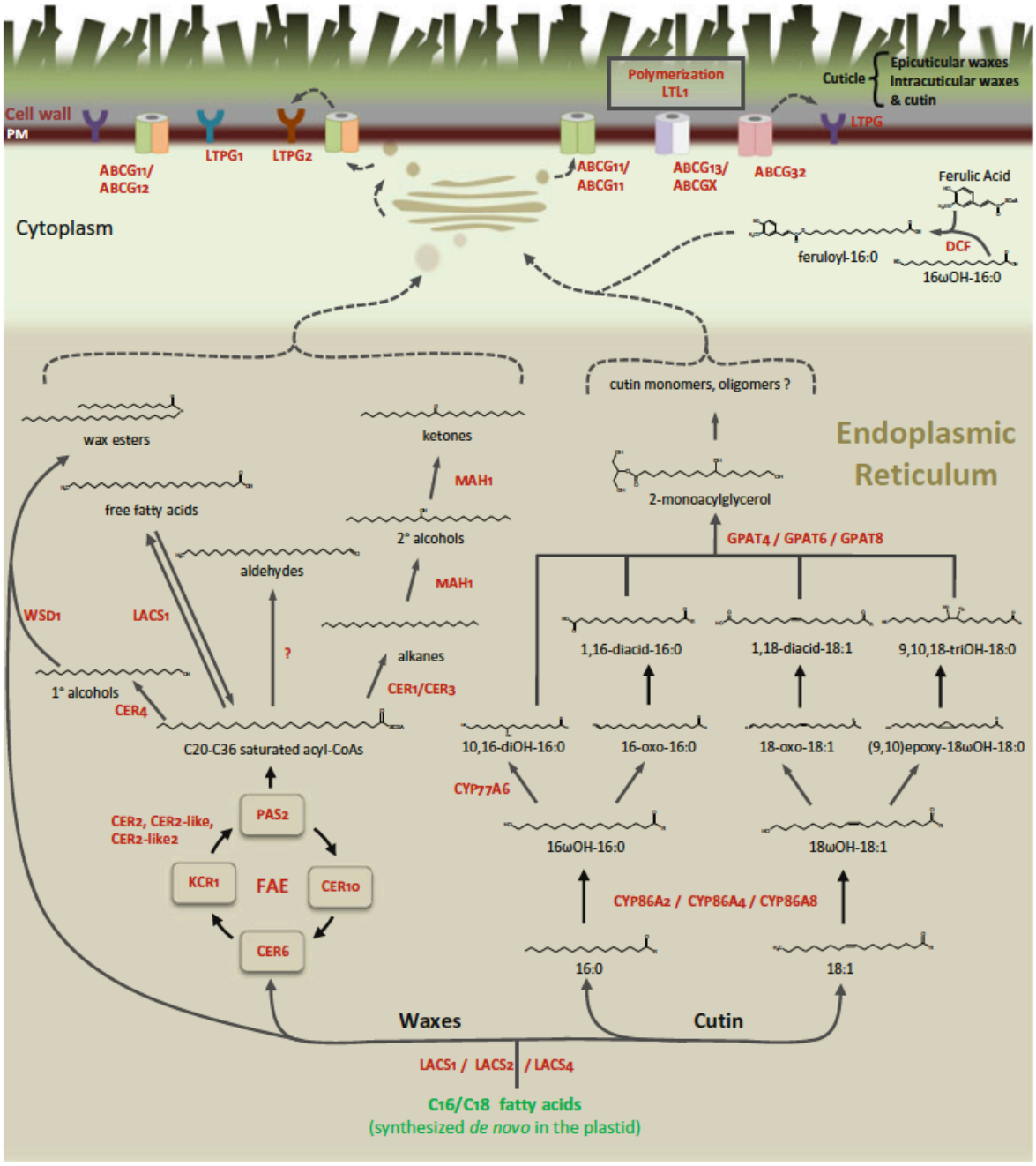
1.3.1 An overview of the cuticle

The cuticle is an extracellular structure produced by the plant epidermis and can be considered as the outermost part of the plant. Structurally, the cuticle is composed of hydrophobic materials, mainly waxes and cutin polymers, which are arranged in a continous layer and generally have hydrophobic properties (Delude et al., 2016). The mains functions of the cuticle are tightly linked to its location at the interface between the plant and its environment. As such, the hydrophobic cuticle allows for resistance to dessication by limiting water losses from the plant, as well as controlling the movement of other molecules and gasses (Lu et al., 2012; Wang et al., 2011). The other main role of the cuticle is to protect the plant against external biotic threats, acting as barrier against pathogens (Reina-Pinto and Yephremov, 2009).

In addition to the physiologically important functions listed above, the cuticle also plays key developmental roles. It has been proposed to provide mechanical strength to organs and restrict growth (Domínguez et al., 2011). In addition it prevents post-genital organ fusion (Luo et al., 2007; Weng et al., 2010). Interestingly, organ fusion events involving the formation of an apoplastic continuum between two organs, which can occur either during normal development, or in mutants with defective cuticles, can lead to an apparent loss of epidermal identity in fused regions (Delude et al., 2016). This underlines the fact that the presence of a functional cuticle is tightly linked to the acquisition and maintenance of epidermal identity and morphology (Delude et al., 2016; Javelle et al., 2011). Consistent with this, a perturbed cuticle metabolism is often linked to epidermal differentiation defects, including defects in patterning epidermis specific cells such as stomatal guard cells and and trichomes (Bergmann, 2004; Bird and Gray, 2003; Marks et al., 2009).

Finally, it should be noted that the hydrophobic cuticle can be defined as an apoplastic diffusion barrier. Another apoplastic diffusion barriers in plants is the

Casparian strip, a zone of highly modified cell wall which forms a net-like structure, sealing the apoplast of the root endoderm in the water-absorbing zone of the root. The Casparian strip forces soil water taken up by the plant, and the dissolved minerals that it contains, to cross the membrane, thus allowing the selective exclusion of undesireable molecules, and allowing the concentration of essential nutrients within the plant body (Barberon et al., 2016; Pfister et al., 2014). Intriguingly extensive parallels can be drawn between the cuticle and the Casparian strip, both in terms of their biogenesis and their functions (Nawrath et al., 2013). These parallels will be developed further later in this thesis.



1.3.2 Cuticle structure and biosynthesis

The plant cuticle is an epidermis specific, continuous layer of lipophilic material found on almost all the aerial surfaces of plants. It is composed of cutin, a fatty acid-derived polymer, which is both impregnated with, and covered by, epicuticular waxes (recently reviewed in Yeats and Rose, 2013). During cuticle biosynthesis, C16/C18 fatty acids produced in the plastids of epidermal cells are transported to the endosplasmic reticulum (ER). There they either enter the cutin biosynthetic pathway to form cutin monomers, or they are used to form Very Long Chain Fatty Acids (VLCFAs) through the activity of the Fatty Acid Elongase complex, which are subsequently modified to form the various alchohols and esters that make up epicuticular wax. I have chosen not to enter into the details of these biosynthetic pathways are they are not directly relevent to the work in this thesis, preferring to present the biosynthesis steps in the form of a Figure (Figure 9) taken from Delude et al., 2016 (a book chapter of which I am a co-author).

Figure 9 (Left) Schematic representation of the process of cutin and wax biosynthesis and export in plant epidermal cells taken from Delude et al., 2016. Proteins in red are those described in Arabidopsis thaliana. Wax biosynthetic pathways: Very long chain fatty acyl-CoAs are synthesized by the Fatty Acid Elongase (FAE) complex from C16-C18 fatty acids imported from the plastid. VLCFAs are then modified via two distinct biosynthetic pathways to generate the aliphatic compounds of waxes; the alcohol forming pathway resulting in the formation of primary alcohols and wax esters, and the alkane forming pathway producing aldehydes, alkanes, secondary alcohols and ketones. Cutin biosynthetic pathways: Addition of a hydroxyl group at the terminal or mid-chain position of C16 and C18 is catalyzed by members of the cytochrome P450 family (CYPs). Formation of dicarboxylic acids (DCA) from ω-hydroxyacids (ω-OH) may involve oxydoreductases. Intermediates are annotated "COOR", where R could be H, CoA or glycerol, since the exact substrate of P450 and the order of reactions remains unclear. Esterification of ω-OH and DCA to glycerol-3-phosphate by glycerol-3-phosphate acyl-CoA transferase (GPATs) produces sn-2-monoacylglycerol. Incorporation of phenolic components into the cutin polymer requires BAHD-type acyltransferases. Extracellular polymerisation is performed by cutin synthases. Export of precursors to the cuticular matrix: Cuticle precursors produced in the endoplasmic reticulum (ER) reach the plasma membrane (PM) via the Golgi apparatus through the secretory pathway. Export of cuticle compounds from the plasma membrane is carried out by ATP-Binding Cassette (ABCs) transporters and glycosylphosphatidylinositol-anchored Lipid Transfer Proteins (LTPGs)

The biosynthesis of the components of cutin and epicuticular wax shown in Figure 9 are perhaps the best understood steps of cuticle biogenesis. The formation of a functional cuticle subsequently requires the multi-step loading of waxes and cutin monomers into the extracellular space at the epidermal surface. As developing structures (leaves, stems…) expand, cuticle components must be continuously exported and "intercalated" with the existing cuticle in order to maintain cuticle integrity. How this process occurs remains relatively mysterious, and involves the transport of the highly hydrophobic components of the cuticle out of the cell and across the highly hydrated cell wall matrix.

A recent study has implicated Golgi-derived vessicles in the transport of cuticle components between the ER and the plasma membrane (PM) (McFarlane et al., 2014). Once at the membrane, ATP-Binding Cassette (ABC) transporters ensure the export of cuticular material, and glycosylphosphatidylinositol-anchored Lipid Transfer Proteins (LTPGs) are thought to play a role in transporting them to the cuticular matrix. Cuticular wax export is thought mainly to be carried out by ABCG11/12 (which can heterodimerize), LTPG1 and LTPG2 (Bird et al., 2007; DeBono et al., 2009; Kim et al., 2012; McFarlane et al., 2010; Pighin et al., 2004), while Cutin is exported mainly through ABC11, ABCG13, ABCG32 and LTPGs which have not yet been characterised (Bessire et al., 2011; Panikashvili et al., 2011). Another mystery involves how cuticle components are integrated into the outer face of the epidermal cell wall. The junction between the cell wall and the cuticle is not distinct; the outermost cell wall layers being merged with the innermost cuticular layer and thus composed of a mix of cellulose, pectin, cutin and waxes. Whether cuticle components are covalently linked to other cell wall polymers is unclear. In addition the process of cutin polymerisation in the cell wall was also relatively mysterious until recent research showed that members of the large family of GDSL-lipases act as cutin synthases (Fich et al., 2016; Girard et al., 2012; Philippe et al., 2016; Yeats et al., 2012). Nonetheless, how cuticular integrity is monitored and maintained during organ growth, and ho cutin components are targetted to the cel wall surface at the subcellular level, remains to be discovered.

1.3.3 The transcriptional control of cuticle formation

Most genes involved in the biosynthesis and transport of cuticle components post-germination show epidermis specific expression suggesting that cuticle biosynthesis is under strict transcription regulation. Several classes of epidermis-specific transcription factors have been shown to be involved in the regulation of cuticle biogenesis (Figure 10), including members of the AP2/EREBP clade such as SHINE (SHN1)/WIN1, SHN2 and SHN3 (Aharoni et al., 2004; Broun et al., 2004). Plants

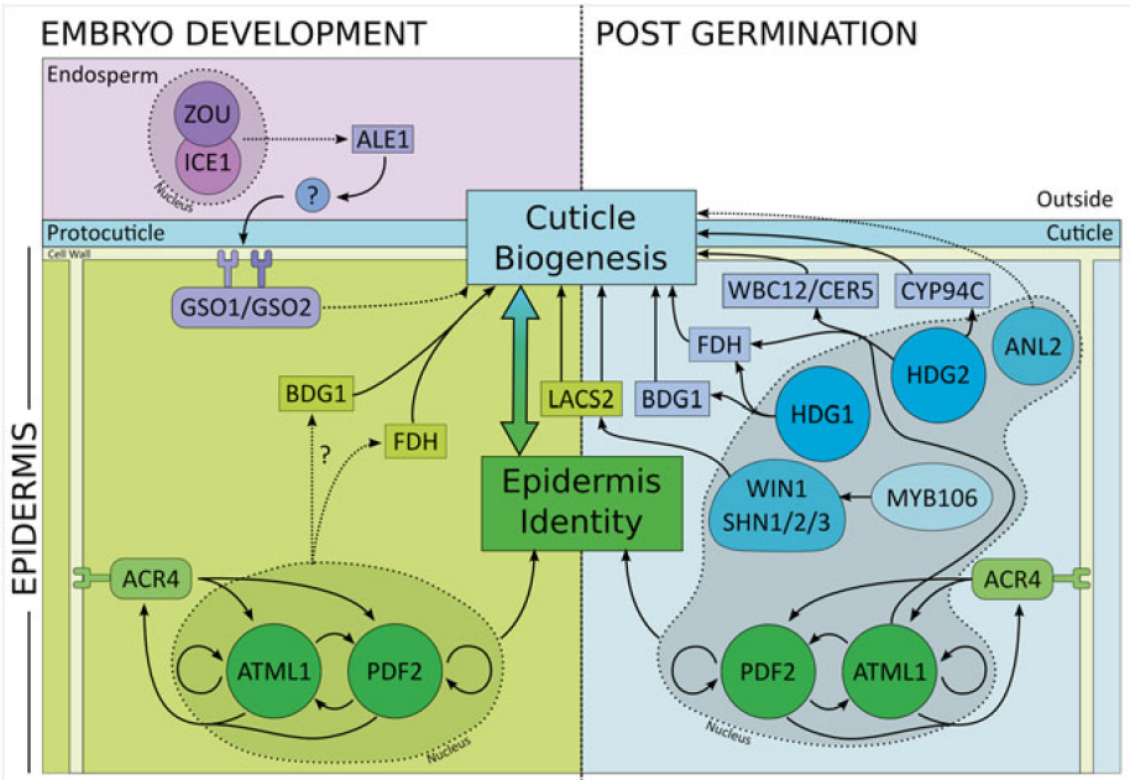


Figure 10 Transcriptional control of cuticle biosynthesis and epidermis specification during development (taken from Delude et al., 2016). Because of their important role in specifying epidermal cell fate it could be argued that the effects of altering HDZIPIV activity on cuticle composition are largely indirect. Nonetheless, several pieces of evidence support a more direct role for members of the HDZIPIV family in the control of cuticle production. Arabidopsis lines ectopically overexpressing *ATML1* and *HDG2*, show strong up-regulation of both *FDH* and *ABCG12* (Takada, 2013). Furthermore the defective trichomes of *hdg2* mutants show reduced cuticle load and reduced expression of *CYP94C*, a gene required for the production of dicarboxylic acids potentially involved in cutin biosynthesis (Marks et al., 2009). Finally, defects in both the tomato HDZIPIV-encoding *CUTIN DEFICIENT2* gene, and its Arabidopsis orthologue *ANTHOCYANINLESS2 (ANL2)* reduce cuticle loads (Nadakuduti et al., 2012).

silenced simultaneously for all three *SHN* genes produce flowers with organ fusions and show changes in the expression of genes involved in not only cuticle biosynthesis but also pectin production and cell elongation, suggesting effects on epidermal cell differentiation (Shi et al., 2011). In further support of a link between epidermal differentiation and cuticle load, the MIXTA-like MYB transcription factor MYB106, which regulates the formation and branching of trichomes, is a positive regulator of WIN1/SHN transcription factors (Oshima and Mitsuda, 2013).

Arabidopsis HDG1, a member of the HDZIP IV family of homeodomain transcription factors to which ATML1 and PDF2 belongs, has been shown to bind the promoter of the cutin-related genes *BDG* and *FDH* , while *HDG1* chimeric repressor plants were shown to have a defective cuticle and leaf fusion events (Wu et al., 2011). In Arabidopsis, the HDZIP IV gene family comprises 16 members, of which several have been shown to be involved in both epidermal specification and differentiation (Abe, 2003; Depège-Fargeix et al., 2011; Javelle et al., 2010; Marks et al., 2009; Nadakuduti et al., 2012; Nakamura et al., 2006; Roeder et al., 2012; San-Bento et al., 2014; Takada, 2013; Vernoud et al., 2009). HDZIP IV proteins appear to play a key role in regulating both epidermal fate specification and cuticle biogenesis. As previously described, loss of function of two family members, *ATML1* and *PDF2*, leads to loss of epidermal cell fate during early embryogenesis (Abe, 2003; San-Bento et al., 2014) and ectopic expression of *ATML1,* and/or of the closely related *HDG2* gene leads to the ectopic formation of stomata in internal tissues (Peterson et al., 2013; Takada, 2013).

Despite this evidence, how the activity of HDZIP IV proteins is integrated with the activity of other key cuticle-regulating transcription factors, such as WIN/SHINE proteins, remains rather unclear, as does how cuticle biogenesis is initiated in developing embryos.

1.3.4 A signalling pathway involved in maintaining embryonic cuticle integrity.

In terms of cuticle biogenesis, plant embryos are unique from two important points of view. Firstly, they arise from gametophyte cells, and their cuticle, like the epidermal identity of the cells on which it appears, therefore arises *de novo* post fertilization. This is in contrast to the situation after early embryogenesis when most, if not all epidermal cells arise from anticlinal divisions of existing epidermal cells, and are thus “pre-equipped” with an inherited cuticle bearing surface (Javelle et al., 2011).

Secondly, not only does the embryonic cuticle arise de novo, but it also arises in a position where it is not juxtaposed by another cuticularized surface. There is no evidence in the literature that the endosperm produces a cuticle at its junction with the developing embryo. The embryonic cuticle, and thus the apoplastic separation of the embryo from the endosperm, therefore arises in developmentally unique circumstances since in all other aerial plant organs the outer, cuticle bearing surfaces of all epidermal cells juxtapose the equivalent surfaces of other epidermal cells. The juxtaposed cuticles of these cells play a seminal role in preventing post genital fusion of epidermal surfaces, no matter how tightly they are packed together in developing buds.

The unique circumstances surrounding angiosperm embryonic cuticle formation might be predicted to demand the deployment of specific molecular and cellular mechanisms permitting the correct definition and positioning of the nascent cuticle, and the successful separation of the embryo from surrounding endosperm tissues. Consistent with this idea, recent research has uncovered a seed-specific signalling pathway, involving both endosperm and embryonically expressed components, which is necessary for the formation of a functional embryonic cuticle, and for physical separation of the embryo and endosperm, in Arabidopsis (Figure 1.10). To date, five components of this pathway have been identified, as described earlier. The first are the distantly related bHLH transcription factors ZOU/RGE1 (which is expressed specifically in the embryo surrounding endosperm (Kondou et al., 2008; Xing et al., 2013; Yang et al., 2008), and ICE1/SCREAM which acts as a heterodimer with ZOU (Denay et al., 2014). Both *zou* and *ice1* mutants fail to form a functional embryonic cuticle (Denay et al., 2014) and mutant embryos fail to physically separate from the surrounding endosperm (Yang et al., 2008). Interestingly however, cuticle components appear to be present at the surface of *zou* mutant embryos, and, importantly, the expression of neither epidermal markers (Denay et al., 2014; Xing et al., 2013; Yang et al., 2008) nor genes involved in cuticle biosynthesis (our unpublished results) is strongly affected in either mutant background. The third gene involved in the pathway, *ALE1* is also expressed specifically in the embryo-surrounding endosperm, but encodes a Subtilisin-like serine protease called ABNORMAL LEAF SHAPE1 (ALE1), which is necessary for normal embryonic surface formation and endosperm/embryo separation (Tanaka et al., 2001). ALE1 is predicted to act in the secretory pathway or apoplast, and therefore represents a non-cell autonomous component of the pathway. The expression of ALE1 is almost completely abolished in *zou* and *ice1* mutants (Denay

et al., 2014; Xing et al., 2013; Yang et al., 2008). Consistent with the fact that ZOU and ALE1 act in the same genetic pathway their mutant phenotypes show no additivity in double mutants (Xing et al., 2013). Finally, two genes encoding the closely related receptor kinases *GSO1* and *GSO2*, and which show strong expression in developing embryos, act redundantly to promote the production of a functional embryonic cuticle and embryo/endosperm separation (Tsuwamoto et al., 2008). Double *gso1 gso2* mutants show remarkably similar phenotypes to *ale1* mutants and non-additive genetic interactions with both *ale1* and *zou* mutants, confirming the likely participation of these RLKs in a signalling processes involving communication between the developing embryo and endosperm necessary for normal embryonic cuticle biogenesis (Waters et al., 2013; Xing et al., 2013).

In addition to these pathway components, another mutant has appeared in the recent literature which have phenotypes strongly reminiscent of those involved in the above pathway. The first is defective in a gene called *FRIABLE1(FRB1) (Neumetzler et al., 2012),* which is annotated in the public database as containing a potential GDP-fucose protein O-fucosyltransferase domain, although this homology appears weak. Loss of function *frb1* mutants are reported to show post-germination modifications in cell wall composition in seedlings including alterations in galactose- and arabinose-containing oligosaccharides, in pectin methylesterification, in cell wall associated extensins and in xyloglucan microstructure. Importantly, however, the seed phenotype published for *frb1* mutants is effectively identical to that of *gso1 gso2* double mutants, with defects in embryo elongation and seedling cuticle integrity (Neumetzler et al., 2012).

AIM OF THE THESIS

The main goal of my thesis work was to deepen our understanding of the molecular basis controlling the production of an intact embryonic cuticle in *Arabidopsis thaliana*. Given the identities of the genes already known to be involved in the process, we hypothesized the existence of one or more signalling peptides involved in the communication process between the embryo and the endosperm allowing for cuticle integrity. Using a gene candidate approach, I therfore investigated the potential implication of several different peptides. Uncovering the function of one candidate, which we named CERBERUS (CRS) has been the main focus of my PhD. The second chapter of my PhD provides a functional characterization of CRS during seed development. One of the receptor-like kinases involved in cuticle integrity signalling in the Arabidopsis embryo, GSO1, has recently been shown to be involved in regulating the integrity of a second apoplastic barrier in the plant, the Casparian strip (Pfister et al., 2014). This has very recently opened up the possibility of studying key regulators of Casparian strip integrity in the context of the developing seed. These investigations are the subject of the third chapter of my PhD. Finally, in the fourth chapter, I will focus on the function of FRIABLE1 (FRB1) in embryonic cuticle formation.

2 THE ENDOSPERM-SPECIFIC CERBERUS PEPTIDE IS INVOLVED IN EMBRYONIC SURFACE FORMATION

2.1 Foreword

The following manuscript and annexes focus on the functional study of the STIG1-family signaling peptide CERBERUS (CRS). As highlighted in the introductive chapter, embryonic cuticle formation in *Arabidopsis* is regulated by an endosperm-embryo signaling pathway. The main genetic actors in this pathway being, on the endosperm side, the bHLH transcription factor ZOU (acting as a heterodimer with ICE1), which regulates the expression of ALE1, a subtilisin-like serine protease. Subtilisin-like serine proteases are known to be necessary for the endoproteolytic cleavage of several plant signaling peptides. However the substrate of ALE1 has not yet been identified. On the embryo side, genetic studies show that the two redundantly acting LRR-RLKs GSO1 and GSO2 act redundantly in the same pathway as ALE1 and ZOU. At the start of my thesis no ligands for GSO1 and GSO2 had been identified. In this context, we hypothesized that one or more peptide ligands cleaved by ALE1 and perceived by GSO1 and GSO2 should be involved in this signaling pathway to ensure endosperm/embryo communication. Finding such ligands was the main focus of my thesis.

2.1.1 How does the embryonic cuticle form ?

As described at the end of the introductive section, the processes underlying embryonic cuticle formation are far from being well established during Arabidopsis embryogenesis. However it is clear that cuticle biogenesis occurs *de novo* on the surface of the embryonic cuticle. Before investigating the genetic control of embryonic cuticle establishment, it was therefore necessary to unveil the dynamic of cuticle biogenesis around the embryo during embryogenesis. TEM analysis of the embryo/endosperm interface at different developmental stage is presented in Figure 11. The images were produced as part of a collaboration with researchers at the University of Bordeaux, and I was responsible for the production of the plant materials used. In addition I travelled to Bordeaux to oversee the staging and harvest of siliques, and was involved in their fixation. Imaging was carried out by Lysiane Brocard at the Bordeaux Imaging Centre (BIC). These data form part of a manuscript (Creff et al.) which is currently under revision and which is not include in the thesis, of which I am a co-author. Figure 11.A-D shows various stages of development of the cuticle of wild-type embryos ranging from the 2-4 -cells stage to the torpedo stage.

The cuticle is an electron dense structure and is visible as a dark layer in these pictures because the samples were stained with Osmium tetroxide, a lipophilic contrasting agent. At the 2-4 -cell stage (Figure 11A), the cuticle is absent and the embryo (bottom of the picture) is surrounded by a thick cell wall. The cytoplasm of the coenocytic endosperm can be observed in a thin layer on the other side of the cell wall. At the globular stage (Figure 11B), dark cuticle-like material is deposited in a patchy manner around the embryo. Discontinuities in the structure can clearly be observed. At the heart stage (Figure 11C), the cuticle is already continuous and stays that way at torpedo stage (Figure 11D, image taken between the developing cotyledons and thus showing two adjacent cuticularized epidermal surfaces).

In conclusion, the embryonic cuticle starts to be deposited at around the globular stage of embryogenesis and, importantly, is already intact by the heart stage of embryogenesis in wild-type embryos.

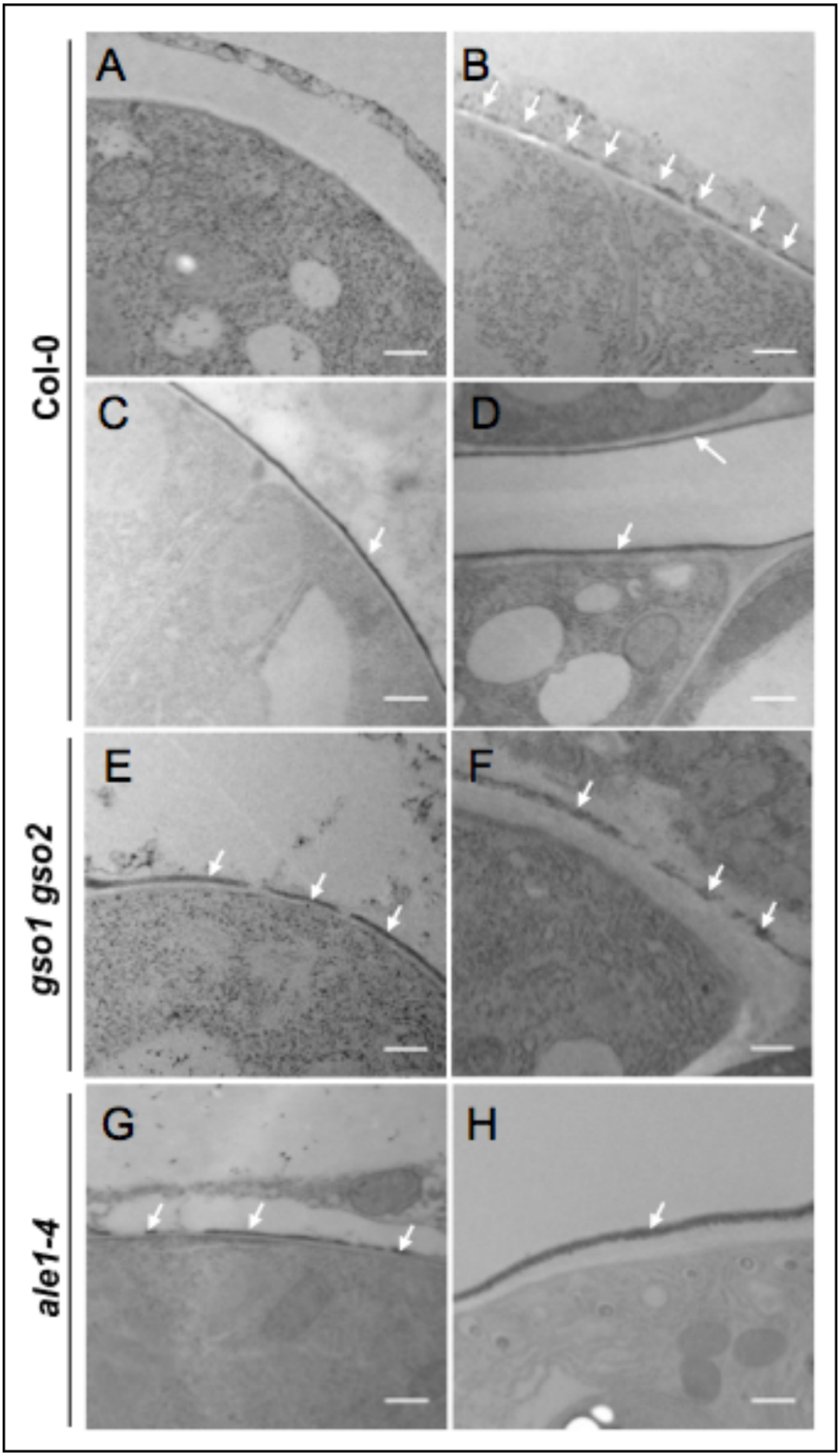


Figure 11. TEM section analysis of Col-0 (A-D), *gso1 gso2* (E,F) and *ale1-4* (G,H) mutants. Developmental stages: 8-cells (A), globular stage (B), heart (C,E,G) and torpedo (D, F H).The cuticle in shown with white arrows. Scale bar: 100nm

Similar studies were carried out in *gso1-1 gso2-1* double mutants, concentrating on the heart and torpedo stages when the cuticle is intact in wild-type embryos. In this background, the cuticle is still produced, but discontinuities persist at heart (Figure 11E) and torpedo stage (Figure 11F), likely explaining the defects in cuticle permeability that have been observed in these mutants (Tsuwamoto et al., 2008; Xing et al., 2013). In addition, the cuticle appears less condensed in *gso1-1 gso2-1* mutants than in wild-type, particularly at the torpedo stage. In the *ale1-4* mutant, the cuticle is also discontinuous at heart stage (Figure 11G) although interestingly, it shows fewer defects at the torpedo stage (Figure 11H).

The conclusion from this study is that the cuticle defects observed in mutants of the GSO1/GSO2/ALE1 signaling pathway are due to a discontinuous cuticle rather than to an absent cuticle. Further experiments carried out in the context of the same study back up this conclusion. For example measurements of cutin load on the cotyledons of young seedlings from mutant and wild-type backgrounds show no significant differences. In addition, the expression of genes known to be involved in cuticle biosynthesis was investigated by transcriptomic analysis, and subsequently by *in situ* hybridization. As expected the expression of these genes was restricted to the embryonic epidermis but again, no differences were observed between mutant and wild-type plants (Creff et al., in revision). Transcriptome studies also showed a remarkably strong overlap between the genes mis-regulated in *gso1-1 gso2-1* and *ale1-4* backgrounds, providing independent evidence supporting our genetic data suggesting that GSO1, GSO2 and ALE1 act in the same signaling pathway. Finally, and rather intriguingly it was found that although the *ale1-4* mutant phenotype is considerably weaker by embryo maturity than that of *gso1-1 gso2-1* mutants (Xing et al., 2013), like the early cuticle phenotype, the early "transcriptional" phenotype in *ale1-4* mutants was almost as severe as that of *gso1 gso2* double mutants. This could suggest that ALE1 is only necessary for early GSO1/GSO2-mediated signaling, and that other factors are required at later developmental stages.

Based on these results, we thus propose a model in which GSO1 and GSO2 are involved in perceiving signals, which alert the embryo to the presence of breaks in the cuticle structure, and permit these breaks to be repaired as the cuticle is generated and expands. Given the spatial localization of other pathway components, and that fact that cuticle biosynthesis probably occurs from the embryo epidermis, it seemed likely that potential GSO1/GSO2 ligands would be produced in the endosperm.

2.1.2 Identification of CERBERUS

The preliminary experiments leading to the identification of CERBERUS (CRS) are not described in the following manuscript and will therefore briefly be described here. A candidate gene approach was used, with candidates selected based on two critera, spatial expression/regulation and receptor identity.

The first approach, and that which ultimately resulted in the identification of CRS, consisted of postulating that the transcription factor ZHOUPI might transcriptionally regulate a functional module involving both ALE1 and the elusive peptide, ensuring a spatially coordinated action of both elements. Following this reasoning, we exploited available transcriptome data (Xing et al., 2013) and looked for peptide-encoding genes that were strongly downregulated in *zou-4* mutant seeds compared to wild-type seeds. Two genes fitted this criterion: a gene encoding a STIG1-like peptide (*CRS)* and *EPIDERMAL PATTERNING FACTOR 2 (EPF2)*, a gene encoding a peptide involved in stomatal development and which has previously been shown to bind to the RLK ERECTA and related receptors (Hunt and Gray, 2009; Lee et al., 2012a, 2015).

The second approach was based on the identity of the GSO1 and GSO2 receptors and their closest homologues. In the LRR-RLK XI family, GSO1 and GSO2 are very closely related at the sequence level to the PEPR1 and PEPR2 receptors (Yamaguchi et al., 2010). Since PEPRs have been shown to bind to the AtPEPs peptides during defence responses (Krol et al., 2010; Yamaguchi et al., 2010), we hypothesized that evolutionary proximity, and thus structural similarity, might mean that GSO1 and GSO2 perceive similar ligands. I therefore selected PROPEP-encoding genes showing expression in seeds and for which mutants were available. These were *AtPROPEP1, AtPROPEP4* and *AtPROPEP6*. The *in silico* expression patterns (Le et al., 2010) of all candidate genes is shown in Figure 12.

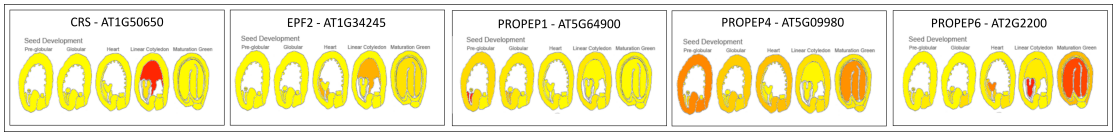


Figure 12. Expression patterns of the candidate peptides during seed development with a compartmental resolution. The color palette from yellow to red indicates the relative expression, with the red being the highest level of relative expression.

I ordered available insertion alleles for each of the above mentionned genes, obtained homozygous mutant plants, and conducted toluidine blue (TB) staining experiments on etiolated seedling to test for the integrity of the embryonic cuticle in these mutants. The results are shown in Figure 3. Col-0 is used as a negative control with its intact cuticle and *ale1-4* as a positive control since it is known to have a defective, permeable cuticle. Etiolated cotyledons are yellow, as they do not produce chlorophyll. TB being a hydrophilic dye, does not readily enter the cotyledons of Col-0 seedlings due to the presence of the intact cuticle (Figure 13A). In the *ale1-4* mutant context, the cuticle is defective and therefore the TB enters the cotyledons, turning them a green/blue colour (Figure 13B). Testing the candidate peptide mutants, only the *crs-1* mutant showed a green/blue coloration (Figure 13C), the *propep1, propep4, propep6* and *epf2* mutants having no visible defects. From this data, I decided to focus my work

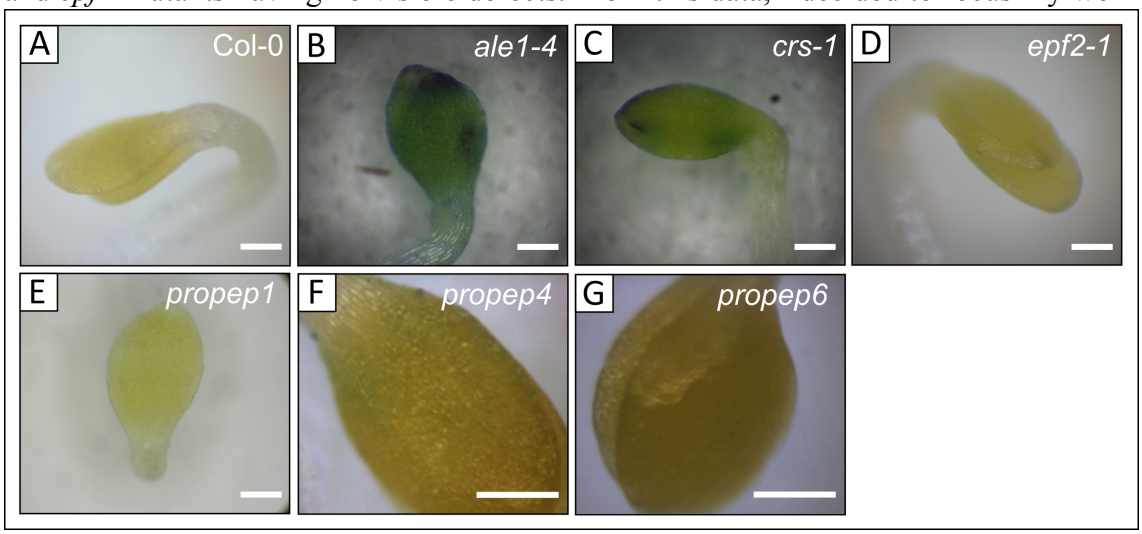


Figure 13 Toluidine blue coloration on etiolated cotyledons of controls: (-) Col-0 (A), (+) *ale1-4* (B) & various mutants for the candidate peptides considered: Col-0 (A), *ale1-4* (B), *crs-1* (C), *epf2* (D), *propep1* (E), *propep4* (F) & *propep6* (G). Scale bar 200µm

on CRS, a peptide whose function has never been investigated to date. However, it is important to highlight that these experiments do not allow us to formally exclude a redundant role for the PEP peptides in ensuring embryonic cuticle integrity, and this subject would merit revisiting in the future.

The next part of this first results chapter has the following structure: I will first present a manuscript which is currently being prepared for submission, and whose main subject is the production of a newly described structure around the embryo, the embryo "sheath", which I have shown to be controlled by both ZOU and CRS. The basic

functional characterization of CRS is undertaken in this manuscript, as well with the description of its spatio-temporal expression characteristics and genetic interactions with the other known member of the signaling pathway. For clarity reasons, the figure numbers are labelled Figure M#. The last part of the chapter comprises descriptions of ongoing investigations of CRS behavior *in vivo* and structure/function analysis.

2.2 Manuscript on CERBERUS and the Embryo Sheath

2.2.1 General informations

Short title: Control of embryo sheath formation

Corresponding author: Gwyneth Ingram

ZHOUPI and CERBERUS mediate embryo/endosperm separation by promoting the formation of an extra-cuticular "sheath" at the embryo surface.

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One sentence summary : Deposition of the embryo sheath, a novel endosperm-derived extra-embryonic structure, depends upon the endosperm-specific transcription factor ZHOUPI and a novel STIG1-like peptide CERBERUS, and is necessary for the separation of the embryo from the endosperm during seed development.

Author contributions : G.I. supervised the experiments; S.M. designed and performed most of the experiments with help from N.D. and C.F.; S.M and G.I. analysed the data ; S.C. and A.C. provided technical assistance to S.M. and G.I.; Z.N. provided a construct for CRISPR experiments. L.B. carried out immunogold-labelling experiments; G.I. conceived the project; S.M. and G.I. wrote the article with contributions from all the authors.

Funding information: S.M. and A.C. were funded by a grant from the French National Research Agency (ANR-13-BSV2-0002, INASEED). S.M. was also supported by a doctoral grant from the Rhône-Alpes region.

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2.2.2 Abstract

Seed development involves the concomitant development of two zygotic compartments, the embryo and the endosperm. Post-fertilization the endosperm expands rapidly as a coenocyte and then cellularises. Subsequently, the embryo grows invasively through the endosperm, which breaks down. How interactions between the growing embryo and the degenerating endosperm are regulated, and in particular how the physical separation between these two compartments is achieved and maintained to allow embryo growth, remains poorly understood. Here we characterise a novel structure, the embryo sheath, which forms on the surface of the embryo as the embryo starts to elongate. We show that the sheath is deposited outside the embryonic cuticle, and incorporates material rich in extensin-like epitopes that originates in the endosperm. Consistent with these observations we show that sheath production is dependent upon the activity of ZHOUPI, an endosperm-specific transcription factor necessary for endosperm degradation, embryo growth, embryo-endosperm separation and normal embryo cuticle formation. We identify a novel secreted peptide from the STIG1-like family, CERBERUS, whose expression is dependent upon ZOU activity. We show that CERBERUS is necessary both for the formation of a normal embryo sheath, and for normal embryo-endosperm separation. Finally, we show that although the production of sheath material in the endosperm is independent of the activity of the receptor-like kinases GSO1 and GSO2, they are nonetheless required for sheath deposition at the embryo surface. Our results uncover the likely existence of a complex dialogue between the embryo and the endosperm during early seed development.

2.2.3 Introduction

Angiosperm seed development is a complex process necessitating the co-ordinated development of three structurally and genetically distinct compartments: the maternal seed coat and the zygotic endosperm and embryo. These three tissues are arranged one inside the other, suggesting that they must communicate both chemically and physically during seed growth. The endosperm surrounds the developing embryo and plays two critical roles in seed development, the first being to grow, and in so-doing to generate space within the maternal seed coat for nutrient storage, and the second being to act as a sink tissue, absorbing nutrients from the mother plants and transferring them to the developing embryo either during seed development, or upon germination (Berger, 2003; Li and Berger, 2012; Olsen, 2004).

In Arabidopsis, the endosperm is a largely transient compartment, which grows rapidly as a coenocyte after fertilization, cellularizes, and then degenerates to be replaced by the embryo as it expands. The interaction between the embryo and the endosperm during this stage of development is relatively poorly understood, but involves the physical invasion of one tissue (the endosperm) by a second (the embryo). This type of interaction between two plants tissues is relatively rare during normal development. Another obvious example is the movement of the pollen tube through the tissues of the style and transmitting tract during fertilization (Cheung et al., 2010). Interestingly, both of these interactions involve the movement of one tissue through a genetically distinct neighbouring tissue.

The endosperm specific bHLH transcription factor ZHOUPI/RETARDED GROWTH OF EMBRYO1 (RGE1) (Kondou et al., 2008; Yang et al., 2008), acting as a heterodimer with a second bHLH called ICE1 (Denay et al., 2014), has been shown to regulate the embryo/endosperm interaction in Arabidopsis. To date, ZOU has been shown to be required for two partially separable processes. The first is endosperm breakdown. ZOU regulates the expression of a range of cell wall modifying enzymes in the endosperm, and endosperm cell wall softening appears to be necessary to permit crushing of the endosperm during embryo expansion (Fourquin et al., 2016). As a result the mature seeds of *zou* mutants contain a large body of persistent endosperm in addition to the specialized outer endosperm cell layer, which is all that remains at seed maturity in wild-type seeds. The second function of the ZOU/ICE1 complex is to participate in the formation of an intact embryonic cuticle. This function appears to be achieved, at least in part through the regulation of the expression of ALE1, a subtilisin serine protease acting in an inter-compartment signalling pathway involving the receptor-like kinases GSO1 and GSO2 (Tanaka et al., 2001; Tsuwamoto et al., 2008; Xing et al., 2013). Like *ale1* mutants, *zou* mutant embryos produce a discontinuous cuticle on the surface of the developing embryo (Tanaka et al., 2001; Yang et al., 2008).

In addition to the two phenotypes described above, *zou* mutants exhibit a third phenotype, namely a strong adhesion of the embryonic surface to surrounding endosperm cells. During plant development normal cuticle formation is generally considered to play an important role in preventing the fusion of developing organs to

their neighbours (Delude et al., 2016), and the embryo/endosperm adhesion phenotype of *zou* has therefore been attributed to defects in the embryonic cuticle. However, although the cuticle of *zou* mutant embryos is defective, it is none the less present over most of the embryonic surface. Interestingly an abnormal deposition of endosperm debris on apparently intact zones of cuticle has been reported on *zou* mutant seedlings (Xing et al., 2013), suggesting that embryo/endosperm adhesion defects may not be entirely attributable to cuticle discontinuities.

Here we reveal that ZOU is required for the production of an extra-cuticular endosperm-derived structure (which we will call the embryo sheath) at the surface of the embryo. We show that a unique, endosperm-specific cysteine-rich peptide, whose production is ZOU-dependent, is necessary for the biogenesis of the embryo sheath and is necessary for normal progression of the embryo through the endosperm tissue. We propose that the embryo sheath provides a separation/lubrication function facilitating the movement of the embryo relative to the degenerating endosperm.

2.2.4 RESULTS

2.2.4.1 The deposition of a "sheath" covering the developing embryo is absent in *zou* mutants.

Previous studies have shown that the surface of the mature embryo of Arabidopsis is covered with a continuous structure which is detected by the "anti-extensin" antibodies JIM12 (Smallwood et al., 1994) and LM1 (Lee et al., 2012b; Smallwood et al., 1995) We analysed the deposition of this structure during embryogenesis in the seeds of wild-type plants and *zou* mutants. We could first detect deposition of this structure at the late heart stage of embryo development, when embryo separation from the endosperm first becomes apparent, in a thin layer covering the embryo surface (Figure M1A-C,G-J). In addition we observed labelling of vesicle-like structures in some of the endosperm cells immediately juxtaposing the embryo Figure M1A-C and Supplementary Figure M1. A similar pattern was detected throughout the rest of embryo development, with labelling detected at the embryo surface, and in the adjacent endosperm, but never in the developing embryo Figure M1(G-J) and Supplementary Figure M1. Reticulate labelling was also observed in the apparently empty space between the endosperm and the embryo. This labelling pattern strongly suggests that the material making up the LM1/JIM12 labelled "sheath" is produced by the endosperm and deposited on the surface of the embryo as it develops.

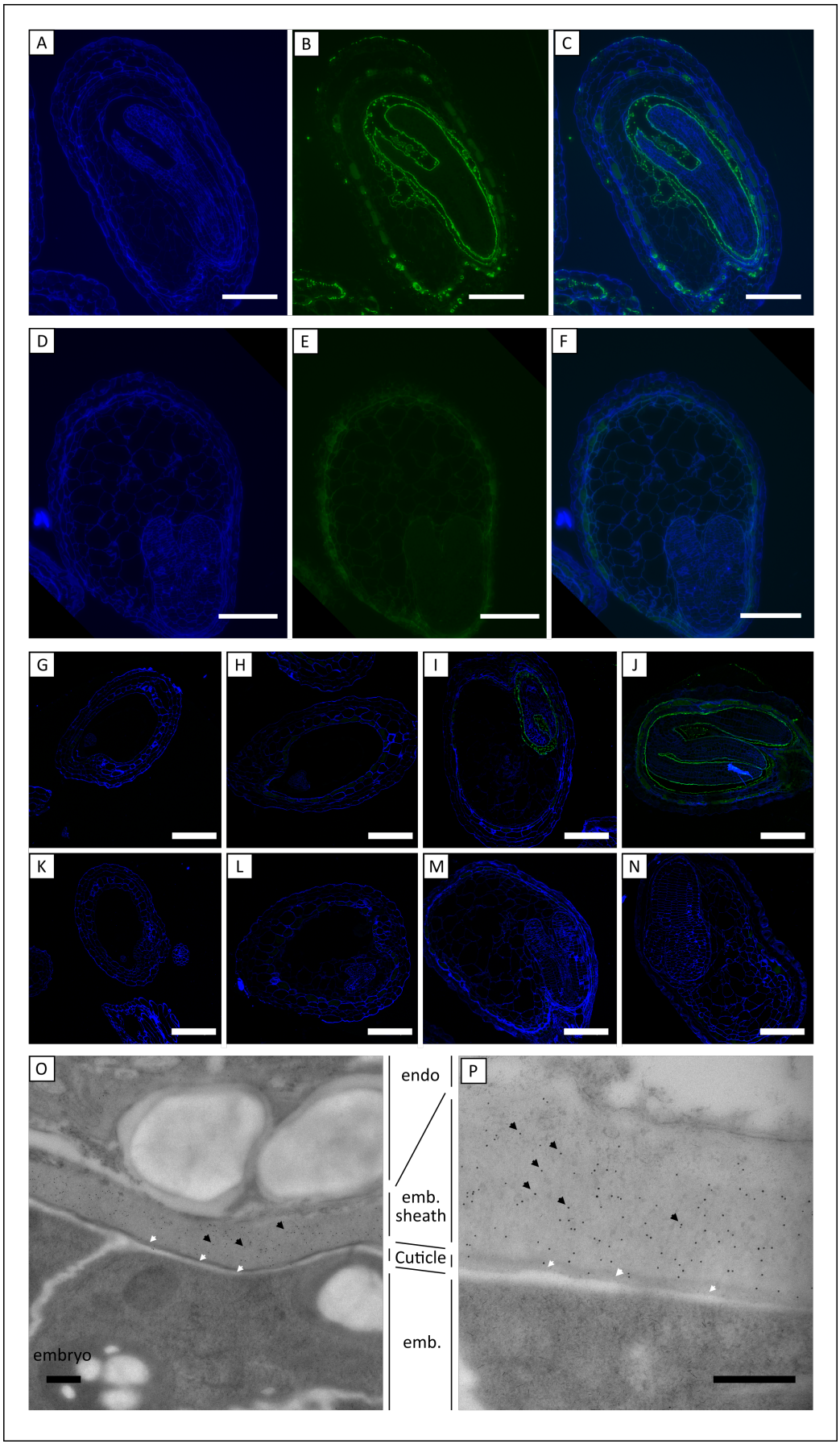


Figure M1: The developing Arabidopsis embryo is surrounded by an extracuticular sheath-like structure, the formation of which i ZOU dependent. (A-N) Fluorescent immunolabelling with the JIM12 antibody. (A-C) Torpedo stage Col-0 seed. (D-F) Torpedo stag *zou-4* seed. (A,D) Calcofluor staining (B,E) JIM12 signal (C,F) Merge. (G-N) Developmental time-course of embryo sheath depositio (G-J) Col-0, (K-N) *zou-4*. Stages: (G,K) Globular stage, (H-L) Heart stage, (I,M) Torpedo stage, (J,N) Fully . Scale bar: 100µm. (O,P TEM images of Immunogold labelling with the JIM12 antibody on torpedo stage Col-0 seeds at the embryo-endosperm interface. Scal bar 100nm. The embryonic cuticle is indicated by white arrows, the embryo and endosperm are labeled. Gold particles are seen a black spots and some are indicated by black arrows.

When immunolocalisations were performed under identical conditions in a *zou-4* mutant background, we were unable to detect labelling with LM1 or JIM12 in either the endosperm or at the embryo surface at any point during seed development (Figure M1D-E,K-N and Supplementary Figure M1), suggesting that the production of the sheath depends upon ZOU function. Furthermore, as previously described we observed no separation of the embryo from the endosperm in *zou-4* mutants.

To ascertain more clearly the relative positions of the embryonic cuticle and the material within the embryonic sheath, we performed immuno-gold labelling experiments on wild-type embryos at the early torpedo stage. These revealed that the JIM12 and LM1 antibodies react strongly with an electron-translucent layer of material that is located between the embryonic cuticle and the endosperm (Figure M1O-P and Supplementary Figure M2), supporting immunofluorescence data, and suggesting that this structure originates from the endosperm.

2.2.4.2 ZOU activity is necessary for the expression of CERBERUS, a unique plant peptide with a C-terminal proline-rich domain

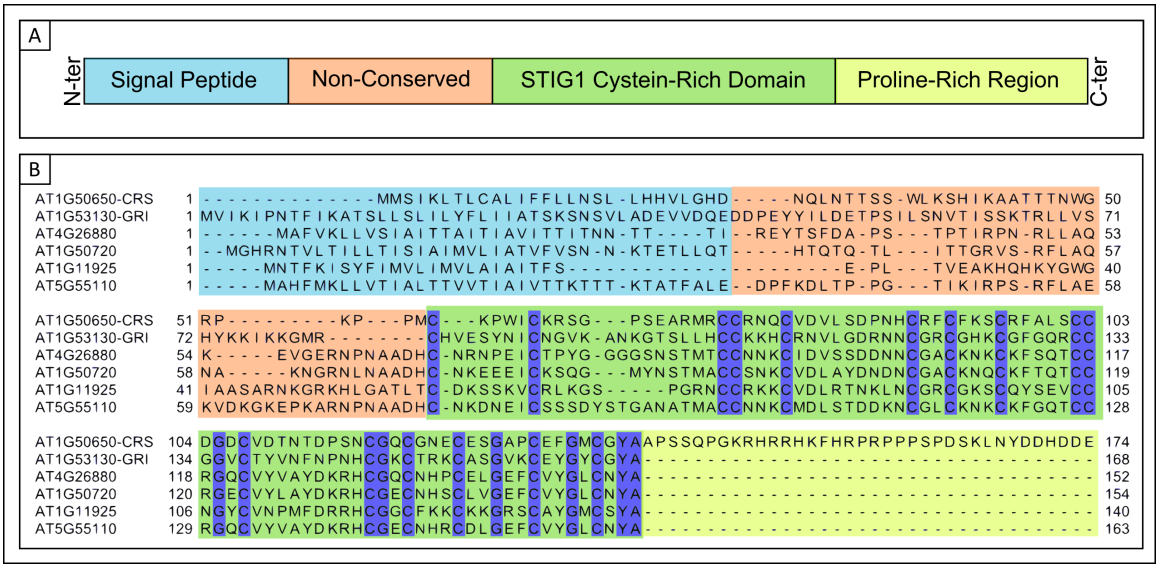


Figure M2: CERBERUS structure and alignment. (A) Subdivision of CRS protein sequence into different domains based on alignment data. (B) Alignment of CRS protein sequence with the other STIG1-Like *Arabidopsis* proteins. The conserved residues in the STIG1 domain are highlighted in dark blue.

Extensins are a class of hydroxyproline-rich glycoproteins (HRGPs) found in plant cell walls (Lamport, 1973; Liu et al., 2016; Tierney and Varner, 1987). Transcriptional analysis of *zou-4* mutant seeds compared to wild-type seeds (Xing et al., 2013) revealed a number of potential ZOU-regulated genes encoding predicted proline-rich secreted peptides. The most strongly down-regulated in this class, *AT1G50650*, encodes a cysteine-rich peptide of the STIG1 family. We named this peptide CERBERUS (CRS) after the mythical guardian of the underworld who separates the dead from the living. STIG1-like peptides are named after the founding member of this protein family STIGMA-SPECIFIC PROTEIN 1, which has been shown to be involved in regulating the production of stigma exudate secretion in tobacco, petunia and tomato (Huang et al., 2014; Tang et al., 2004; Verhoeven, 2005). Based on sequence homologies with the other members of the STIG1 family in *A thaliana*, we defined four different domains composing CRS (Figure M2A). (1) A signal peptide predicted to be necessary for targeting to the secretory pathway; (2) A non-conserved region showing very low similarity with the other family members from *Arabidopsis*; (3) The cysteine-rich STIG1 domain and (4) a domain unique to CRS, harboring a stretch of basic residues followed by a proline enriched sequence (Figure M2B).

To test the evolutionary relevance of the CRS-specific proline-rich region, we performed a phylogenetic analysis on all the STIG1-like peptides from representative sequenced genomes (https://phytozome.jgi.doe.gov/pz/portal.html). Basing the phylogenetic analysis using only the conserved STIG1 domain, the CRS-like peptides (defined by the presence of the C-ter proline-rich sequence) fall into a distinct clade, separate from other STIG1-like proteins found in Arabidopsis and other species (Supplementary Figure M3). Proteins from this clade appear to be present in most eudicots but no CRS-like STIG1-like peptides are found in monocots. Alignment of the CRS-specific C-terminal domain from CRS-like peptides across the eudicots does not show any highly conserved motifs, but this domain always contains an array of several proline residues, and SP motifs, possibly indicating the presence of hydroxyproline substitutions in the mature form of the peptides (Shi et al., 2015).

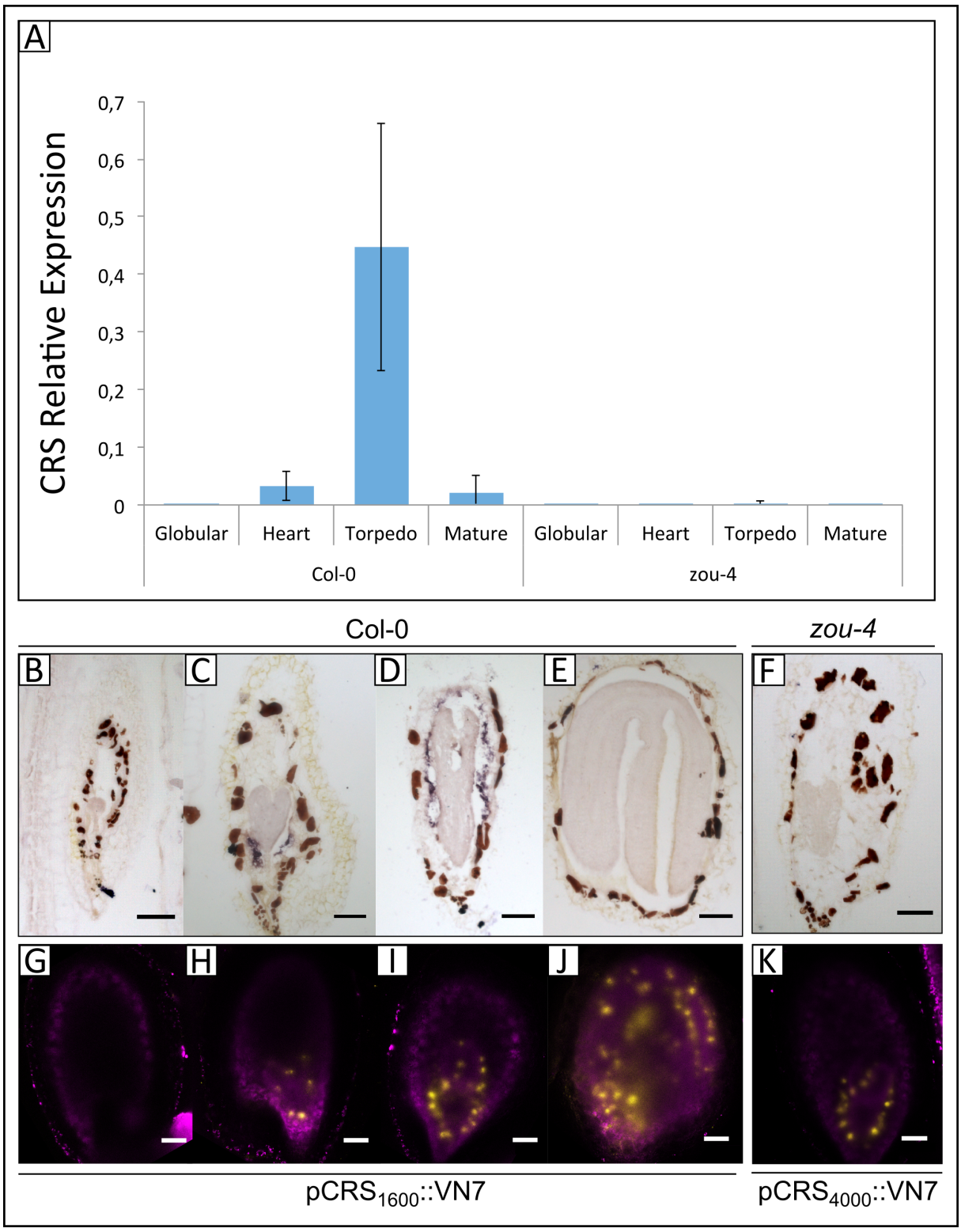


Figure M3: CRS expression analyses: (A) Expression of *CRS* relative to that of *EIF4* in the Col-0 and *zou-4* ; Error bars represent standard errors. Three biological replicates were used. (B-F) *In Situ* hybridization experiments. Developmental stages: (B-E) Col-0, (F) *zou-4* mutant. (B) Globular, (C) Heart, (D,F) Torpedo, (E) Mature. (G-J) Confocal imaging of $pCRS_{1600}$::VN7 and $pCRS_{4000}$::VN7 harbouring seeds. Nuclei are in yellow. Developmental stages: (G) Globular, (H) Late heart, (I,K) Torpedo, (J) Mature. Scale bars : 100µm

We investigated the spatio-temporal pattern of *CRS* expression through *CRS* mRNA and promoter activity studies. Public microarray data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Le et al., 2010) indicates a mainly seed-specific expression for *CRS*, with strong specificity for the endosperm (Supplementary Figure M4). Weak expression is also observed in some root cell types in *in silico* data (Brady et al., 2007). RT-qPCR and *in situ* hybridizations against *CRS* mRNA transcripts confirmed this expression pattern. Temporally, *CRS* expression initiates at heart stage, increases during embryo growth and decreases as the embryo reaches its final size (Figure M3A). Spatially, CRS expression is restricted uniquely to the endosperm cells immediately surrounding the embryo (Figure M3B-D).

CRS promoter activity was studied using either the 1600bp ($pCRS_{1600}$) or the 4000bp ($pCRS_{4000}$) upstream sequences of *CRS* driving the expression of YFP (VENUS) carrying a N7 nuclear localization tag (referred to as VN7). VN7 accumulation in transgenic plants was endosperm specific for both $pCRS_{1600}$*::VN7* (Figure M3G-K) and $pCRS_{4000}$*::VN7* (Figure M3K), suggesting that the 1600bp upstream sequence is sufficient to drive *CRS* expression. The signal was first observed around the base of the embryo at the heart stage and then surrounded the embryo during its growth (Figure M3 G-J). Consistent with *in silico* data we also observed weak expression of the *CRS* promoter in an internal cell layer of the expanding root (Supplementary Figure M5).

The control of *CRS* expression by ZOU, which had previously been suggested by RNA-seq experiments (Xing et al., 2013), was investigated using *zou-4* mutants (Yang et al., 2008). *CRS* transcripts could not be detected in *zou-4* mutants by RT-qPCR during seed development (Figure M3A). Consistent with this result, *in situ* hybridizations with a *CRS* antisense probe detected no transcripts in torpedo stage *zou-4* seeds (Figure M3F). To further understand the regulation of *CRS* expression by ZOU, we tested whether transcription from CRSpro1600 could be activated by direct binding of heterodimers of the ZOU and ICE1 using a Physcomitrella transactivation system as described previously (Denay et al., 2014) for the promoter of the direct ZOU target gene *RGP3*. No transactivation was observed (data not shown). Taken together, these results show that *CRS* expression is restricted in the embryo-surrounding endosperm, starting at the heart stage and peaking at the torpedo stage before disappearing once the endosperm

is lysed. Moreover, consistent with the relatively late onset of CRS expression, our data indicate that although *CRS* expression is dependent upon ZOU activity, *CRS* is probably not a direct target of the ZOU/ICE1 transcription factor complex.

2.2.4.3 CERBERUS is required for normal embryo growth and embryonic cuticle integrity

To investigate *CRS* function in the seed, we generated a series of knock-down and knock-out mutants. A publicly available insertion line with a T-DNA insertion in the *CRS* promoter (Figure M4A) was found to have residual expression of *CRS* in the

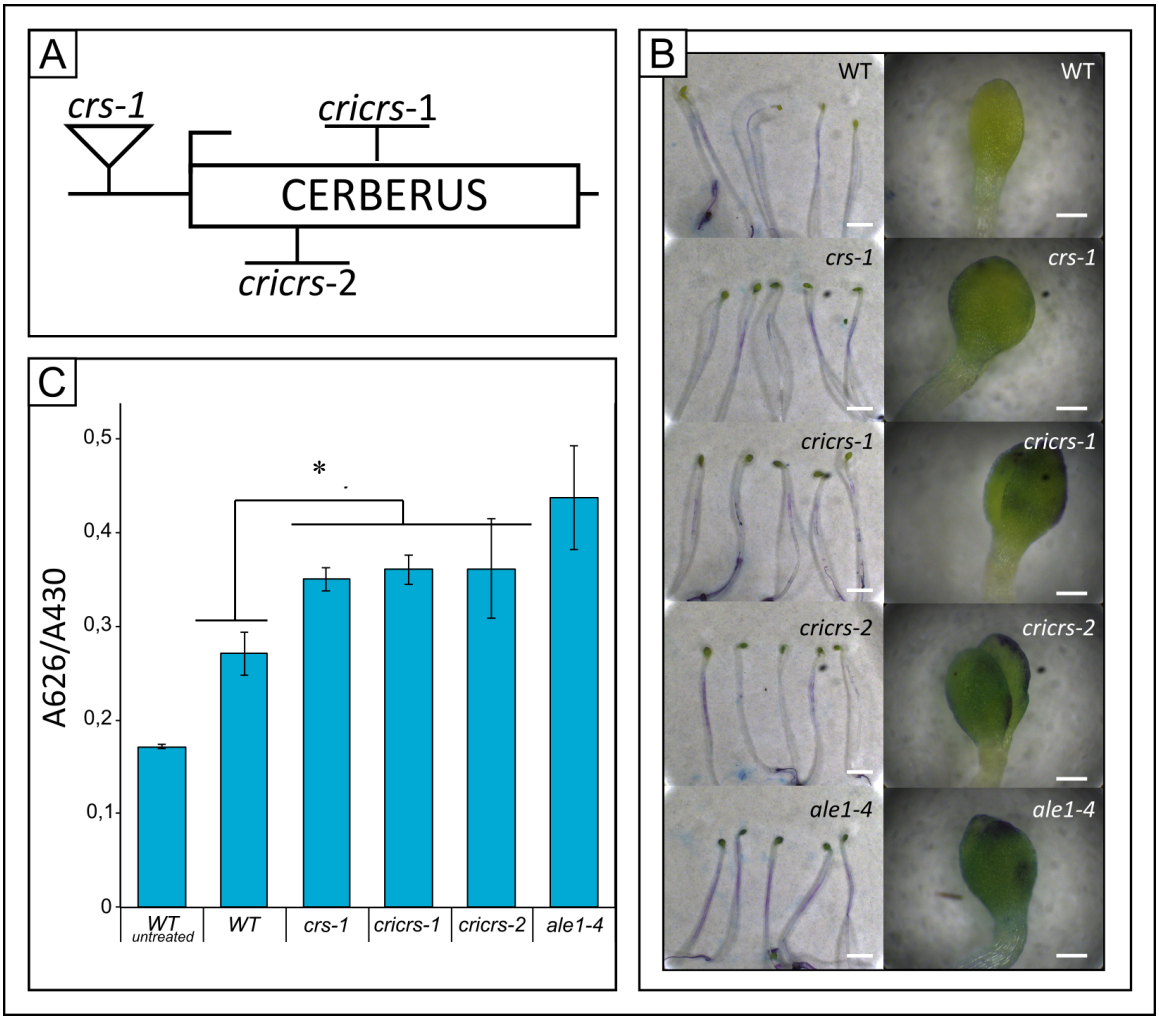


Figure M4: Phenotypic analysis of the effects of loss of CRS function on cuticle permeability. (A) Cartoon representation of the *crs* mutant alelles generated for this study. (B) Evaluation of toluidine blue permeability experiment on etiolated seedlings from *crs* mutant backgrounds. Col-0 is used as a negative control and *ale1-4* as a positive control. (C) Toluidine blue permeability quantification in the mutants. Error bars represent standard deviation From 3 biological replicates. Black star indicates statistically significant difference.

seed (Supplementary Figure M6). We termed this knock-down allele *crs-1*. To generate independent knock-out alleles of *CRS*, we used CRISPR/Cas9 technology to target the *CRS* genomic sequence (Peterson et al., 2016; Schiml et al., 2016). We obtained two independent alleles termed *cricrs-1* and *cricrs-2* corresponding to frame-shift inducing insertions of 1bp in both cases (Figure M4A and We performed both qualitative and quantitative Toluidine Blue (TB) permeability assays on cotyledons to test for cuticle integrity in mutants (Denay et al., 2014; Xing et al., 2013). *ale1-4* seedlings, which are known to be defective in embryonic cuticle formation (Tanaka et al., 2001, 2004), were used as positive controls for TB uptake in these assays. A blue coloration of the yellow etiolated cotyledon indicates a defective cuticle in qualitative assays. After treatment, WT cotyledons were still yellow, whereas *ale1-4* cotyledons were blue/green in colour. *crs-1, cricrs-1* and *cricrs-2* mutants all show a slight blue coloration (Figure M4B). Toluidine blue uptake quantifications were consistent with these observations, showing significant differences in uptake between WT cotyledons and all *crs* alleles (Figure M4C). All *crs* alleles showed the same level of TB uptake, with a slightly stronger effect observed in *ale1-4*, suggesting that CRS, like ALE1, is necessary for embryonic cuticle integrity. Supplementary Figure M7). Mutants defective in embryonic cuticle formation such as *ale1-4* and *gso1-1 gso2-1* have been reported to show an unusual seed shape phenotype (Xing et al., 2013). These phenotypes are thought to be due to abnormal adhesion between the embryo and the endosperm/testa during seed development (Tsuwamoto et al., 2008). Consistent with their weak cuticle defect, *crs* mutants had a low penetrance seed shape phenotype (Figure M5E). 3,5% (+/- 1,2%; n_{tot}=1538) of the seeds of *crs-1* were misshapen compared to 0,06% (+/- 0,11) (N=1614) in wild-type. The CRISPR alleles *cricrs-1* and *cricrs-2* both produced misshapen seeds at a respective frequency of 4,5% (+/- 0,2%; n_{tot}=1314) and 3,7% (+/- 0,6%; n_{tot}=1280). They are therefore equivalent alleles in terms of seed shape phenotype penetrance. The seed-shape phenotype in *crs* mutants is not identical to that in *gso1-1 gso2-1* mutants. Most misshapen seeds in *crs* mutants have a twisted appearance almost as if the embryo has been “knotted” (Figure M5A-B). Clearing of developing seeds showed that these phenotypes are due to an apparent adhesion of the embryo to the endosperm/testa in the zone of the seed furthest from the micropyle, which then forces the embryo to elongate into a looped structure (Figure M5C-D). Although this

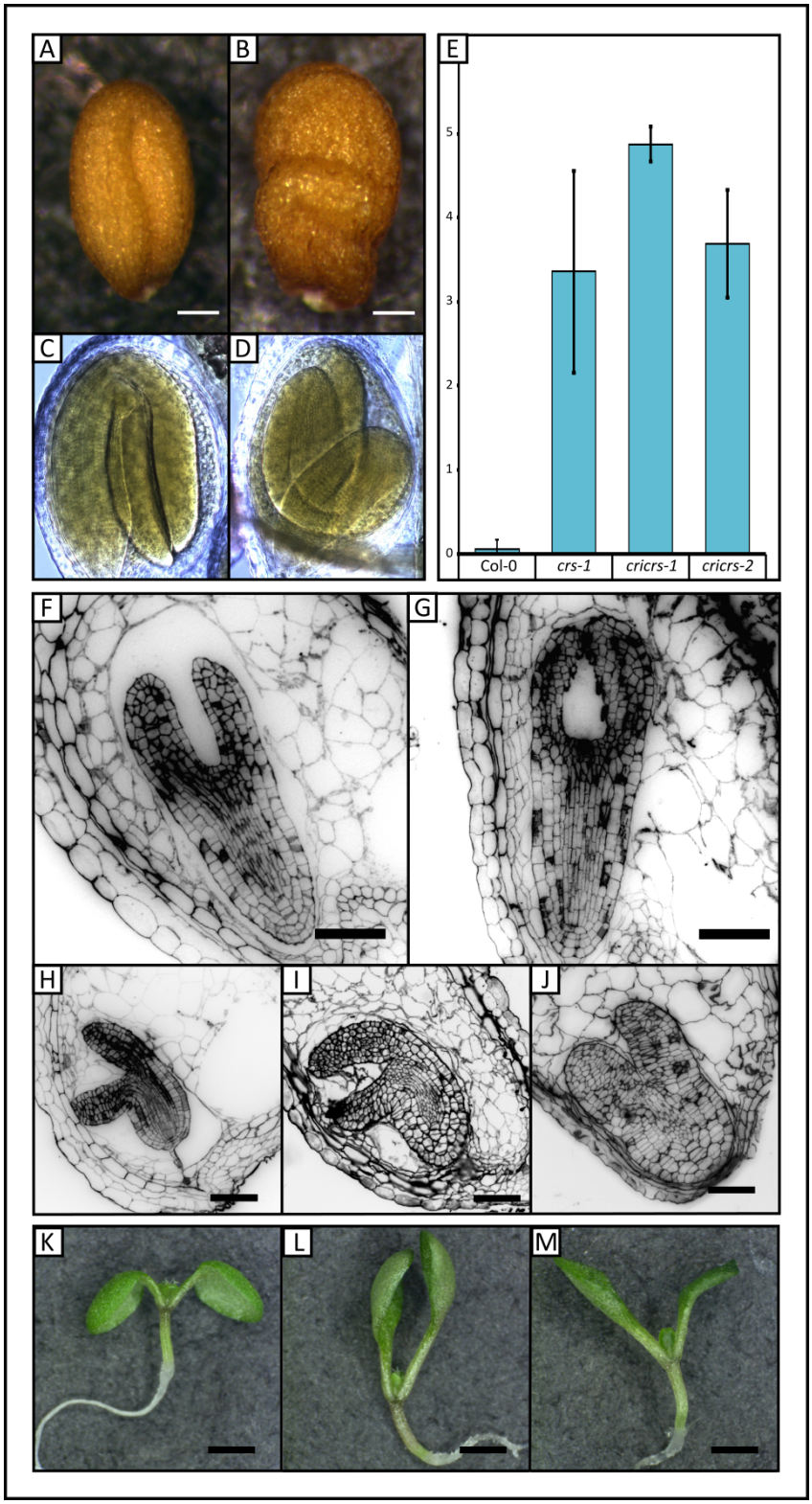


Figure M5: Seed shape and endosperm phenotypes caused by loss of *CRS* function. (A) Col-0 mature seed. (B) Knotted *cricrs-1* mature seed. (C-D) Clearings of developing seeds showing equivalent phenotypes (C) Col-0 seed, (D) *cricrs-1* seed. (E) Penetrance of the seed shape phenotype: % of misshapen seeds observed in the different *crs* mutants analysed in this work, and in wild-type plants. (F-J) Endosperm/embryo adhesion visualized with thin sections stained with calcofluor. (F) Col-0, (G) *cricrs-1*, (H) *ale1-4,* (I) *gso1 gso2* and (J) *zou-4.* Scale bar = 50µm (K-M) Seedling cotyledon cupping phenotype. (K) Col-0, (L) *cricrs-1*,(M) *ale1-4*. Scale bar: 2mm.

phenotype is not observed in *gso1-1 gso2-1* mutants, a small proportion of *ale1-4* seeds show the same "knotted" phenotype. The position of the embryo adhesion to surrounding tissues suggests that it occurs later in *crs* seeds than in those of *gso1-1 gso2-1* and the majority of *ale1-4* mutant seeds.

To understand the seed phenotype of *crs* mutants in more detail we fixed and sectioned developing seeds. In wild-type seeds, from the late heart stage, a gap is found around the embryo, caused by the physical separation of the embryo from surrounding tissues (Figure M5 F). This gap was not visible in *crs* mutants (Figure M5G), or in *zou-4* and *gso1-1 gso2-1*. In *ale1-4* mutants the gap is also less obvious than in wild-type seeds (Figure M5H-J) These results support the idea that that the seed shape defects in *crs* mutants, as in *gso1-1 gso2-1*, *zou-4* and *ale1-4* mutants, are likely due to abnormal adhesion of the embryo to surrounding tissues. Like *gso1-1 gso2-1* and *ale1-4* mutants, *crs* mutants show no persistent endosperm phenotype at seed maturity.

Interestingly, *crs* mutants also show a strong cotyledon phenotype post germination. Cotyledons tend to be cup-shaped, is if the borders of the cotyledon are unable to expand correctly (Figure M5K-L). This phenotype is observed in 37,5% of *cricrs-1* seedlings at 7 days after sowing (n=300). A similar phenotype is has been observed in *gso1 gso2* mutant seedlings (Tsuwamoto et al., 2008), and was observed in *ale1-4* seedlings (Figure M5M), although at a lower frequency (21,2% n=300).

2.2.4.4 Embryo sheath labelling with JIM11/LM1 is dependent upon the production of CRS.

Because *CRS* encodes a peptide with the potential to encode a hydroxyproline-containing protein, we tested whether the reactivity of the embryo sheath to JIM12 is altered in *crs* mutant seeds. We found that JIM12 labelling of the embryo sheath was dramatically reduced, although not completely eliminated in all *crs* alleles tested, suggesting that CRS likely contributes either directly, or indirectly to the production of the epitope recognised by JIM12 in the embryo sheath (Figure M6).

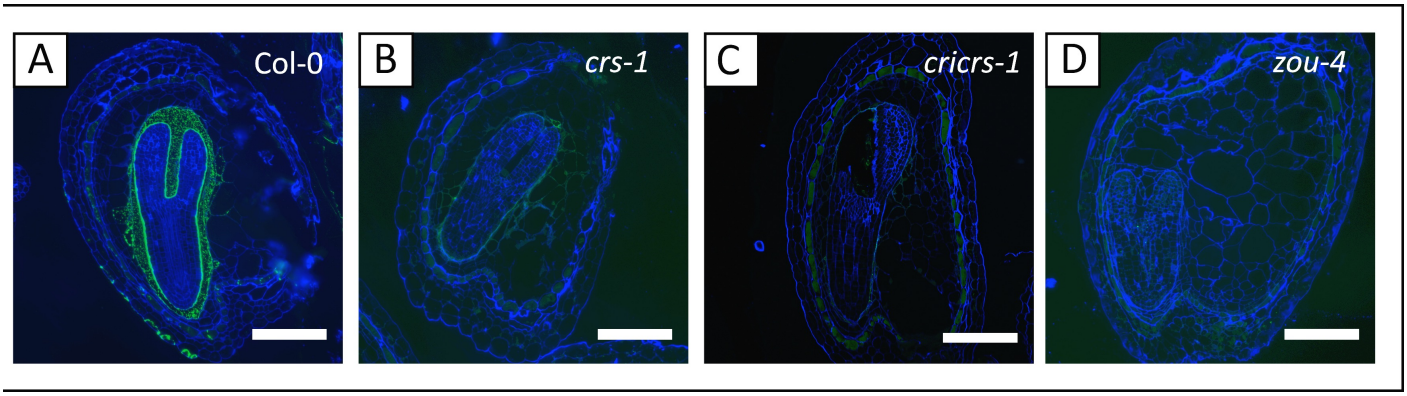


Figure M6: CRS is required for normal production of the embryonic sheath. JIM12 immunolabelling of torpedo stage seed sections. (A) Col-0, (B) *crs-1*, (C) *cricrs-1*, (D) *zou-4* Scale Bar: 100µm.

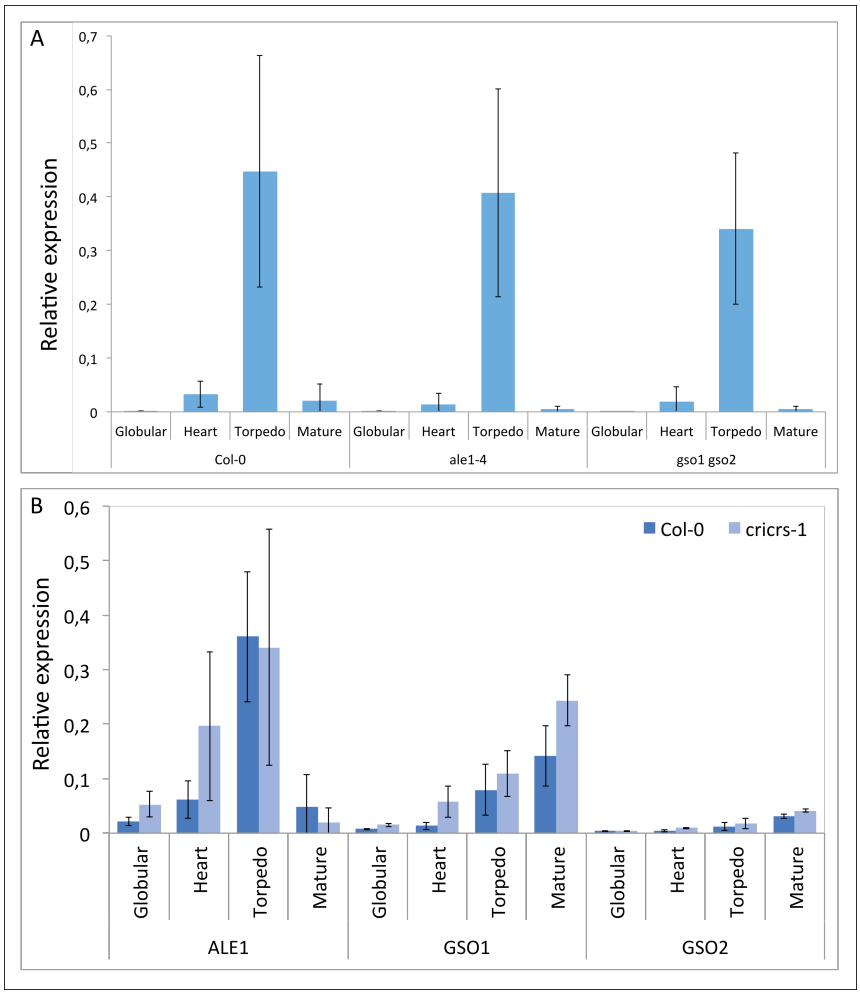


Figure M7: The expression of *CRS* is not affected by loss of *ALE1*, *GSO1* and *GSO2* function and *vice versa.* Expression levels are shown relative to *EIF4* expression. (A) *CRS* expression in Col-0, *ale1-4* and *gso1 gso2* backgrounds at various developmental stages (globular, heart, torpedo, mature). (B) *ALE1*, *GSO1* and *GSO2* expression in Col-0 and *cricrs-1* mutants. Error bars represent standard errors. Three biological replicates were used.

2.2.4.5 CRS is not transcriptionally regulated by the ALE1/GSO1/GSO2 signalling pathway.

The embryo adhesion and cuticle integrity phenotypes of *crs* mutants suggest that *CRS* could acts in the same genetic pathway as *GSO1/GSO2* and *ALE1*. To test whether *CRS* expression is dependent upon the activity of *ALE1, GSO1* and *GSO2* we measured the expression levels of *CRS* in *ale1-4* and *gso1-1 gso2-1* mutant seeds Figure M7A. Consistent with previous transcriptome results (Xing et al., 2013), we found that *CRS* expression is dependent upon the activity of neither ALE1 nor GSO1 and GSO2. Furthermore, the expression of *ALE1, GSO1* and *GSO2* is unaltered in *crs* mutants (Figure M7B). These results suggest that *CRS* does not form part of a transcriptional cascade downstream of ALE1 GSO1 GSO2 signalling. To further understand the implication of *CRS* in the ALE1/GSO1/GSO2 signalling pathway, we generated triple *gso1-1 gso2-1 cricrs-1* mutants, which showed identical seed shape and cuticle permeability phenotypes to *gso1 gso2* double mutants. Interestingly however double *cricrs-1 ale1-4* mutants that showed a seemingly additive phenotype, with a higher proportion of misshapen seeds than observed in either of the two single mutants, suggesting that they may act in parallel in this signalling pathway (data not shown).

2.2.4.6 GSO1, GSO2 and ALE1 affect the deposition of the embryo sheath, but not the production of JIM12/LM1 epitopes in the endosperm.

To further understand the relationship between ALE1 GSO1 GSO2 signalling and CRS function, we carried out immunolocalisation on *gso1-1 gso2-1* and *ale1-4* mutant seeds. Unlike the situation in *zou-4* mutants, which entirely lack labelling with JIM12/LM1 in the zygotic compartment, and *crs* mutants, in which the intensity of this labelling is very strongly attenuated, we found that *gso1-1 gso2-1* and *ale1-4* mutant seeds showed strong JIM12 labelling in the endosperm surrounding the embryo. In *ale1-4* mutant seeds labelling of the embryo sheath was similar to that observed in wild-type, although it was occasionally patchy, especially in the cotyledons, whilst in *gso1-1 gso2-1* mutant seeds, no labelling at all could be detected at the embryo surface, and signal remained entirely localised within the cells of the embryo surrounding

endosperm, within an apparently vesicular compartment. These results suggest that GSO1 and GSO2 function to mediate the deposition of the embryo sheath on the embryonic surface.

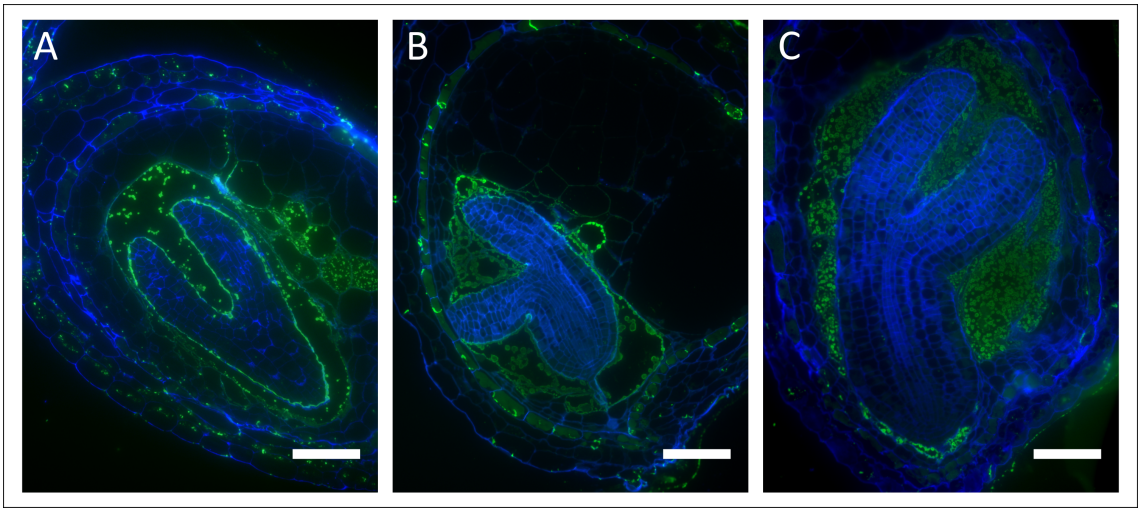


Figure M8: Embryo sheath material is not deposited normally on the surface of *gso1 gso2* mutant embryos. JIM12 immunolabelling (green) in (A) Col-0, (B) *ale1-4* (C) *gso1 gso2*. Scale bar: 50µm. Calcofluor labeling is shown in blue.

2.2.5 Discussion

Here we present ultrastructural, phenotypic and genetic data showing that the unique plant-specific peptide CERBERUS plays an important role in the formation of a previously undescribed structure, which we have called the embryo sheath, at the embryo surface. Our results suggest that CRS is necessary for the production of a component of this structure that reacts strongly with the "anti-extensin" antibodies JIM12 and LM1. The fact that CRS contains potential sites of proline hydroxylation in its unique C-terminal domain is consistent with the hypothesis that CRS could itself be detected by JIM12 and LM1 in the embryo sheath. Furthermore, our results show perfect correlation between the expression of functional CRS (spatially, temporally, and in the various mutant backgrounds investigated) and the presence of JIM12/LM1-reactive material in the developing endosperm. However formal proof that CRS is indeed the target of JIM12/LM1 in seeds remains lacking to date.

The presence of JIM12/LM1 reactive material at the wild-type embryo surface has been previously described at Arabidopsis embryo maturity (Lee et al., 2012b) and is intriguing in the light of our results as it suggests that material is actively secreted by the endosperm onto the embryo surface. Ultrastructural studies of the developing seeds

in *Solanum* and maize have produced images suggesting of active and directional secretion of material from the endosperm via large vesicles (Briggs, 1993, 1996; Schel et al., 1984), although the identity of this material remains unclear. Interestingly, in their highly informative study comparing cell wall composition in the mature seeds of Arabidopsis, a close relative *Lepidium campestre*, and the much more distantly related angiosperm tobacco, Lee *et al* noted that although LM1 detected an epitope specifically at the embryo surface in all three species, labelling with JIM12 was specific to Arabidopsis, suggesting that these two antibodies detect non-identical epitopes (Lee et al., 2012b).

Based on the presence of a clearly defined predicted secretion signal, CRS is proposed to encode a secreted protein, and another family member, the tomato STIG1 protein, has been shown to be secreted from stigmatic tissues and to bind to the surface of invading pollen tubes (Huang et al., 2014; Tang et al., 2004). Interestingly STIG1 appears to be processed during or after secretion in tomato, petunia and tobacco, so that in addition to the N-terminal secretion signal, the non-conserved domain of the protein is lost leading to the production of a mature peptide consisting of the conserved cysteine rich STIG1 domain (Huang et al., 2014; Verhoeven, 2005). Although loss of function of STIG1 leads to an increased production of stigmatic exudate, the expression of STIG1 appears to stimulate pollen tube growth *in vivo*. This is likely not simply a mechanical effect of exudate quantity, since a recent detailed study has shown that the bacterially expressed STIG1 domain of the STIG1 protein is sufficient to stimulate pollen tube growth *in vitro* (Huang et al., 2014; Tang et al., 2004). Importantly this stimulation is dependent upon the expression of the POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 2 LePRK2, the extracellular domain of which directly binds the STIG1 protein leading to increased REDOX-potential in growing pollen tubes. Thus the STIG1 domain is likely a functional ligand for LePRK2. Interaction of STIG1 with the extracellular domain of LePRK2 has been shown to depend upon specific amino acids within the conserved cysteine rich domain of STIG1. The STIG1 domain of tomato STIG1 also mediates interaction with Phosphatidylinositol 3-Phosphate at the pollen-tube surface, an interaction that is also required for the stimulation of pollen tube growth (Huang et al., 2014).

Interestingly, another member of this class of peptides, GRIM REAPER (GRI) has also been shown to act as a ligand for an RLK, although in this case an 11 amino acid peptide produced by AtMETACASPASE-9-mediated processing of the non-conserved N-terminal domain that was shown to bind the extracellular domain of the Arabidopsis POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5) (Wrzaczek et al., 2015). This association triggers ion leakage and cell death. Importantly, the PRK5 ligand domain of GRI is not conserved either in CRS or other Arabidopsis family members.

The fact that both STIG1 and GRI are processed to form RLK ligands raises the question of whether CRS, or a processed product of CRS, could also act as an RLK ligand. An obvious possibility is that CRS could act as a ligand in the signalling pathway comprising the RLKs GSO1/GSO2 and the subtilisin protease ALE1. The possibility that CRS acts as a ligand for GSO1 and GSO2, and/or that CRS is an ALE1 substrate cannot be excluded. However, the expression of *CRS* initiates relatively late in seed development compared with that of *ALE1*. More importantly, we found that the phenotypic consequences of loss of *CRS* function, in terms of production of embryonic sheath material, are not identical to those of *ale1* and *gso1 gso2* mutants. Importantly, we found that CRS was involved in the production of JIM12 antigen in the endosperm, whereas ALE1 and GSO1/GSO2 are not involved in this process. In this context it is particularly interesting to note the high penetrance of the "cupped cotyledon" phenotype in *crs* mutants compared to ale1 mutants. This phenotype may be a consequence of the abnormal adhesion of endosperm material to the surface of the cotyledons (as previously observed in *zou* mutants), which could mechanically constrain the expansion of the cotyledon boundaries (Yang et al., 2008). The fact that *crs* mutants show a significantly stronger cotyledon cupping phenotype than *ale1* mutants correlates with the stronger sheath and separation phenotype observed in *crs* mutants at the cytological level. Consistent with this idea the cotyledon cupping phenotype is also prevalent in *gso1 gso2* double mutants in which the release of sheath materials onto the embryonic surface is defective. Taken together with the seemingly additive genetic interaction observed between *crs* and *ale1* mutants, from the point of view of embryo sheath formation, it therefore seems likely that CRS acts essentially in parallel with ALE1, GSO1 and GSO2. How the process of cuticle deposition (which is initiated well before CRS expression initiates) affects, or is affected by sheath formation, remains an important subject for further investigations.

Interestingly, although the expression of *CRS* and *ALE1* in the developing endosperm is mutually independent, the expression of both genes depends upon the activity of the ZOU/ICE1 transcription factor complex in the seed (Yang et al., 2008). We have, however, not been unable to show direct binding of ZOU/ICE1 heterodimers to the promoters of either *CRS* or *ALE1* (unpublished data), suggesting that their regulation is likely indirect. For *ALE1* this idea is further supported by weak expression observed during very early seed development in *zou* mutant seeds, at stages before ZOU protein fusions can be detected in wild-type seeds (Xing et al., 2013; Yang et al., 2008). Such expression is not observed for the direct ZOU target RGP3, expression of which is never detected in the endosperm of *zou* mutants (Denay et al., 2014; Fourquin et al., 2016). In the case of *CRS,* expression of this gene in the root, where ZOU is never expressed (Yang et al., 2008), supports the idea that regulation is not directly ZOU dependent. The basis for ZOU/ICE1 regulation of the expression of these genes thus also remains to be resolved, although it is tempting to speculate that this could be associated with "cell wall thinning" functions of ZOU in the endosperm which eventually lead to endosperm disintegration (Fourquin et al., 2016).

2.2.6 Materials and Methods.

2.2.6.1 Plant materials and growth conditions

All plant materials used in the project were in the Columbia (Col-0) background. Several genotypes described in the work have been previously published as follows: *zou-4* (Yang et al., 2008), *ale1-4* (Xing et al., 2013), *gso1-1 gso2-1* (Tsuwamoto et al., 2008), *ice1-2* (Denay et al., 2014). *crs-1* is a T-DNA insertion allele (GABI_824G07) from the GABI-Kat collection (Kleinboelting et al., 2012) and was obtained from the Nottingham Arabidopsis Stock Centre (NASC). CRISPR alleles of *CRS* were obtained as described below. Genotyping of *crs* mutants was carried out using primers listed in Supplementary Table 1)

Unless otherwise specified, chlorine gas-sterilized seeds were sown on Murashige and Skoog (MS) agar plates with 0.5% sucrose, stratified for 2 days in the dark at 4°C, grown under long day conditions (21°C, 16h light/8h dark) for 10 days, and then transferred to soil under standard long-day conditions (21°C, 16h light/8h

dark). To obtain synchronous seed material, newly opened flowers were marked with threads each day for two weeks.

For toluidine blue staining of etiolated seedlings, chlorine gas-sterilized seeds were spread uniformly on 15 cm MS plates with 0.5% sucrose and 0.4% Phytagel (Sigma) (pH 5.8) and stratified for 2 days in the dark at 4°C. After stratification seeds were transferred to a growth chamber and incubated for 6h under continuous light followed by 4 days in the dark. For quantified toluidine blue assays, seedlings were not returned to the dark, and were grown for 10 days prior to assaying (as described in (Xing et al., 2013).

2.2.6.2 Generation of new CRS alleles using CRISPR/Cas9 technology.

Independent CRISPR edited *crs* alleles were generated using two different strategies. The *cricrs-1* allele was generated using the protocol and vectors described by by Schiml et al., 2016, while *cricrs-2* was generated using the protocol and vectors described by Peterson et al., 2016. For *cricrs-1, pDE-CAS9-cricrs1*, the CRISPR-Cas9 containing vector used to induce the mutation was generated as follows. The 5'-CAAACGGTCCGGACCATCAG-3' PAM sequence was cloned by restriction into the *pEN*-Chimera vector, giving the *pEN-Chimera-cricrs1* vector. Gateway LR recombination was then performed using *pEN-Chimera-cri1* and *pDE-Cas9*, to produce the plant transformation vector *pDE-Cas9-cricrs1*. For *cricrs-2,* the 5'-ACTTAACACAACCTCGTCA-3' PAM sequence was cloned in the *pMOA33* vector, resulting in the plant expression *pM3U-cricrs2* vector. Both vectors were transformed into the Col-0 background. Primary transformants were generated and screened for the presence of gene editing by amplifying *CRS* genomic DNA with CRS_F and CRS_R, and sequencing the resulting PCR products using the using the CRScriSeq primer. Homozygous plants were identified in the T2 generation using the same technique. Primer sequences are shown in supplementary Table 1. To remove the Cas9 encoding cassette, homozygous plants were backcrossed to Col-0, and homozygous mutants lines lacking the *Cas9* cassette were identified by lack of resistance to appropriate antibiotics. The Cas9 cassette absence was checked for by PCR using the primer SS42 and SS43 (Supplementary Table 1)

2.2.6.3 Generation of other transgenic lines.

The $pCRS_{1600}$::VN7 and $pCRS_{4000}$::VN7 lines were generated in the Col-0 background. The expression vector containing the $pCRS_{1600}$::VN7 and $pCRS_{4000}$::VN7

constructs were produced as follows. The 1600bp and 4000bp upstream sequences of CRS were amplified using the primers listed in supplementary Table 1. They were cloned by restriction in the pENTR5'-MCS vector. Triple LR reactions were performed using the pENTR5'-pCRS$_{1600}$ or pENTR5'-pCRS$_{4000}$ in combination with pENTRgene-VENUS-N7, pENTR3'-Mock and the pBART (Gleave, 1992) destination vector. The resulting plant expression vectors were transformed into plants using Agrobacterium-mediated plant transformation using the floral dip method (Logemann et al., 2006) , and at least 4 independent transformation events were analysed for each line.

2.2.6.4 In situ hybridization.

DNA template was amplified from CRS gDNA using CRS_F and CRS_R_+ primers and subsequently cloned into pTOPO ZeroBlunt vector (Invitrogen). The *CRS* antisense probe was amplified from genomic DNA using CRS_F and SP6 primers (Supplementary Table 1). Digoxigenin-labelled RNA probes were produced and hybridized to tissue sections following standard procedures. Briefly, siliques were opened, fixed overnight in ice-cold PBS containing 4% paraformaldehyde, dehydrated through an ethanol series and embedded in Paraplast Plus (Mc Cormick Scientific). 8 mm sections were cut and immobilized on coated slides (Menzel-Gläzer SUPERFROST ULTRA PLUS^{R} (Thermo Scientific). Sections were dewaxed and hydrated, treated with 2x saline sodium citrate (20 min), digested for 15 min at 37°C with proteinase K (20 mg/ml) in 50mM Tris-HCl, pH 7.5, 5mM EDTA), treated for 2 min with 0.2% glycine in PBS, rinsed, postfixed with 4% paraformaldehyde in PBS (10 min, 4°C), rinsed, treated with 0.25% w/v acetic anhydride in 100mM triethanolamine (pH 8.0 with HCl) for 10 min, rinsed and dehydrated. Sections were then hybridized under coverslips overnight at 50°C with RNA probes (produced using DIG RNA labelling kit (Roche)) diluted in DIG easy Hyb solution (Roche) following the manufacturer's instructions. Following hybridization, the slides were extensively washed in 0.1x saline sodium citrate and 0.5% SDS at 50 °C (3 h), blocked for 1 hour in 1% blocking solution (Roche) in TBS and for 30 minutes in BSA solution (1% BSA,0.3% Triton-X-100, 100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$), and then incubated in a 1/3000 dilution of in alkaline phosphatase-conjugated antidigoxigenin antibody (Roche) in BSA solution for 2 h at RT. Sections were extensively washed in BSA solution, rinsed

and treated overnight in the dark with a buffered NBT/BCIP solution. Samples were rinsed in water before air drying and mounting in Entellan (Sigma).

2.2.6.5 Microscopy

Developing live seeds were imaged by opening siliques and removing the replum (with seeds) into a drop of water. Seeds were then gently covered with a coverslip. Roots were imaged by colouring with Propidium iodide (1mg/mL) (Sigma) and mounting in water. Embryos were imaged by gently bursting seeds between slide and cover-slip in water. Confocal imaging was carried out using a Zeiss LSM700 and a LSM710. Light/fluorescence microscopy imaging was carried out using a Zeiss axioimager 2. Pictures of dry seeds and seedlings were obtained using a Leica MZ12 dissection microscope fitted with an AxioCamICc5.

2.2.6.6 Quantitative gene expression analysis

Intact siliques were frozen in liquid nitrogen and total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma). Total RNAs were digested with Turbo DNA-free DNase I (Ambion) according to the manufacturer's instructions. RNA was reverse transcribed using the SuperScript VILO cDNA synthesis Kit (Invitrogen) according to the manufacturer's protocol. PCR reactions were performed in an optical 384-well plate in the QuantStudio 6 Flex System (Applied Biosystems), using FastStart Universal SYBR Green Master (Rox) (Roche), in a final volume of 10 µl, according to the manufacturer's instructions. The following standard thermal profile was used for all PCR reactions: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Data were analysed using the QuantStudio 6 Flex Real-Time PCR System Software (Applied Biosystems). As a reference, primers for the *EIF4A* cDNA were used. PCR efficiency (E) was estimated from the data obtained from standard curve amplification using the equation $E=10^{-1/slope}$. Expression levels are presented as $E^{-\Delta Ct}$, where $\Delta Ct=Ct_{GOI}-Ct_{EIF4A}$. Primers are listed in Supplementary Table 1. All Q-RT-PCR experiments were performed using three independent biological replicates.

2.2.6.7 Immunofluorescent labelling of seeds.

Single seeds were fixed in ice cold PEM buffer (50 mm PIPES, 5 mm EGTA, and 5 mm MgSO4, pH 6.9) containing 4% (w/v) paraformaldehyde. Samples were placed under vacuum (3 x 30 minutes on ice), rinsed in PBS, dehydrated through an ethanol series, and infiltrated with increasing concentrations of LR White resin in absolute ethanol (London Resin Company) over 8 days before being polymerized in

100% resin in Beem capsules (Electron Microscopy Sciences) at 60°C. 1.0 µm sections were cut using a glass knife on a Leica RM6626 microtome. Sections were incubated in PBS containing 3% (w/v) milk protein and a 10-fold dilution of antibody hybridoma supernatant (Molecular probes) for 1 h, washed and incubated with a 100-fold dilution of fluorescein linked secondary antibody for 1 h in the dark. Samples were washed and counterstained with filtered Calcofluor White M2R (fluorescent brightener 28; Sigma-Aldrich) at 0.25 µg mL−1 and mounted in VECTASHIELD (Eurobio).

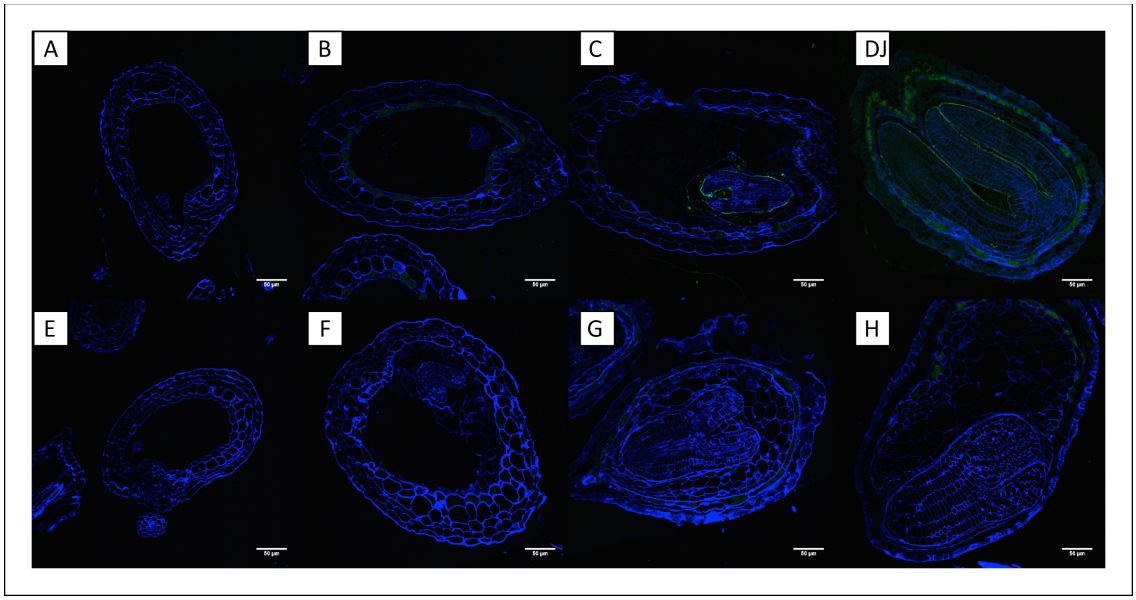
2.2.6.8 Immunogold labelling

Single seeds were fixed by high-pressure freeze-substitution using an EM PACT1 device (Leica Microsystems) with a carrier depth of 200µm. Single seeds were dissected, deposited in the carrier filled with BSA 20% (w/v) in 0.5X MS and immediately frozen. Freeze-substitution was carried out in acetone containing Glutaraldehyde 0,5%, Uranyl acetate 0,1% and OsO_4 2% at -90°C for 48h followed by a temperature ramp of 3°C per hour to -50°C. Samples were washed 3 times with pure acetone and 3 times with pure ethanol. Samples were progressively embedded in Lowicryl HM20 resin at -50°C (2h at 25%, 2h at 50%, overnight at 75%, 3x2h at 100%). Polymerisation was carried out progressively under UV illumination for 48h at -50°C followed by 48h 20°C. Ultra-thin (90nm) sections were made with a Leica UC7 ultramicrotome and placed on grids. Grids were placed on 30µL drops of filtered water (for 2 x 5 minutes), and then PBSTB (PBS1X, 0.2%Tween, BSA 1%) for 15 mins. Grids were then transferred to 20µL drops of PBSTB containing a 1:10 dilution of primary antibody for 1h. Grids were washed on 30µL drops of PBSTB (for 4 x 5 minutes). Grids were then transferred to 20µL drops of PBSTB containing a 1:30 dilution of 10 nm gold-linked anti-rat secondary antibody (Tebu) for 1h. Grids were washed on 30µL drops of PBSTB (for 4x5 minutes). Grids were then transferred to 20µL drops of 0.1% Glutaradehyde for 1 min before washing on 30µL drops of filtered water (for 4x5 min). Grids were imaged at 120kV using an FEI TEM tecnai Spirit with 4kx4k eagle ccd.

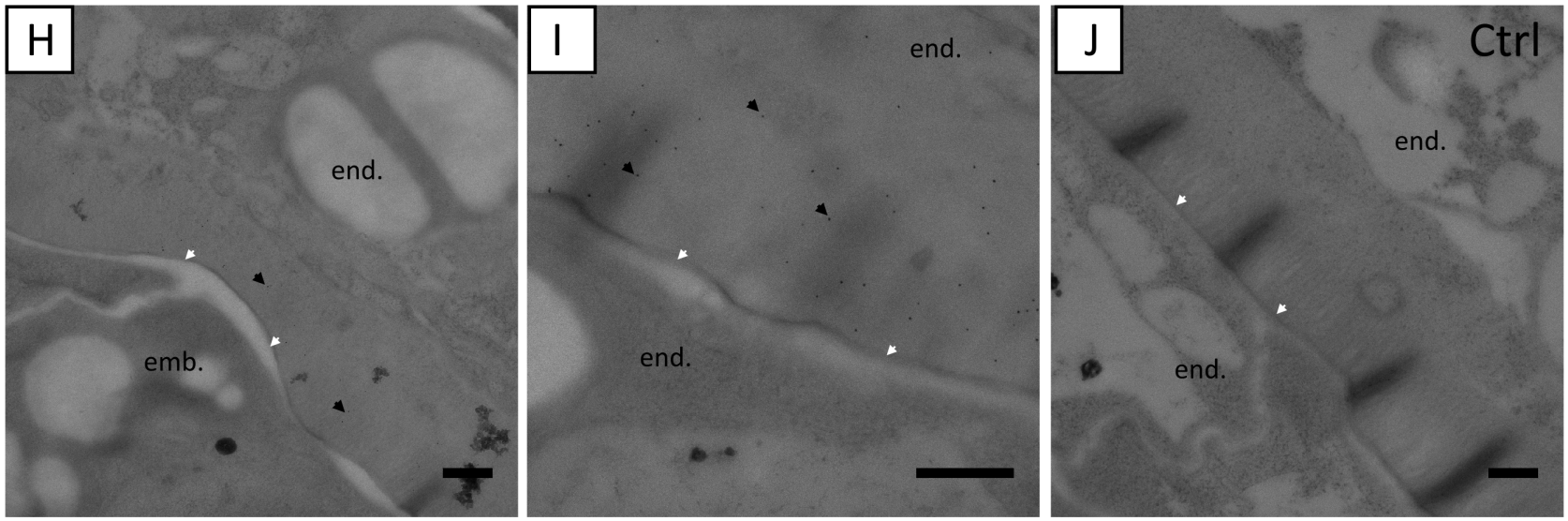
2.2.7 Acknowledgements

We would like to thank the European Arabidopsis Stock Centre (NASC) for providing seed and the plant culture and the logistics and secretarial teams at the RDP for their support. This work was financed by a grant from the French National Research Agency (ANR-13-BSV2-0002, INASEED) and a doctoral grant from the Rhône-Alpes region.

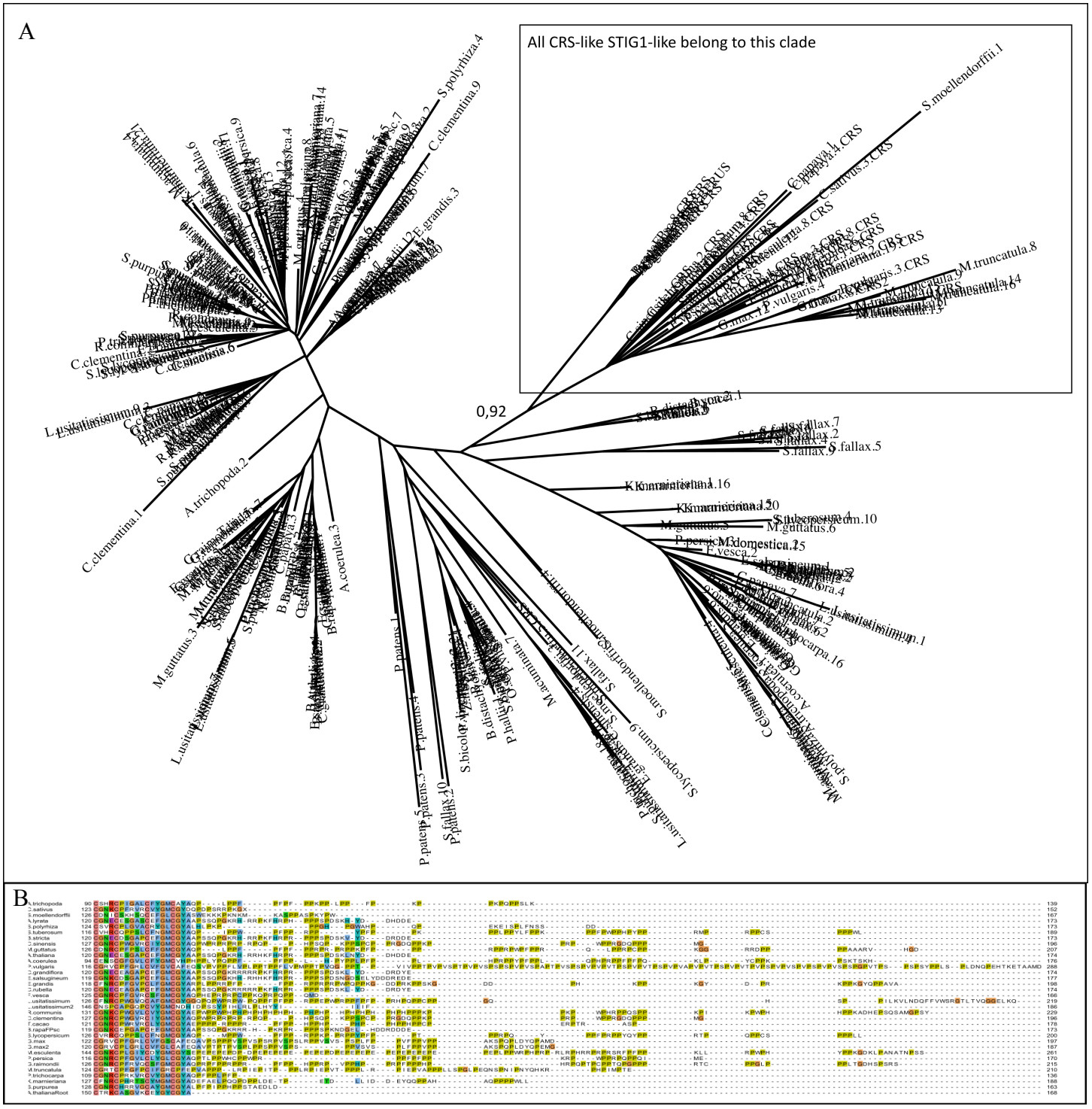
2.2.8 Supplementary Figures



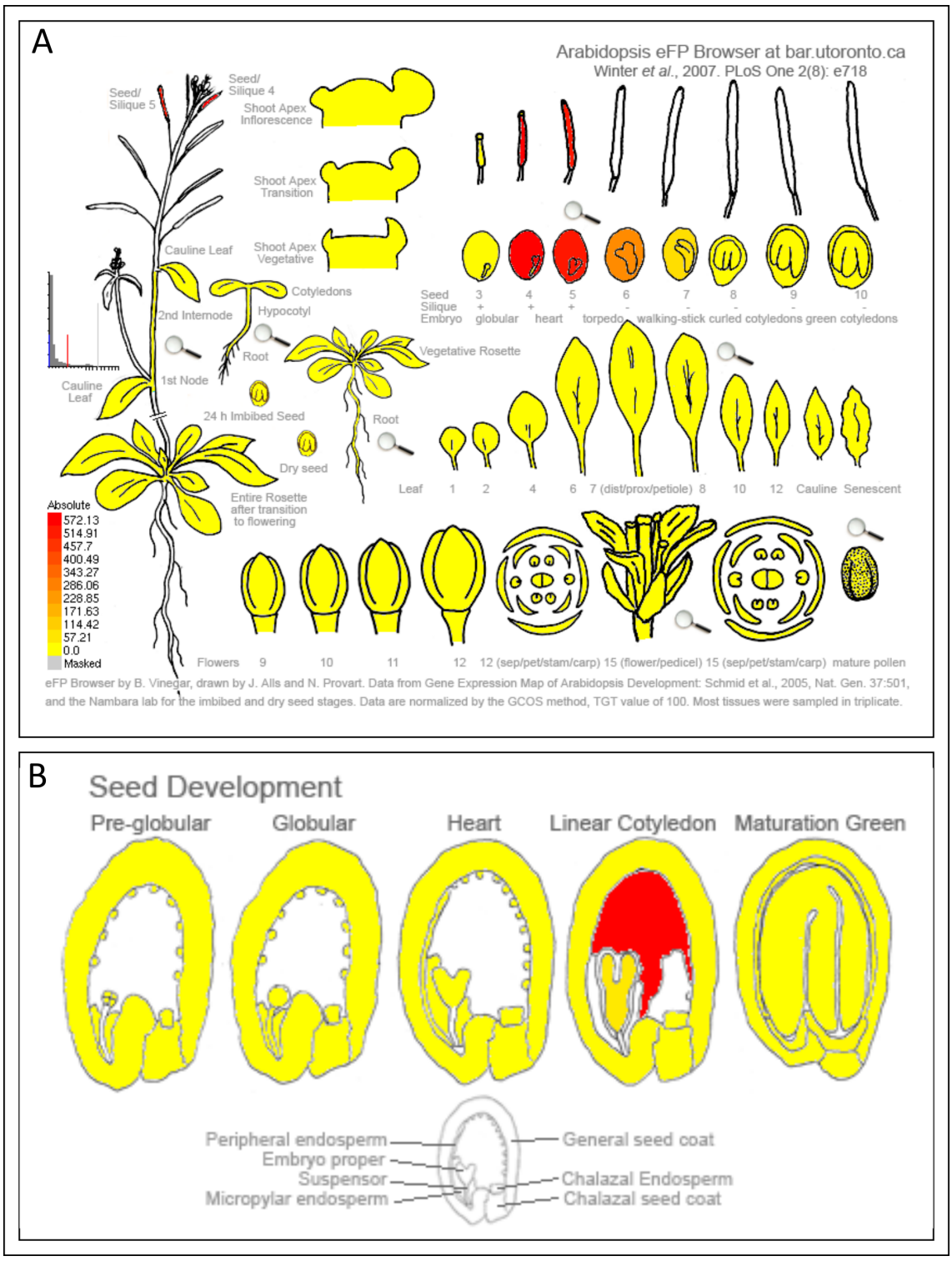
Supplementary Figure M1: Fluorescent immunolocalisation experiments using the LM1 antibody. (A-D) Col-0, (E-H) *zou-4*. Stages: (A,E) Globular stage, (B,F) Heart stage, (C,G) Torpedo stage, (D,H) Mature stage. Scale bar: 50µm. LM1 labelling is shown in green, and calcofluor staining in blue.



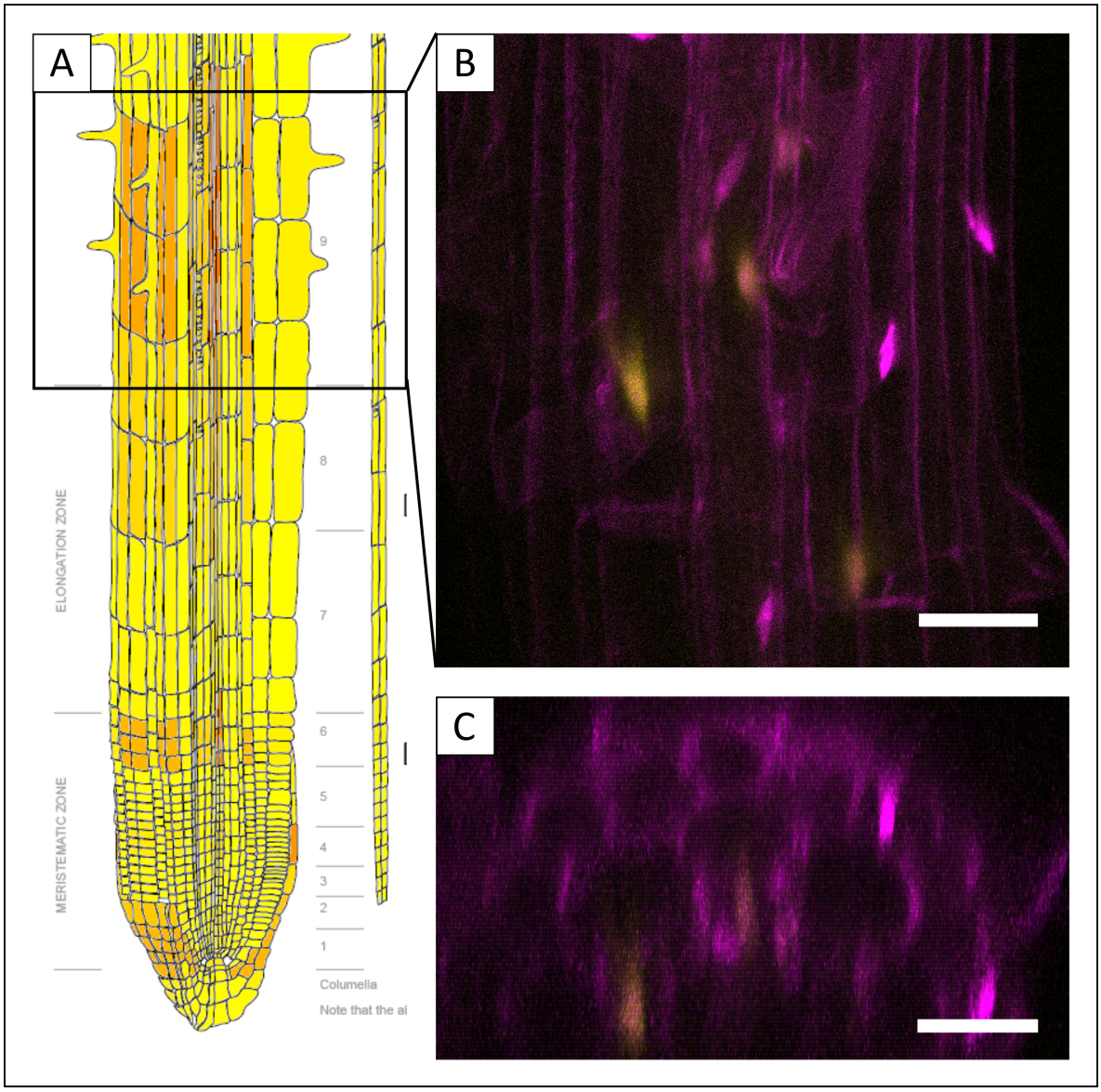
Supplementary Figure M2: TEM images of immunogold labelling with the LM1 antibody and negative control. (H,I) TEM images of Immunogold labelling with the LM1 antibody on torpedo stage Col-0 seeds at the embryo-endosperm interface. The embryonic cuticle is indicated by white arrows, the embryo and endosperm are labeled. Gold particles are seen as black spots some are indicated by black arrows. (J) Control without primary antibody (control for both JIM12 and LM1) labelling). Scale bar 100nm.



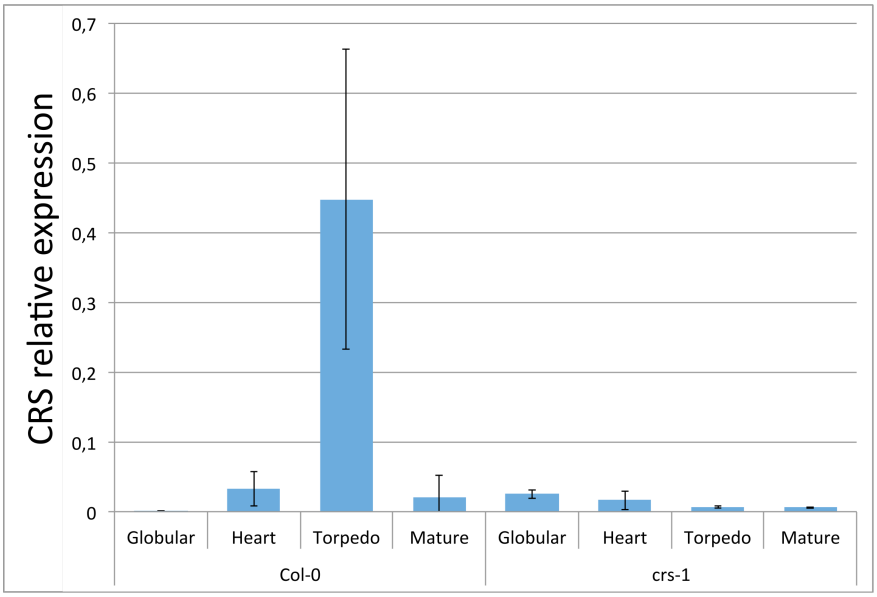
Supplementary Figure M3. CRS phylogenetic analyses. (A) Phylogenetic tree with all STIG-1 domain containing proteins from various species. The box indicate the CRS-like clade. CRS is indicated by a blue arrow. (B) Alignement of the CRS-like peptides proline-rich C-ter domain from various species. Yellow indicates proline residues.



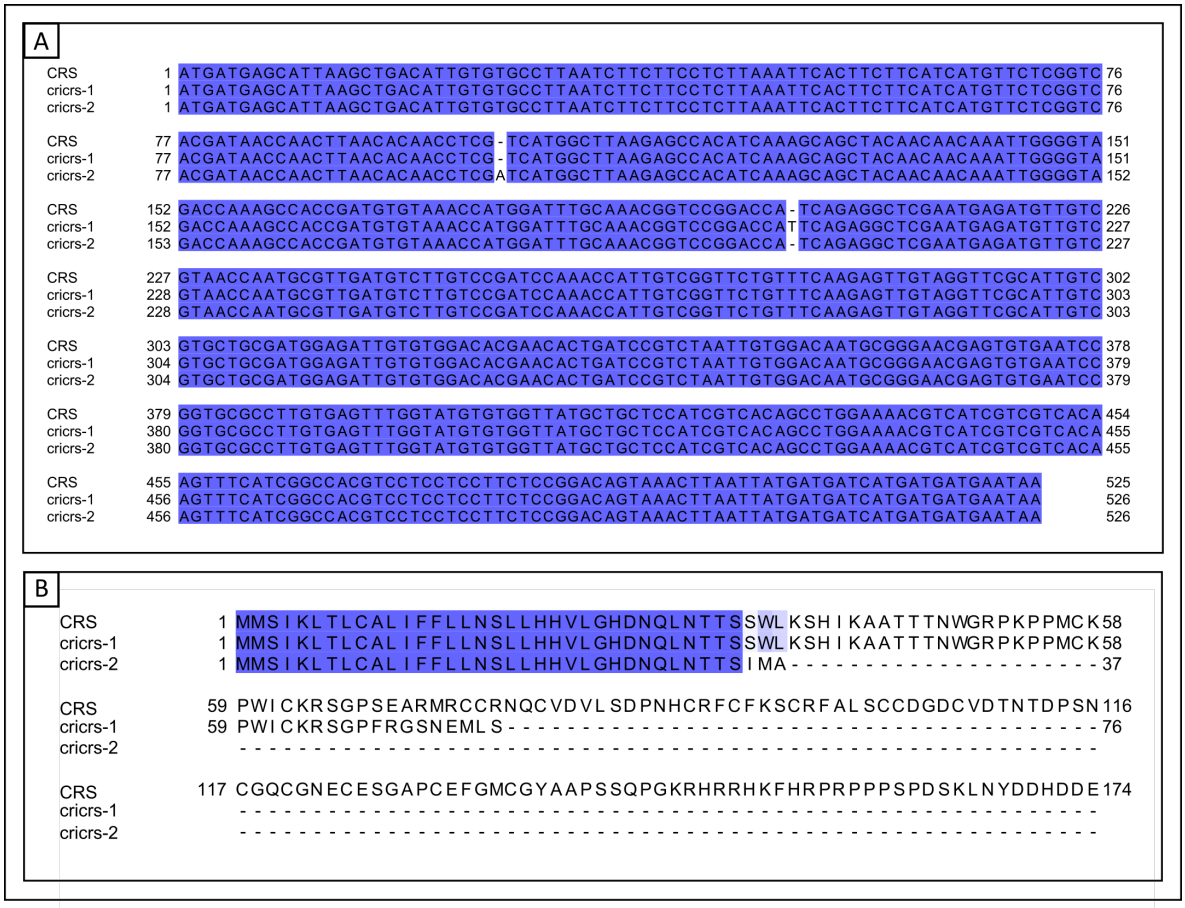
Supplementary Figure M4: CRS *in silico* expression data. (A) Arabidopsis general developmental map. High expression is indicated in red. (B) Seed specific expression data from laser micro-dissection of the different seed compartments at different stage (Le, et al, 2013; Winter et al, 2007) .



Supplementary Figure M5: CRS promoter activity in the root. (A) In silico data showing expression of CRS in the developing root (Winter et al., 2007) (B,C) Yellow: VN7, Magenta: PI staining of cell walls. (B) Longitudinal Stack of confocal images. (C) Transverse stack of resliced confocal images. Scale Bar: 20µm



Supplementary Figure M6: Expression of *CRS* relative to that of *EIF4* in Col-0 and *crs-1* mutant seeds at the developmental stages indicated. Error bars represent standard errors. Three biological replicates were used.



Supplementary Figure M7: Alignment of *CRS* cDNA and predicted encoded protein sequences for WT and *cricrs* alleles. (A) DNA sequences. (B) Corresponding protein sequences.

Primers list	
CRS_F	ATGATGAGCATTAAGCTGACATTG
CRS_R_+	TCACCACGTCACGCTTTATTA
pCRS1600_F_BamHI	CACAGGATCCTATTAAAAACTTAGTCCCGCAGTA
pCRS4000_F_BamHI	CACAGGATCCCTTCACATAATTGAGCTCCAA
pCRS_R_Xho1	CACACTCGAGTGAGATGACTTTGTGATGATGA
SP6	TATTTAGGTGACACTATAG
CRScriSeq	AAGCTGACATTGTGTGCCTT
SS42	TCCCAGGATTAGAATGATTAGG
SS43	CGACTAAGGGTTTCTTATATGC
qPCR	
GSO1-qPCR-F	TGG TTT GCA TGC TCT TAT GG
GSO1-qPCR-R	CAG TCG CCT TCA ACG AGT AA
GSO2-qPCR-F	CGA GTA TAT GGC GAA TGG AAG
GSO2-qPCR-R	CAA GAA CCT CCT TTT TCT TCG TAT
ZOUD3F	GCTGACTATCTGTGGGAATG
ZOUD3R	AACTCGGATTTACCTGTGCT
ALE1Fex7	TCCTAGATTCGGCGATTATGTTG
ALE1Rex8	GCCACTCCTCTCGTGTCTCTAAA
EIF4A-F	TTCGCTCTTCTCTTTGCTCTC
EIF4A-R	GAACTCATCTTGTCCCTCAAGTA
crs_F	GCGCCTTGTGAGTTTGGTAT
crs_R	GATGACGTTTTCCAGGCTGT

Supplementary Table M1: Primers used in the study.

2.3 The Cellular behavior of CERBERUS

The manuscript presented in the previous section of this chapter described the spatio-temporal and functional characterization of the CERBERUS peptide. However, this work has not addressed important questions regarding the *in vivo* comportment of CRS and how its structure and behaviour ensure its function. In this work, I aimed to investigate the *in vivo* localization of CRS through fusion protein analysis.

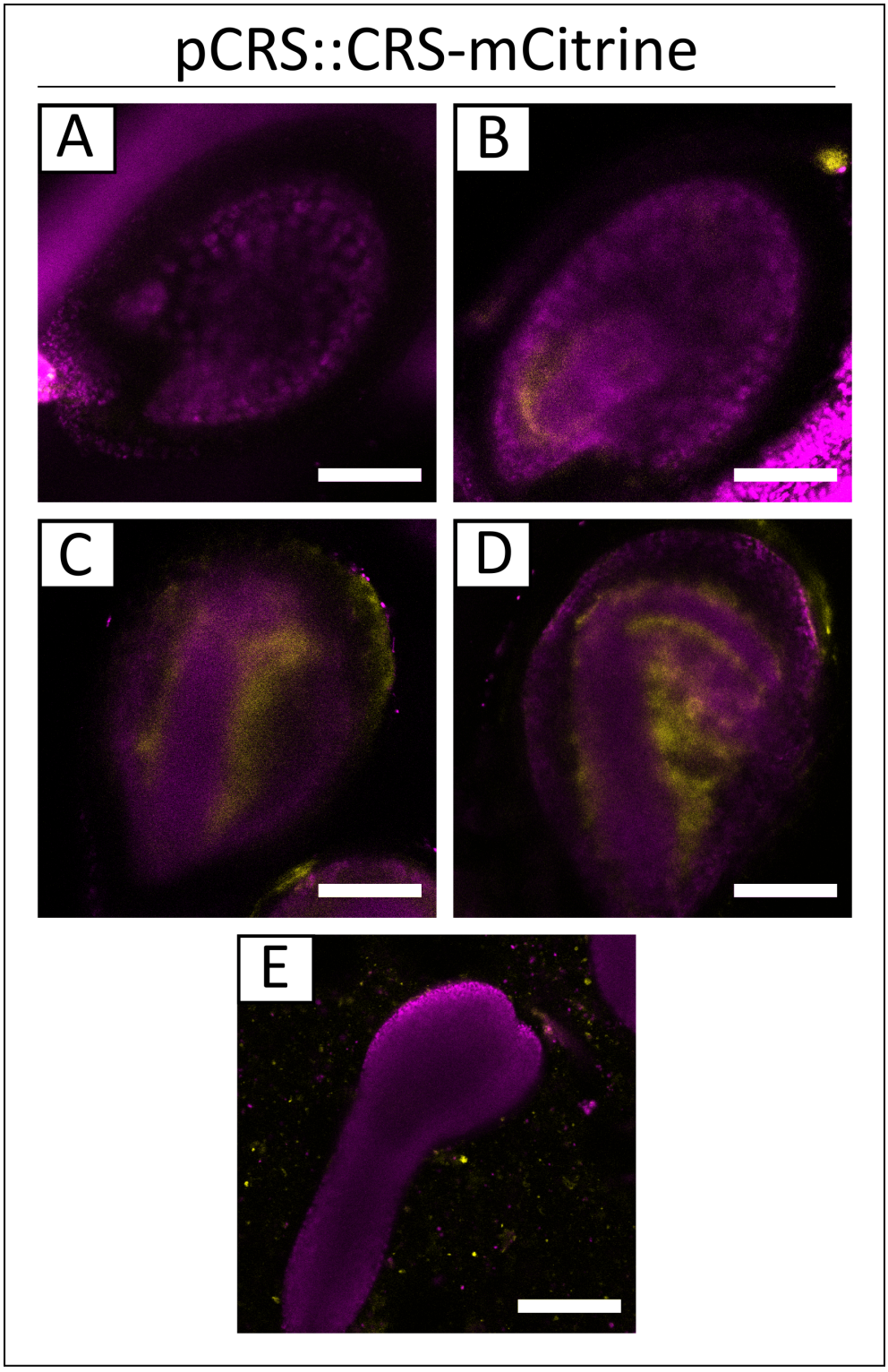


Figure 14: Confocal imaging of *pCRS::CRS-mCitrine* expressing seeds. CRS-Citrine fluorescence is in yellow and autofluorescence revealing the seed structures is shown in magenta. (A-D) Developmental time-course covering seed development. Stages: (A) Globular, (B) Late heart, (C) Torpedo, (D) Bent/Mature. (E) Embryo removed from the seed context. Scale bar 100 µm.

2.3.1 Cerberus is found in the apoplastic compartment in developing seeds

To visualise CRS protein localisation in the developing seed, *pCRS1600::CRS-mCitrine* lines were generated and CRS-mCitrine localization was observed during seed development using confocal microscopy. Consistent with our *in situ* hybridization and promoter-fusion expression studies, fluorescence was detected in the endosperm surrounding the embryo starting at the heart stage and continues to be detectable surrounding the embryo at the torpedo and bent/mature stages (Figure 14A-D).

As discussed in the previous section of this chapter, the CRS protein harbours a predicted signal peptide (http://www.cbs.dtu.dk/services/SignalP/), which should be involved in targeting CRS to the apoplastic space. If CRS is present in the endosperm apoplast, diffusion within the apoplastic compartment is to be expected. However our studies (Creff et al, in revision and introduction to this chapter) suggest that an intact embryonic cuticle surrounds the developing embryo from the heart-stage onwards. Furthermore, two published studies (Beeckman et al., 2000; De Giorgi et al., 2015) have shown the presence of a cuticle-like structure separating the endosperm from the seed coat at both early and later stages of Arabidopsis seed development. Importantly however, the physiological properties of these cuticles as diffusion barriers have not been investigated. We were therefore interested in seeing whether the CRS-mCitrine fusion protein was capable of moving between seed compartments.

Unfortunately, the highly refractive tissues of the developing seed coat do not allow for high quality confocal imaging of the zygotic compartments of the intact seed. The signal is always blurry and subcellular resolution is technically impossible to achieve for now. By extracting torpedo-stage embryos from the seed, we could however observe that the mCitrine signal was not detectable in the embryo and therefore conclude that CRS-mCitrine does not diffuse across the endosperm/embryo interface (Figure 14E). In contrast, and to our considerable surprise, we consistently observed that the cell walls of the inner integument of the testa appeared to show CRS-mCitrine fluorescence (Figure 15). To test whether this signal was originating from transgene expression in the testa or was due to proteins diffusing from the endosperm, we observed seeds generated by crossing *pCRS1600::CRS-mCitrine* transgenic pollen onto

WT female plants. In these seeds, the testa being entirely maternal in origin, the transgenic construct can only be expressed in zygotic tissues, i.e. the embryo and the endosperm. In these seeds we still systematically observed signal in the testa, indicating diffusion across the endosperm/testa cell wall origin (Figure 15C-D). These results confirm two things. Firstly, (bearing in mind that the endosperm and testa are symplastically isolated) that CRS-mCitrine is located in the apoplastic space of the endosperm. Secondly, that CRS-mCitrine is able to diffuse from the endosperm to the testa, but not from the endosperm to the embryo (at least at the torpedo stage) suggesting that the apoplastic "barriers" between the three seed compartments have differing physiological properties.

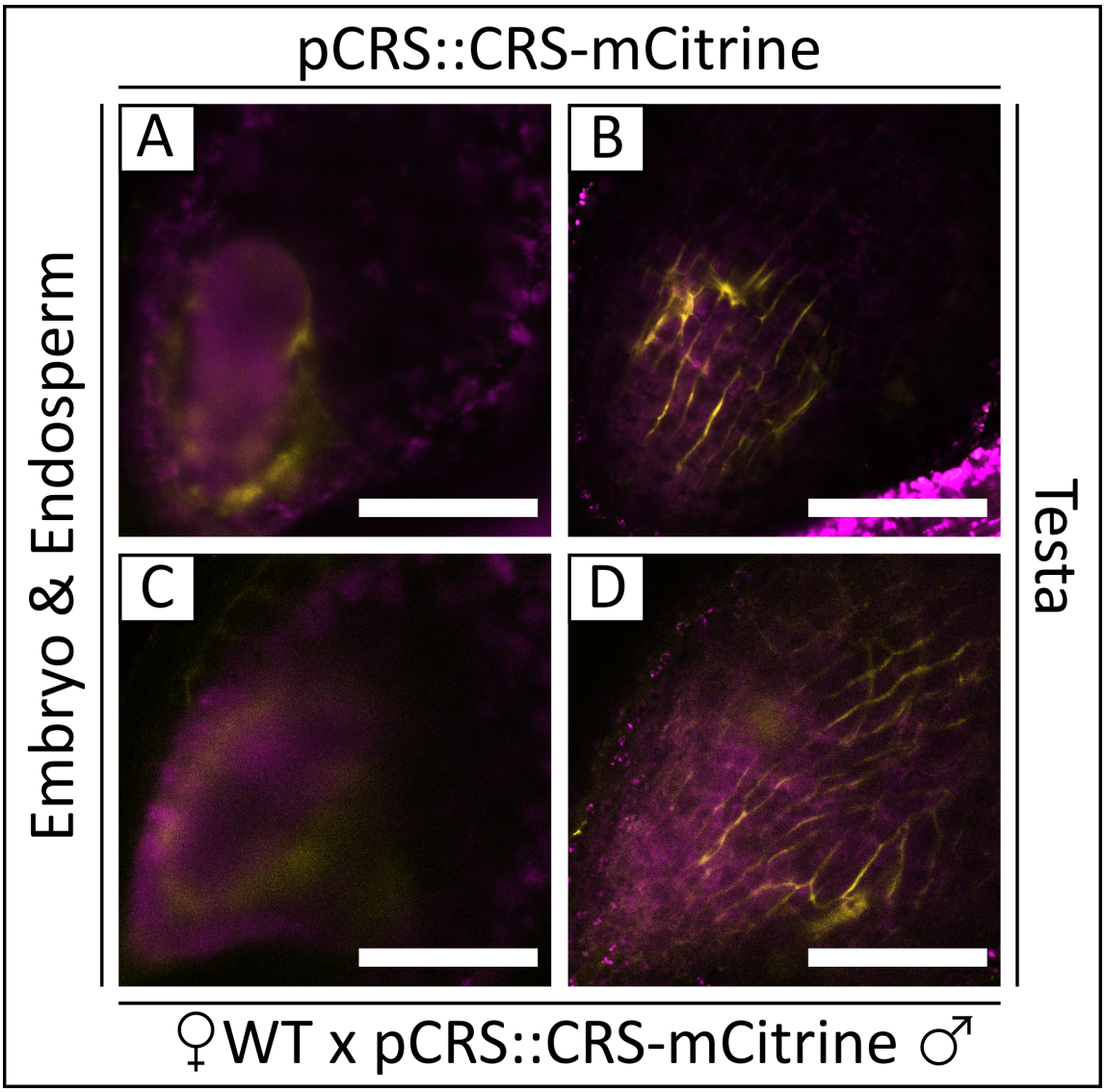


Figure 15: Confocal imaging of CRS-mCitrine in the endosperm and the testa. (A,B) pCRS1600::CRS-mCitrine self fertilized seeds. (C-D) Female WT crossed with male pCRS1600::CRS-mCitrine generated seeds. (A,C) Focal plane: Endosperm & Embryo, (B,D) Focal plane: Testa. Scale bar: 100µm.

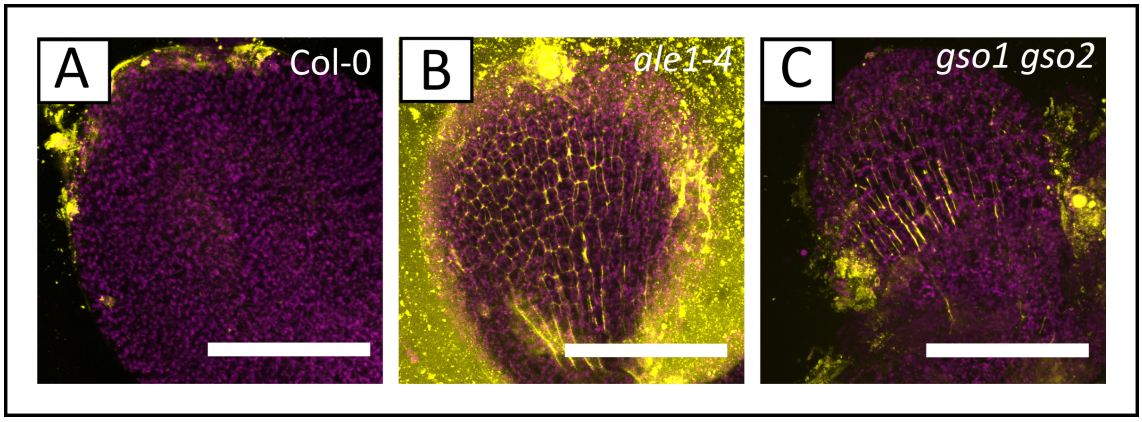


Figure 16: CRS-mCitrine behaviour in the cotyledons of dissected embryos from WT, *ale1-4* and *gso1 gso2* mutants. (A) Col-0, (B) *ale1-4* (C) *gso1 gso2.* Scale bar : 100µm.

Whether or not CRS plays a role in testa development has not been investigated in the context of my thesis. However, we realized that these transgenic lines represent an interesting tool for use in investigating the properties of the embryonic cuticle as a diffusion barrier and thus to probe the cuticular defects observed in *ale1-4* and *gso1 gso2* mutants. To do this, we generated both *ale1-4* and *gso1 gso2* lines harboring the *pRGP3::CRS-mCitrine* construct (Figure 16). The *RGP3* gene is a direct target of ZOU/ICE1 (Denay et al., 2014) and the *RGP3* promoter has exactly the same expression profile as that of $pCRS_{1600}$ (see Figure 17).

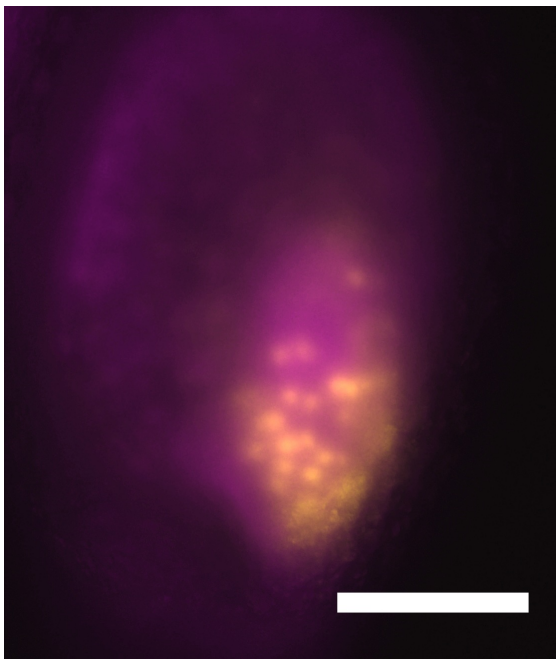


Figure 17 : *pRPG3::VENUS-N7* expressing seed at the beginning of the torpedo stage. Nuclear-localized VENUS-N7 signal (yellow) is restricted to the endosperm surrounding the developing embryo. Scale bar 100µm

As observed previously in $pCRS_{1600}$*::CRS-mCitrine* lines, when wild-type embryos were removed from seeds, we could not observe signal within the embryo (Figure 16A). Occasionally some signal is observed on cotyledon surfaces, particularly

at the tips of the cotyledons. This is also observed in *pCRS1600::CRS-mCitrine* lines and we attribute this to endosperm residues. Interestingly, we observed a different localization for CRS-mCitrine in the mutants (Figure 16B-C). In both the *ale1-4* and *gso1 gso2* backgrounds we can observe apoplastically located signal within the embryo. External yellow signal corresponds to endosperm debris generated while dissecting the embryos. These experiments demonstrate that cuticle discontinuities correspond to changes in permeability during embryo development in these mutant backgrounds.

2.3.2 How does Cerberus behave at the protein level?

The results shown above suggest strongly that CERBERUS is present in the apoplast of the developing endosperm. Although the functionality of the CRS-mCitrine transgene has not yet been confirmed using complementation (currently underway). We nonetheless were keen to answer two further questions. The first being whether CERBERUS is indeed a secreted protein, and the second being whether CERBERUS is a substrate of ALE1.

In order to assess whether CRS is a secreted protein several approaches were undertaken. In the first instance CRS, fused C-terminally to GFP or to mCitrine were expressed under the constitutive promoter of the ribosomal protein-encoding gene *RPS5A* as described in the manuscript presented in this chapter. The resulting plants were analysed using confocal microscopy of non-seed tissues. However, we found that the majority of fluorescent signal in these plants was either apparently cytoplasmic or, alternatively located in the vacuole. These two localisations could be observed in different cells of the same sample (for example Figure 18). None of our observations supported an apoplastic localization for the CRS-FP protein fusions.

In parallel we initiated a collaboration with Professor Andreas Schaller, at the University of Hohenheim, Germany. Professor Schaller expressed our *CRS-GFP* fusion construct transiently in tobacco leaves. He then performed apoplastic washes on these leaves and compared the resulting samples with whole protein samples. This experiment showed three things (Figure 19A).

1) That CRS-GFP is present in the apoplastic fluid of tobacco leaves.

2) That the majority of GFP in both apoplastic and whole protein samples is not present as a CRS fusion but as a cleaved version of GFP which could, based on its size, also contain the C-terminal domain of CRS.

3) That whole protein samples contain a higher molecular weight version of CRS-GFP that is not present in apoplastic washes, and that could correspond to a version of CRS without the signal peptide removed.

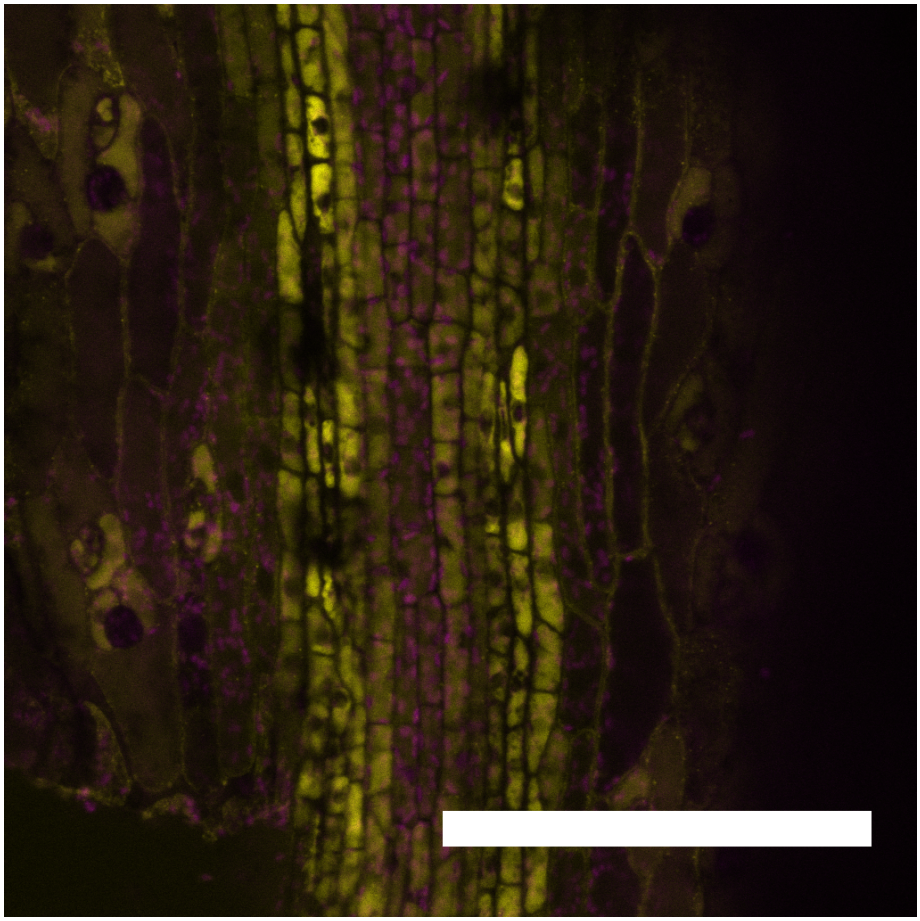


Figure 18: Subcellular localization of pRPS5A::CRS-mCitrine out of the seed context (here, the replum). Localization differs between the central cell- type (apparently vacuolar) and the bordering one (apparently localized in the Cytoplasm/golgi). Scalebar 100µm.

These experiments stimulated us to perform western blots on seeds expressing CRS-FP fusions under the control of endosperm-specific promoters. The results are shown in Figure 19B, and the profile obtained in western blots from three independent constructions resembles very strongly that obtained in apoplastic washes from tobacco.

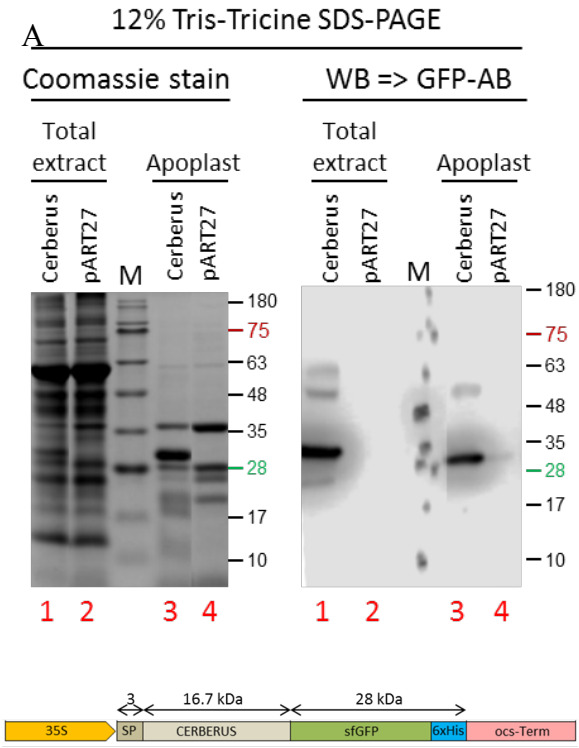
The majority of the fluorescent protein is present as an FP molecule potentially including the extreme C-terminal domain of CRS, although the full-length CRS-GFP fusion (potentially lacking its signal peptide) can also be detected in all lines.

In order to ascertain whether this profile was altered in *ale1-4* mutants, western blots were also carried out on the genetic material shown in Figure 3B. However due to technical difficulties no conclusive results were obtained.

2.4 Discussion

Globally, the results presented in this section support the hypothesis that CRS-mCitrine reaches the endosperm cell wall in the seed context. In lines expressing CRS-mCitrine under either the *CRS* promoter, or the promoter of an endosperm-specific gene, we were able to detect cell wall located CRS-mCitrine/mCitrine in the testa walls, and to show using genetic crossing that this protein originated from the developing endosperm. Similarly apoplastically located protein clearly enters the apoplast of the developing embryo from the endosperm in backgrounds where the embryonic cuticle is defective. Unfortunately, due to the presence of the highly refractive seed coat, subcellular resolution of CRS-mCitrine localization in the endosperm could not be investigated, however the presence of CRS-GFP in transiently expressing tobacco leaves supports the idea that CRS can be secreted in this system.

In contrast, our results in plants stably expressing CRS-mCitrine in Arabidopsis under a ubiquitously expressed endogenous promoter show alternative subcellular localisations, including the vacuole. This result is difficult to explain and merits further investigation. The correct addressing of membrane localized and secreted proteins is known to be dependent upon correct post-translational modification (including glycosylation) in some cases (Łuczak et al., 2008), and it could be that CRS can only be correctly modified in the endosperm of Arabidopsis due to the seed-specific expression of modifying proteins. It has, however also been shown that the C-terminal addition of GFP and other fluorescent proteins to secreted proteins can prevent their secretion, and as a result impact their functionality. Since we have not yet shown complementation of the *crs* mutant phenotype by our transgenes, we cannot exclude this option as an explanation for our results.



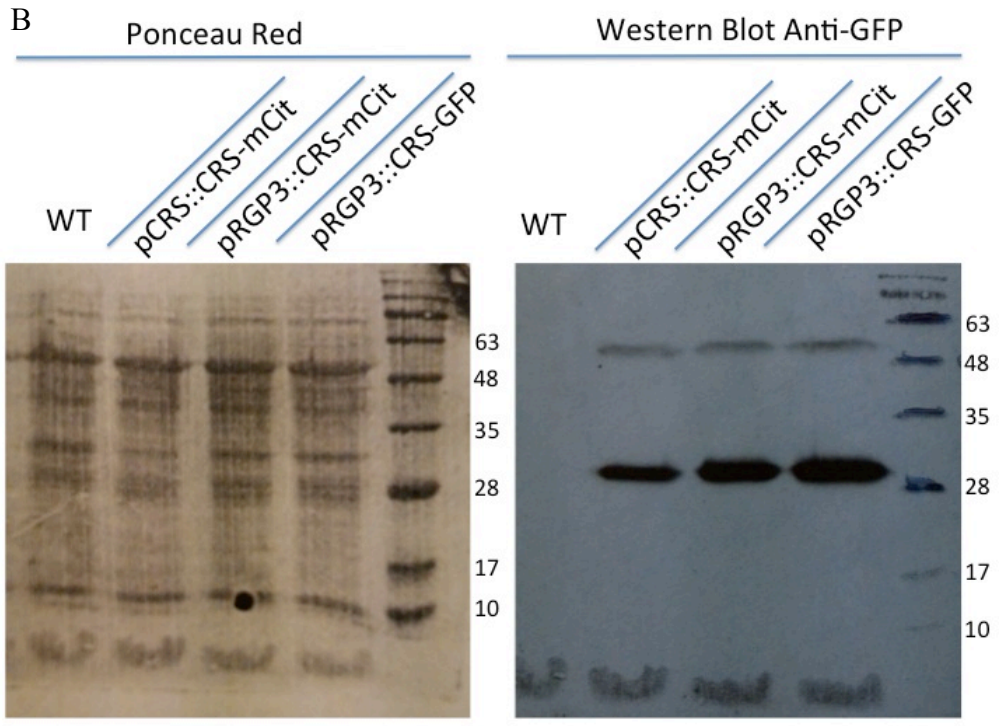


Figure 19: Western blots against transiently and stably expressed CRS-GFP fusion proteins. (A) Western blot against transiently expressed CRS-GFP in tobacco cells. Both total extract and apoplastic wash samples are shown. (B) Stably expressing CRS fused to GFP or CITRINE. Proteins were extracted from developing siliques and detected with anti-GFP antibody. Molecular weights are indicated on the right side of each blot.

A further possibility is, however, more seductive. Some peptides are known to be located inside cells and released only upon cell elimination/damage. This is, indeed, the case with the PEP peptides, which are located in the cytoplasm (Bartels et al., 2013). This could be the case for CRS, with the presence of CRS in apoplastic washes from tobacco potentially due to damage inflicted upon infiltration of the leaves.

From the heart stage of development onwards, in wild-type seeds, the endosperm surrounding the developing embryo breaks down. Recent research from the Ingram lab has suggested that endosperm breakdown is mediated by a physical "bursting" of the endosperm cells as the embryo expands (Fourquin et al., 2016). This bursting is made possible by the cell wall softening activity regulated by the ZOU/ICE1 heterodimer. It is therefore possible that CERBERUS is not secreted into the extracellular space, but is released when endosperm cells "burst" as the embryo expands. If CRS release necessitates such a scenario it should be dependent on ZOU function (Kondou et al., 2008; Yang et al., 2008). Unfortunately, we could not directly test this possibility by crossing our *pCRS1600::CRS-mCitrine* and *pRGP3::CRS-mCitrine* lines into a *zou-4* mutant background, because the expression of both *CRS* and *RGP3* is dependent upon ZOU activity. Ongoing experiments are therefore focused on the production of CRS-mCitrine in an endosperm-specific, ZOU-independent, manner in *zou-4* mutants. Once these lines are generated, if no fluorescent signal is detected in the testa (or embryo, given that *zou* mutant embryos have a defective cuticle), we could conclude that release of CRS was ZOU-dependent rather than mediated by active secretion.

Our data and data from our collaborators indicate a potential cleavage of CRS C-ter domain resulting in the production of a 3-kDa peptide fragment fused to the fluorescent protein tag. Further experiments are planned, such as IP-MS of CRS-mCitrine fragment to confirm the presence of the C-terminal domain of CRS. If this cleavage event is confirmed, it would constitute strong evidence for the potential activity of the CRS C-terminal domain as a small peptide, especially in light of the conservation of a related domain throughout most CRS proteins from eudicots. How this cleavage is mediated will also be considered in more detail. Experiments to test whether *in vitro* cleavage can be mediated by ALE1 are currently being undertaken by our collaborators. However, preliminary genetic evidence tends to argue against the fact

that CRS is a substrate of ALE1, because *cricrs-1 ale1-4* double mutants appear to have additive effects on cuticle integrity.

A major aspect of ongoing and future work started during my PhD study involves carrying out a more detailed structure function study of the CRS protein. CRS is likely to be released into the apoplast and therefore has the potential to act as a signal between the endosperm and the embryo. However, the puzzling structure of CRS, which contains a STIG1-like domain with conserved cysteines (characteristic of CRPs) and a C-ter part that may be cleaved (characteristic of SPTM peptides), has not yet been resolved. In order to determine which part of CRS mediates its function in ensuring the formation of the embryo sheath, complementation experiments with various combinations of the different domains identified in CRS are required. Since treating the embryo surface with synthetic peptides is not feasible in the whole seed context, we proposed to generate lines stably expressing chimeric peptides in the *cricrs-1* mutant background.

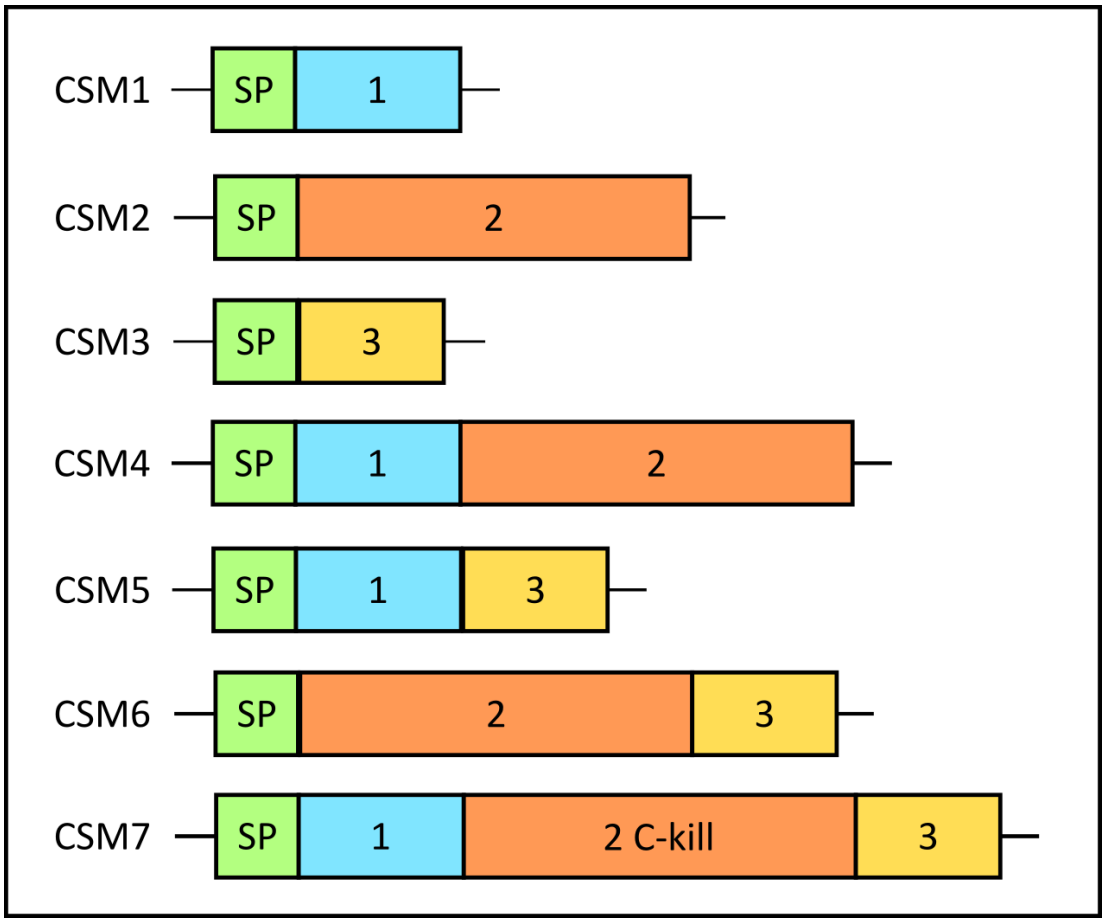


Figure 20: CRS Synthesis modulation constructs. SP: Signal Peptide, 1: Non-Conserved Region, 2 Cysteine Rich Region, 3: Proline Rich Region, 2 C-kill: Cysteine-Rich Region mutated at 4 different cysteines.

The delay in the production of these lines is due to that fact that we wanted to complement a true null alleles (generated by CRISPR-Cas9), and before complementation could be initiated it was absolutely necessary to eliminate the CAS9-containing transgene from the mutants background to avoid the mutation of introduced transgenes.

We have now generated constructions designed to express a range of domain combinations and termed CERBERUS Synthesis Modulation (CSM) transformed plants. These are shown in Figure 6. CSM1-3 correspond to each of the individual domains of CRS and are designed to investigate the potential sufficiency of a given domain in mediating CRS function. For example, if the C-ter domain (3) is cleaved and acts as an SPTM-like peptide, we would not expect to need the other domains in order to complement at least part of the CRS phenotype. CSM4-6 correspond to combinations of two domains. Finally CSM7 correspond to full-length CRS full-length, but with 4 of the conserved cysteines in the STIG1-like domain substituted by alanines and will be used to investigate the functional relevance of the conservation of these cysteines. A wild-type full-length protein has also been included in these complementation experiments. These constructs have been placed under the control of both the *CRS* promoter and the *RPS5A* promoter and transformed into the clean *cricrs-1* mutant background. These experiments should therefore start to produce results in the next few months.

A final, but important point of discussion highlighted by the results presented in this section revolves around the localization of the CRS-mCitrine protein produced in the endosperm. The lack of complementation data for this protein makes it dangerous to draw any hard conclusions for the moment, but it is important to point out that CRS-mCitrine, although it is released into the endosperm apoplast, is not apparently located in an embryo-surrounding sheath structure. This finding argues against the hypothesis that the CRS protein is the source of the epitope detected by the JIM12 and LM1 antibodies in the developing endosperm. However, given that the vast majority of the mCitrine protein expressed in these lines is present in a form that is only slightly larger than that predicted for free mCitrine, we cannot exclude the possibility that the remainder of the CRS protein is localized to the embryo sheath. Ultimately it will be

both interesting and instructive to generate antibodies specifically against the various domains of CRS in order to ascertain their localization at the tissular, cellular and subcellular levels in the developing seed.

3 A CONSERVED APOPLASTIC BARRIER INTEGRITY PATHWAY IN ARABIDOPSIS?

3.1 The Casparian strip

The success of plants in the terrestrial environment relies on a multitude of structural and physiological adaptations that permit survival in conditions that can show extreme spatial and temporal fluctuations. The ability to permit optimal water uptake and movement throughout the plant, whilst actively regulating the ingress of harmful soil minerals into the plant body is critical to plant survival.

Water uptake into plants occurs through the root. Root hairs maximise effective root surface area in the water-absorbing zone. Water uptake is driven by gradients in both chemical (osmotic) and pressure potential, negative pressure within the xylem caused by transpiration being the major force driving water movement under most conditions. For most of the distance between the root surface and the vasculature, soil water can move either through the hydrated cell wall continuum (the apoplastic pathway), or pass through cell membranes and move through the cell

cytoplasm/plasmodesmata (the symplastic pathway). However the apoplastic pathway into the root is blocked at the endodermis, a concentric ring of cells encircling the vascular tissue, by an apoplastic barrier. In Arabidopsis this barrier, called the Casparian strip, is initially composed of lignin (Naseer et al., 2012), and then becomes impregnated by the waterproof cutin-related polymer suberin (Geldner, 2013). By forming as a continuous band around endodermal cells in the water-absorbing regions of the root, the Casparian strip forces water entering the plant to cross cell membranes, permitting the plant to actively regulate solute uptake (for example to actively take up and retain important ions such as K^+, but selectively exclude0 harmful ions such as Na+, which would otherwise build up to toxic levels in plant tissues) (Barberon et al., 2016; Baxter et al., 2009; Geldner, 2013; Pfister et al., 2014). In addition recent results have also shown that the resistance provided by the endodermis to water movement also participates in the build up of negative pressure in the root vasculature and thus affects the force with which water is "sucked" into the root (Pfister et al., 2014).

The path of water movement through plant tissues is not only gated by the Casparian strip, but as described in the general introduction is also regulated at aerial plant surfaces, due to the presence of another apoplastic barrier, the cuticle. The cuticle "waterproofs" the outer face of cells at the plant surface (the epidermis) allowing a precise control of gas exchange (and thus transpiration) though stomatal pores.

To function correctly as barriers, both the Casparian strip and the cuticle must be continuous, as gaps would seriously compromise their function. This integrity needs to be achieved before they are deployed, since integrity defects in either barrier post-deployment could rapidly lead to lethality in suboptimal environments. As described in previous chapters, the GSO1/GSO2/ALE1 pathway plays a key role in ensuring pre-germination integrity of the cuticle. In parallel, research from the group of Professor Geldner, has indicated that despite the compositional differences (lignin/suberin vs cutin) and differences in spatial deposition patterns (discrete circumferential ring vs surface coverage) between the two barriers, integrity of the nascent Casparian strip is apparently dependent upon similar signalling events. In particular GSO1/(also called SCHENGEN3 (SGN3) is required both for the production of a gap-free Casparian strip (Alassimone et al., 2016; Pfister et al., 2014). In both cases, GSO1/SGN3 mediated signalling appears to act specifically to direct the spatial distribution of primary barrier components, although in the case of the Casparian strip the localisation of these components has been shown to be dependent upon to localisation of a protein scaffold

composed of CASP proteins, which predicts and dictates the position of the nascent Casparian strip (Roppolo et al., 2011).

GSO1/SGN3 encodes an LRR RLK which, based on alignment of the extracellular (ligand binding) domain, has clear orthologues throughout the Eudicots. The duplication giving *GSO2* appears only to have occurred in the *Brassicales*. *GSO1/SGN3* is strongly expressed in both the endodermis and in the embryonic epidermis (Pfister et al., 2014; Creff et al in revision). However GSO1/SGN3 protein is localised specifically to a domain on either side of the nascent Casparian strip, whilst in the embryonic epidermis protein is observed throughout the membrane of epidermal cells (Creff et al., in revision). *GSO2* is strongly expressed in the embryonic epidermis (Creff et al in revision) but not in the endodermis and, consistent with this, does not appear to be required for Casparian strip formation (N. Geldner, personal communication). In the case of Casparian strip formation, GSO1/SGN3 has recently been shown to act together with an RLCK (SGN1) (Alassimone et al., 2016), which is localised specifically to the endodermal membrane facing the root cortex. Loss of SGN1 function gives a similar phenotype to loss of GSO1/SGN3 function and genetic interactions support the idea that GSO1/SGN3 and SGN1 act together to ensure Casparian strip integrity. The different subcellular localisations of these proteins means that co-localisation should occur only in the GSO1/SGN3 domain immediately to the cortex side of the Casparian strip. However, a physical interaction between GSO1/SGN3 and SGN1 has not been detected.

The spatial polarity potentially conferred on GSO1/SGN3 signalling by SGN1 makes it is possible to imagine a common mechanism in both the embryo and the endodermis in which apoplastic barrier integrity is monitored during biogenesis by the spatially restricted production of a ligand, the access of which to GSO1/SGN3 (+GSO2 in embryos) is gated by barrier integrity. In the root the ligand would be predicted to originate within the stele, whilst in the seed, it would be predicted to originate within the endosperm.

It is entirely possible that the GSO1/SGN3 receptor perceives the same, or related ligands during Casparian strip formation and embryonic cuticle formation. Until recently however, no *bona-fide* ligands had been identified as being involved in either

process. Very recently however, we obtained access to unpublished results from Professor N. Geldner showing that the TYROSYLPROTEIN SULFOTRANSFERASE (TPST) protein was involved in the formation of the Casparian strip. This suggests that the GSO1/SGN3 ligand might be sulfated, and indeed, even more recent results from the Geldner laboratory have shown that a sulphated peptide (which we will refer to here simply as PEPTIDE-X (PEP-X) for reasons of confidentiality) is the likely ligand of GSO1 during Casparian strip integrity signalling. The *PEP-X* gene has one close orthologue in *Arabidopsis*, which we have called *PEP-X'*. In the last few weeks of my thesis I therefore initiated studies aimed at uncovering whether TPST and the PEP-X peptides could be involved in embryonic cuticle formation.

3.2 Results

3.2.1 TPST is expressed early during seed development

In light of the function of TPST in modifying GSO1 ligands in the Casparian strip context, we studied its possible involvement in the biogenesis of the embryonic cuticle. According to publicly available datasets (Winter et al., 2007), *TPST* is most strongly expressed in the pollen grain (Figure 21A), and has recently been shown to be involved in pollen tube growth (Stührwohldt et al., 2015). However, seed development specific datasets (Le et al., 2010) show a strong relative expression of TPST in the micropylar endosperm at the globular stage of seed development when the cuticle is formed *de novo* (Figure 21B). A weaker expression is notable in the seed coat through all stages of seed development. Initial *in situ* hybridisation experiments support these expression profiles, but need to be repeated due to problems with high background (data not shown).

Scrutiny of available RNA-seq datasets from the laboratory suggests that *TPST* expression is not significantly regulated by the activity of ZOU, ALE1 or GSO1 and GSO2 during seed development (Xing et al., 2013 and Creff et al., in revision), although this needs to be confirmed.

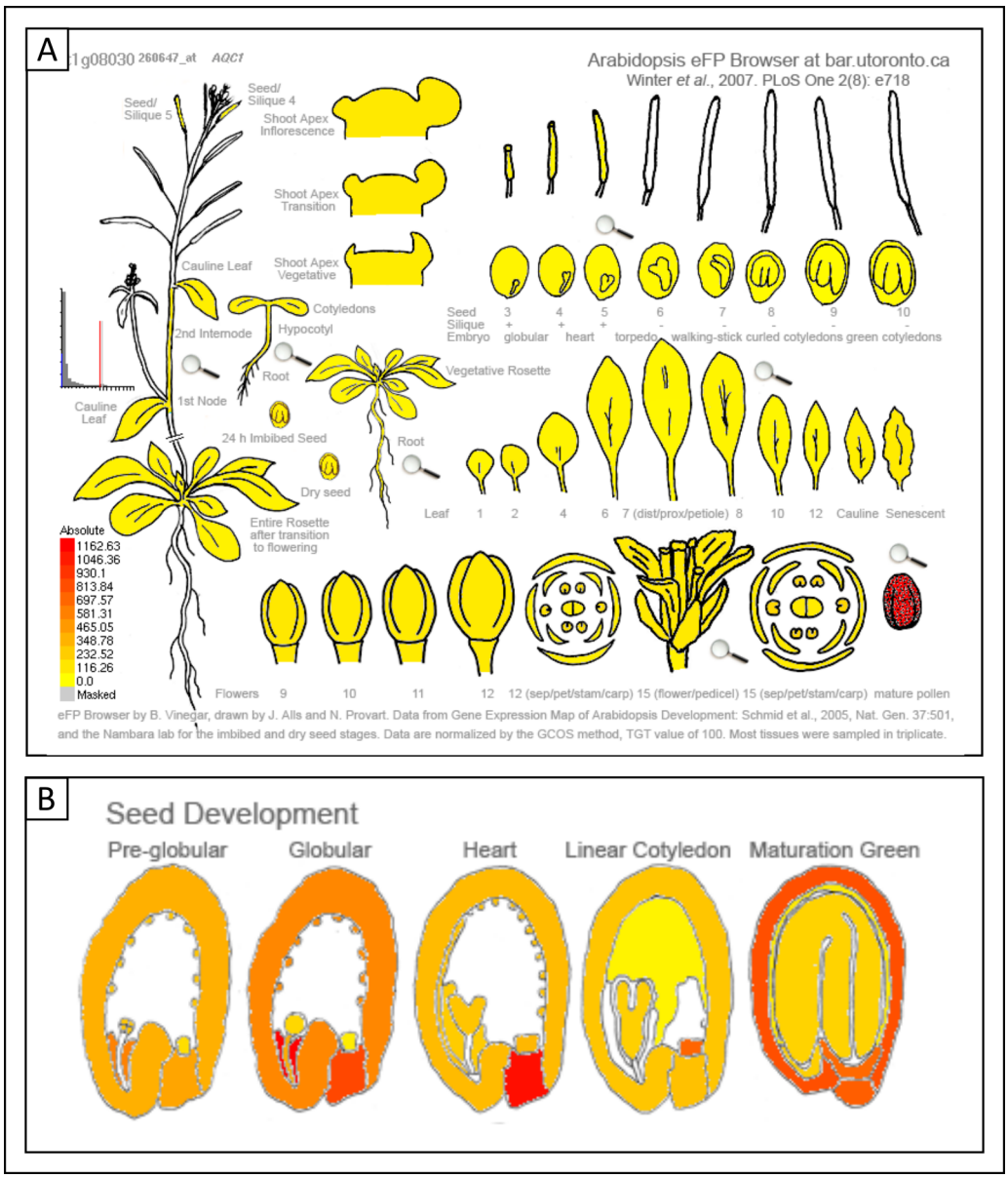


Figure 21: TPST *in silico* expression data. (A) Arabidopsis general developmental map. High expression is indicated in red. (B) Seed specific expression data from microdissection of the different seed compartment at different stages.

3.2.2 TPST is necessary for embryonic cuticle integrity

The spatio-temporal pattern of *TPST* expression resolved, the next logical step was to investigate its potential function in regulating embryonic cuticle formation. Professor Geldner kindly provided two different mutants to study the effects of TPST loss-of-function: A null mutant, termed *tpst-1* which has previously been described in the literature (Komori et al., 2009), and *sgn2,* a weaker mutant allele of *TPST* identified in a screen designed to identify mutants with a defective Casparian strip phenotype (Alassimone et al., 2016). The *sgn2* mutant has not yet been published. We performed toluidine blue assays on etiolated cotyledons from these mutant backgrounds to test the integrity of the embryonic cuticle (Figure 22). Col-0 seedlings were used as negative control, and having an impermeable cuticle they show no penetration of the hydrophilic blue dye and are thus yellow after dye treatments (Figure 22A). *ale1-4* and *gso1 gso2* are also presented as positive controls. As previously described, *gso1 gso2* double mutants have a very strong phenotype reflected by a dark blue coloration (Figure 22E), while *ale1-4* has a milder phenotype, showing a green coloration resulting from a mix of blue and yellow (Figure 22D). TB assays on *sgn2* and *tpst-1* mutants showed a penetration of the dye in both backgrounds (Figure 22B,C). The dye uptake appears qualitatively very weak in *sgn2* and stronger in *tpst-1*, correlating with the strength of the two alleles. To confirm this observation, we quantified the TB uptake in the same mutants as used in the qualitative assay (Figure 22F). A significant difference is observed between Col-0 and the *sgn2* and *tpst-1* mutants, as well as between the two mutants. However, a slight discrepancy was noted with respect to *ale1-4,* which qualitatively appears less defective than *tpst-1* and is found here to be more affected after quantification.

ale1-4 and *gso1 gso2* seeds are misshapen due to a perturbed embryo orientation during seed development originating from cotyledon tip adhesion defects. While *gso1 gso2* seeds have a fully penetrant phenotype, *ale1-4* alleles show approximately 50% of their seeds misshapen. We observed the same seed phenotype (data not shown) in both *sgn2* and *tpst-1,* with fewer seeds affected in *sgn2,* however this proportion has not yet been quantified as we have yet to go through a complete generation to multiply the seeds.

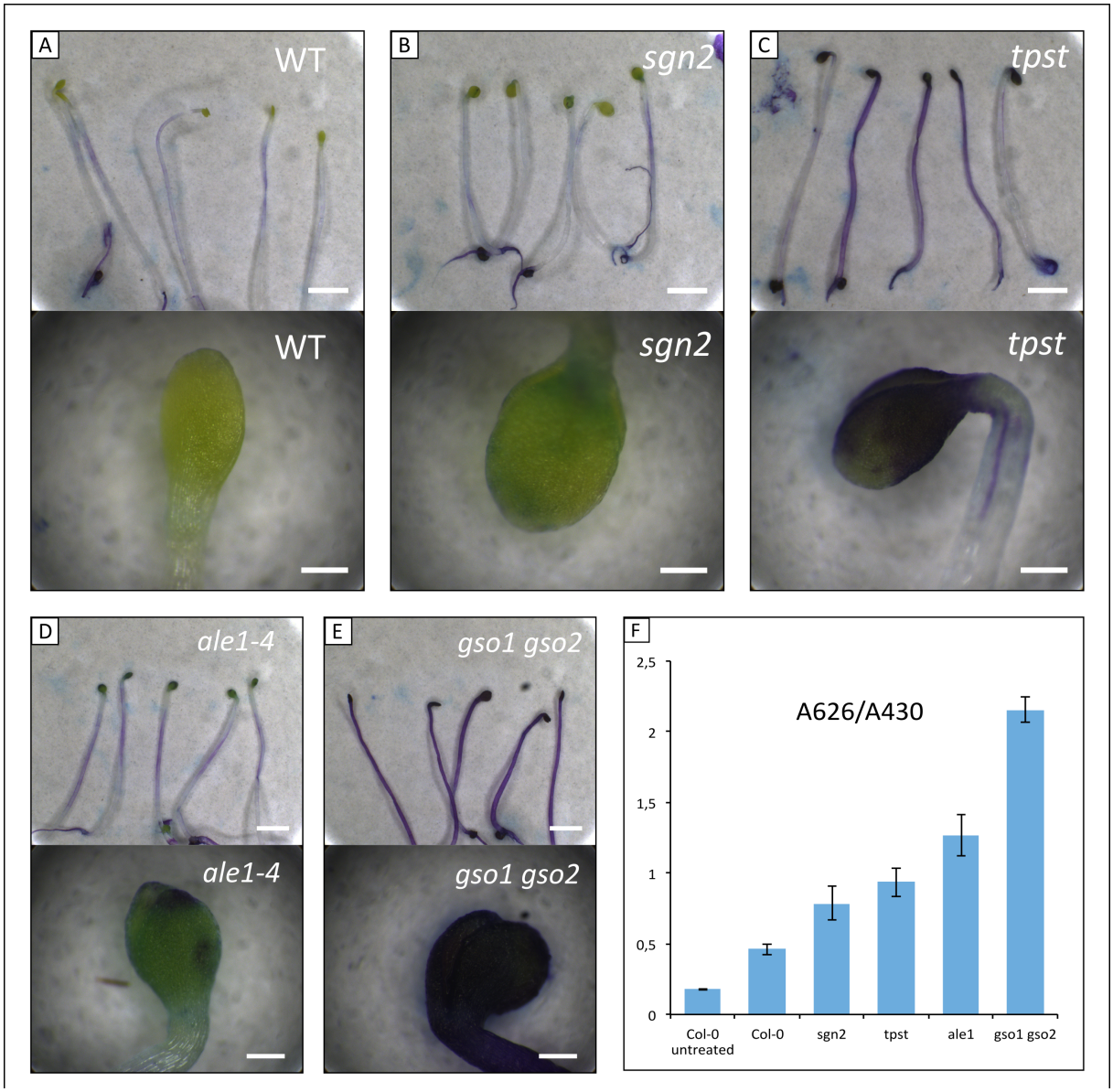


Figure 22: Loss of TPST function affects cuticle integrity: (A-E) Toluidine blue experiment on etiolated cotyledons *of tpst* mutants. Col-0 is used as a negative control and *ale1-4* and *gso1 gso2* as positive controls. (F) Toluidine blue uptake quantification in the mutants.

3.2.3 *PEP-X* and *PEP-X'* expression studies

The data presented above provide strong evidence that TPST is involved in the production of an intact embryonic cuticle. We therefore initiated an investigation on the peptides identified by Professor Geldner as being sulphated by TPST and perceived by GSO1 in the Casparian strip context. Publicly available data were only available for the

expression of *PEP-X* (Figure 23). General development data shows a strong relative expression in the root. A very weak relative expression is reported in both early developing seeds and siliques, hinting at possible expression in the seed (Figure 3A). However, when looking at seed specific datasets, *PEP-X* expression seems localized principally in the chalazal endosperm, and in this tissue only at later developmental stages. To further characterize *PEP-X* spatio-temporal expression pattern, we conducted *in situ* hybridization experiments against PEPX mRNA. We observed no convincing expression in developing seeds compared to controls (data not shown).

Frustratingly, technical issues have prevented the cloning of a template for the *PEP-X'* antisense probe. However we note that although some reads corresponding to *PEP-X* transcripts have been detected in extensive RNA-seq transcriptome analyses of early stage developing seeds carried out in the laboratory (Xing et al., 2013; Creff et al., in revision), no reads corresponding to transcripts from *PEP-X'* were detected, strongly suggesting that this gene is not expressed in the developing seed.

3.3 Discussion

In this chapter we report a role for TPST as a new regulator of embryonic cuticle integrity. Preliminary phenotypic analyses show that *tpst* mutants present seed shape defects and embryonic cuticle defects consistent with those observed in *ale1* and *gso1 gso2* mutants. Based on these results we propose that *TPST* likely functions in the same signalling pathway as *ALE1, GSO1* and *GSO2*. In order to confirm this both *TPST* mutant alleles used in this study have been crossed to other pathway components, and in depth phenotypic, transcriptomic and cytological studies of mutants are planned.

The function of TPST as a TYROSYLPROTEIN SULFOTRANSFERASE provides a tantalising hint suggesting that at least some of the signalling peptides involved in embryonic cuticle integrity signalling could be sulphated. It is, however, interesting to note that the phenotype of the null *tpst-1* allele used in our study is weaker than that of the *gso1 gso2* double mutant, suggesting that either peptide sulfation may not entirely necessary for function, or alternatively that GSO1 and GSO2 perceive multiple ligands during embryo development, some of which are not sulfated.

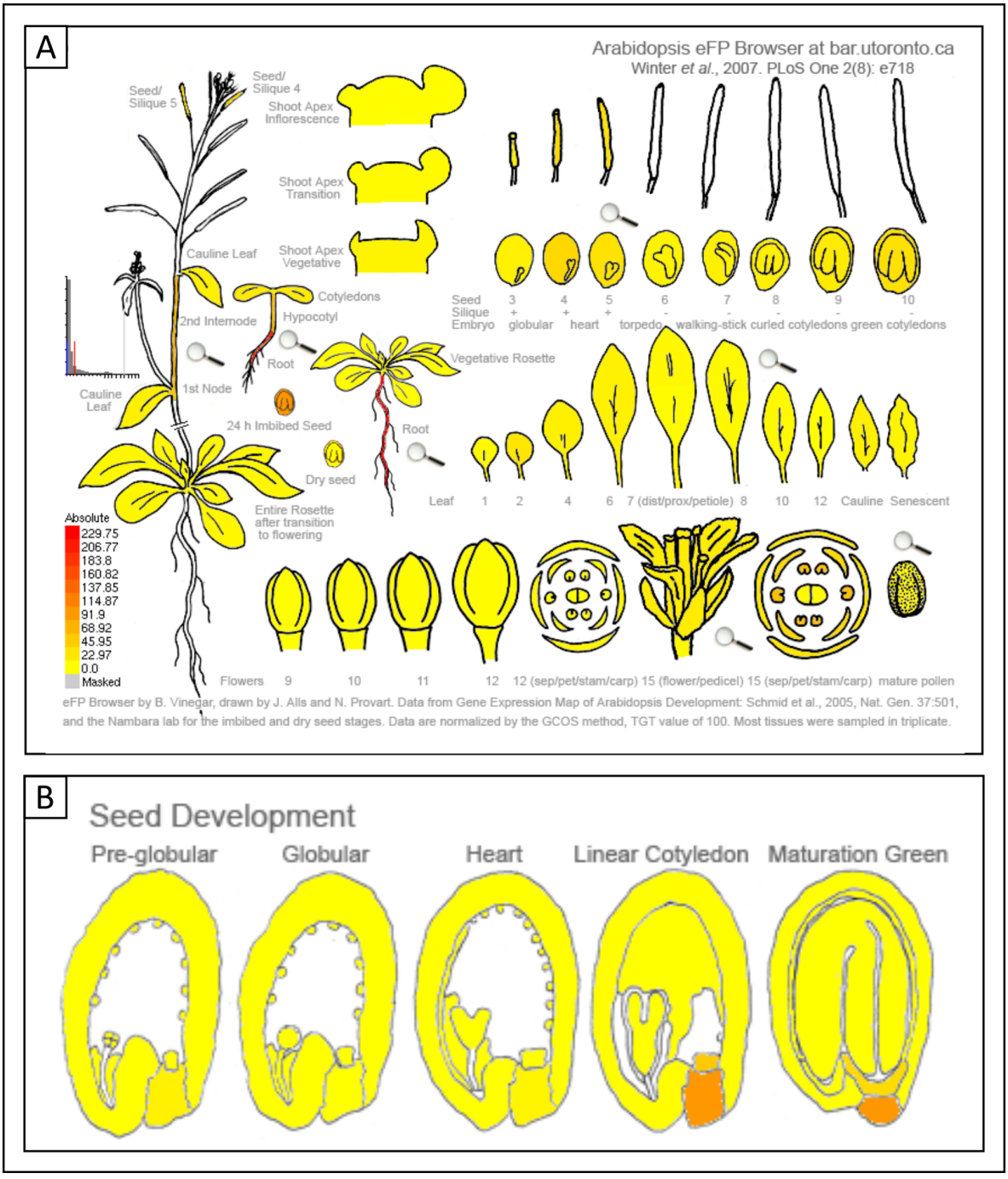


Figure 23: *PEP-X in silico* expression data. (A) Arabidopsis general developmental map. High expression is indicated in red. (B) Seed specific expression data from microdissection of the different seed compartment at different stages.

One obvious possibility is that GSO1 and GSO2 perceive the same ligands in the seed as those perceived by GSO1 in the root. Our very preliminary data regarding the expression of the *PEP-X* and *PEP-X'* genes suggest that they are not strongly expressed during early seed development in silique tissues. However, given the potential long distance mobility of peptides in plants, and the fact that the zygotic compartment of the seed is not apoplastically isolated from maternal tissues at early developmental stages, we cannot exclude a function for PEP-X and PEP-X' in embryo development. The acquisition and phenotypic/genetic analysis of mutants in the *PEP-X* and *PEP-X'* genes will be a key step forward in clarifying the situation. A further important question which will need to be addressed by both genetic and biochemical analysis, is whether the PEP-X and PEP-X' pro-peptides can act as substrates for ALE1.

Another interesting possibility highlighted by the results in this chapter, is that CRS could be a target of sulfation by TPST. Tyrosine residues are present in the CRS protein sequence, including in the C-terminal domain of the protein, and we therefore cannot exclude a role for tyrosine sulfation in permitting CRS activity. Again, genetic analysis will help to clarify the functional relationship between TPST and CRS in the first instance. A targeted mutagenesis approach could be envisaged in order to assess the functional importance of Tyrosine residues in the CRS sequence, while more in depth biochemical analysis of CRS modifications could also be used to address this hypothesis in the longer term. It is, nonetheless, interesting to note that the expression of TPST in the embryo-surrounding endosperm appears to be strongest at the globular stage of embryo development, before the expression of *CRS* initiates.

Finally, and on a more general note, the common roles of GSO1 and TPST in ensuring the integrity of both the Casparian strip and the embryonic cuticle, two biochemically different but functionally analogous structures is of the utmost interest from an evolutionary point of view. In evolutionary terms the seed is a much more recent invention than the Casparian strip. Understanding how the same signalling elements have been recruited to the two different functions will therefore have considerable potential to shed light on how signalling modules can be "recycled" during the evolution of complex multicellular organisms.

4 FRIABLE1 IS A NOVEL COMPONENT OF SIGNALLING PATHWAYS INVOLVED IN EMBRYONIC SURFACE FORMATION

4.1 Introduction

Our studies of the CRS peptide have highlighted the fact that CRS contains a unique and conserved C-terminal domain which is a potential site of glycosylation. In addition, CRS is necessary for the production of a cell-wall epitope in the endosperm which is generally associated with EXTENSINS, a class of hydroxyproline rich glycoproteins. To date, however, mutants in genes usually associated with the glycosylation of hydroxyproline-rich glycoproteins have not been shown to have significant defects in seed development, although an extensive survey has not yet been undertaken.

In contrast, recent work has identified a protein potentially involved in other types of protein glycosylation, which could be implicated in the embryo sheath

deposition pathway. Previous work in the laboratory identified a mutant in the background of a T-DNA insertion line, called *mis-shapen seed sticky seedling (msss)* due to frequent cotyledon fusions. Seeds mutant for *msss* were phenotypically very similar to *gso1 gso2* double mutants, but *msss* acted as a single recessive locus. Like *gso1 gso2* double mutants, *msss* seeds are misshapen due to an apparent adhesion between the endosperm and embryo. Mapping *msss* led to the conclusion that the causal lesion was situated in a group of around 15 genes at the extreme distal tip of chromosome 5. Unfortunately the mutation was caused by the deletion of this genomic region, transgenic complementation experiments using single genes from the region gave no conclusive results, and the project was abandoned (G. Ingram, personal communication). However, more recently Neumetzler et al., 2012 identified one of the genes in this cluster, *FRIABLE1 (FRB1)*, as causing an apparently identical phenotype to that observed in *msss.*

FRIABLE1(FRB1) encodes a unique putative O-fucosyltransferase, a distantly related member of a family of enzymes that, in animals, are involved in the fucosylation of serine and threonine residues, including those found in Epidermal Growth Factor (EGF), a peptide ligand (Verger et al., 2016). The enzymatic activity of FRIABLE1 has not yet been elucidated, although several proteins, including several RLKs of the WALL ASSOCIATED KINASE (WAK) clade, contain EGF-like motifs (Verger et al., 2016). Recently, Verger et al., showed that the cotyledons of *frb1* seedling are coated with material, similar to the situation observed in *zou* mutants (Yang et al., 2008). Furthermore, Verger et al., went on to show that this material was rich in pectin. Recent work from the Ingram lab has shown that pectic epitopes are abundant in the endosperm during its development (Fourquin et al., 2016). It is therfore possible that *frb1* mutants have an embryo/endosperm separation phenotype similar to that observed in mutants of the GSO1/GSO2/ALE1 signalling pathway.

Interestingly, the main focus of the two papers so far published describing *FRB1* is almost exclusively on the fact that *frb1* mutants show defects in cell adhestion in expanding seedlings, although Neumetzler et al., do mention apparent defects in seedling cuticle permeability. Both groups showed changes in cell wall composition in *frb1* seedlings compared to wild-type seedlings, but without making reference to the adhering material on these seedlings described in Verger et al.. Loss of function *frb1* mutants are reported to show alterations in galactose- and arabinose-containing oligosaccharides, in pectin methylesterification and pectin load, in cell wall associated

extensins and in xyloglucan microstructure (Neumetzler et al., 2012; Verger et al., 2016).

We previously hypothesized that apparent cell adhesion defects in *zou* mutant seedlings could be an indirect consequence of defects in expansion caused by the adhesion of endosperm materials to the embryo surface (chapter 2 and (Yang et al., 2008). Consistent with this idea, we noticed that neither *msss* not *frb1* mutants, nor indeed *zou* mutants or *gso1/gso1* mutants, show obvious cell adhesion defects during post-embryonic development (i.e in tissues other than seedling tissues).

In light of these observations, and above all in light of the strong similarity between the phenotypes of *gso1 gso2* double mutants and *frb1* mutants, we hypothesised that *frb1* could play a primary role in the formation of the embryo sheath. The phenotypic and genetic analyses carried out to investigate this possibility are presented in this chapter.

4.2 RESULTS

4.2.1 FRB1 is necessary for a proper embryonic cuticle establishment

To investigate the function of FRB1, we obtained the the *frb1* null allele *frb1-2* described by Neumetzler et al . Defects in cuticle permeabily have already been reported by Neumetzler et al for *frb1-2*, although not studied precisely. We therfore proposed to carry out the functional characterization of FRB1 function in the context of embryonic cuticle formation. The mature seeds of *frb1-2* mutant are mis-shapen, with an identical phenotype to that previously described for *gso1 gso2* and *ale1-4* seeds (Figure 24). This phenotype is fully penetrant, as is the case in *gso1 gso2.* In *gso1 gso2* mutants this phenotype is resulting from the sticking of a cotyledon to the testa at around the heart stage of seed development. We therefore investigated the origin of this phenotype in *frb1-2* mutants using WT, *ale1-4* and *gso1 gso2* seeds as controls (Figure 25A-D). We found that the stuck-cotyledon-induced bending of the embryo is identical in *gso1 gso2, ale1-4* and *frb1-2* mutants.

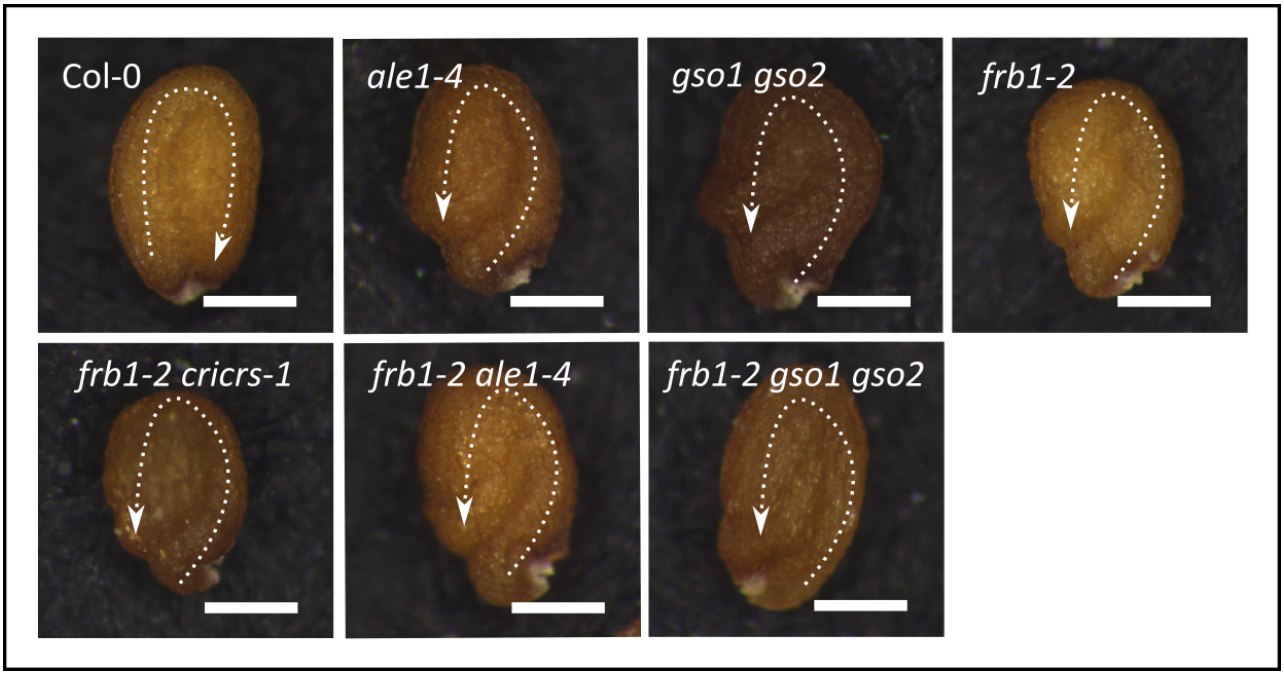


Figure 24: Mature seeds from the following genotypes : Col-0, *ale1-4, gso1 gso2, frb1-2, frb1-2 ale1-4, frb1-2 gso1 gso2.* Scale 100µm

In a subsequent set of experiments we investigated whether the permeability of the cuticle is similarly affected in *frb1-2* and *gso1 gso2* mutants. We therefore performed qualitative toluidine blue assays on etiolated WT, *frb1-2* and other known mutants seedlings (Figure 26A). The dark blue coloration of both *frb1-2* and *gso1 gso2* cotyledons, contrasts strongly with the absence of coloration in those of the WT seedlings, and confirms that *frb1-2* is strongly affected in embryonic cuticle integrity. To confirm this observation, we quantified the uptake of TB, and, consistent with the results of our qualitative assay, the results showed a high level of TB permeability for *frb1-2* mutants, similar to that obtained for *gso1 gso2* mutants and significantly different from that obtained in WT conditions (Figure 26B). Therefore, we conclude that FRB1 plays an essential role in mediating embryonic cuticle formation.

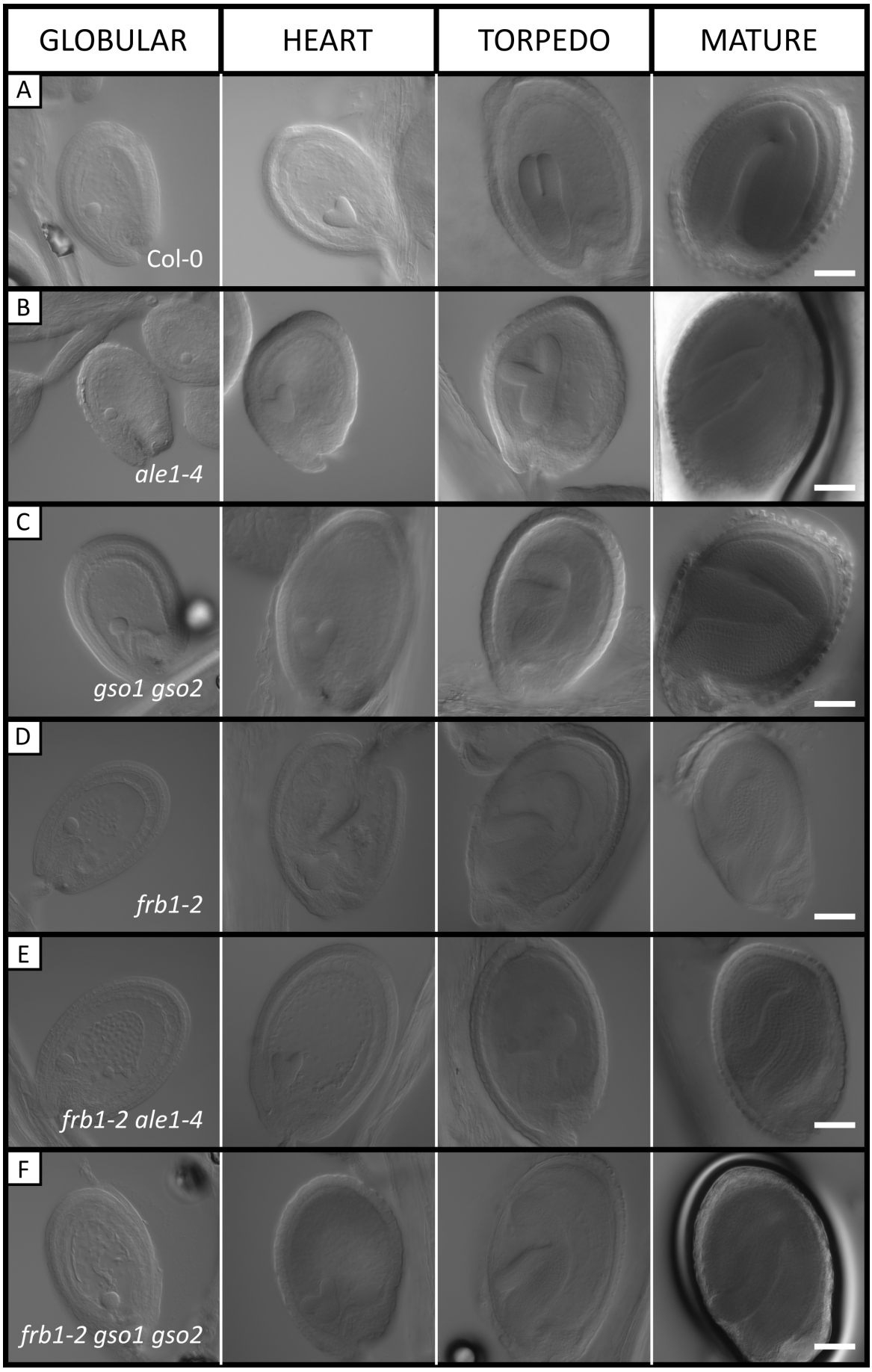
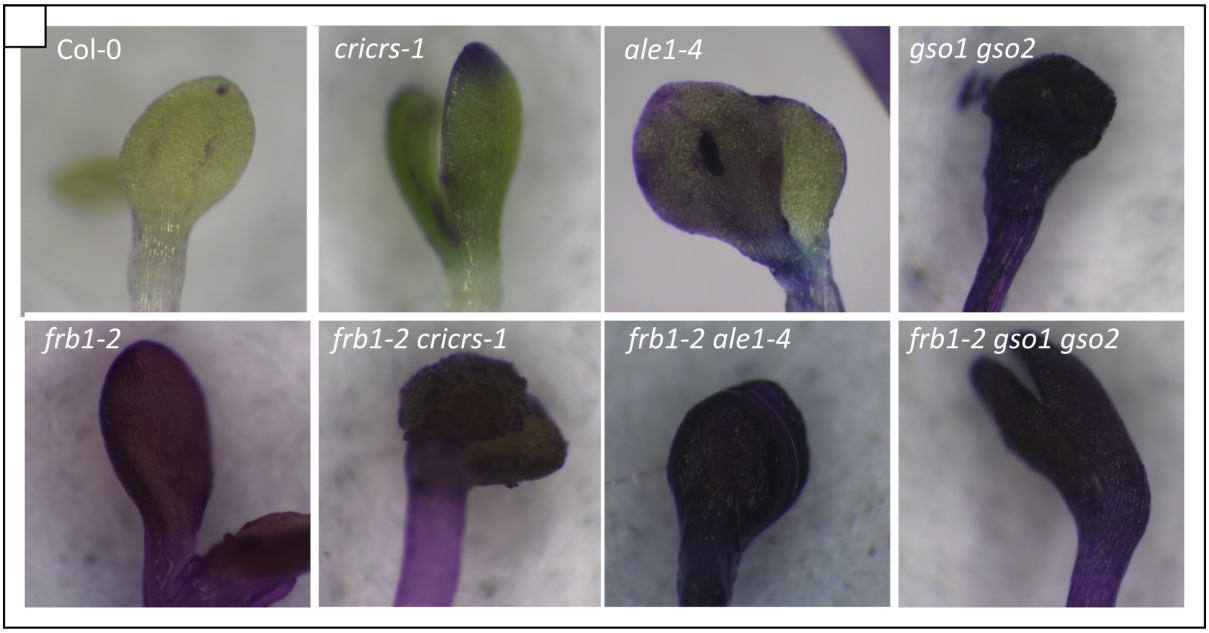


Figure 25: Clearing of developing seeds. For each genotype, the globular, heart torpedo and mature stages are presented. (A) Col-0, (B) *ale1-4*,(C) *gso1 gso2*, (D) *frb1-2*, (E) *frb1-2 ale1-4* (F) *frb1-2 gso1 gso2*. Scale 100µm

A



B

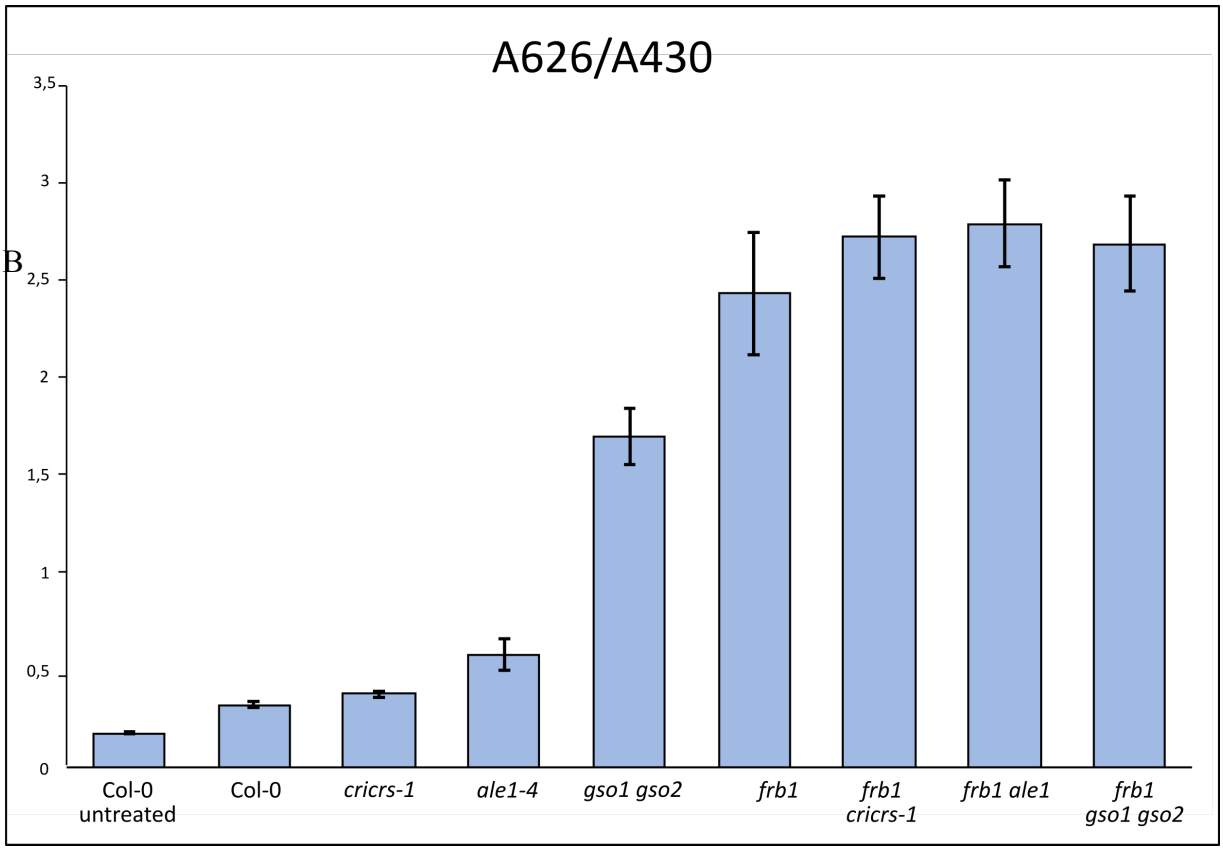


Figure 26: Qualitative and quantitative analysis of cuticle permeability. (A) Toluidine blue assay on various mutants. Scale 100µm. (B) Toluidine blue uptake quantification in various mutants.

4.2.2 FRB1 is expressed during seed development

No expression data for *FRB1* is available in public microarray datasets. However, the expression of the GUS reporter under the control of the *FRB* promoter was studied by Neumetzler et al., 2012, and *FRB1* promoter activity was detected in the cotyledons of very young seedlings, hinting at an embryonic expression. To obtain a seed specific view of *FRB1* expression, we performed *in situ* hybridization experiments targetting *FRB1* mRNA (Figure 27). We observed *FRB1* expression in the developing embryo throughout seed development from as early as the globular stages (Figure 27A). Later, the signal remains in the embryo, with a seemingly slightly darker signal in the outer layer of the embryo, which suggests a potential preferential expression of *FRB1* in the epidermis (Figure 27B-D). This pattern is slightly reminiscent of the expression patterns of *GSO1* and *GSO2* in developing embryos (Creff et al., in revision), although the epidermis specificity of *FRB1* expression is considerably less marked.

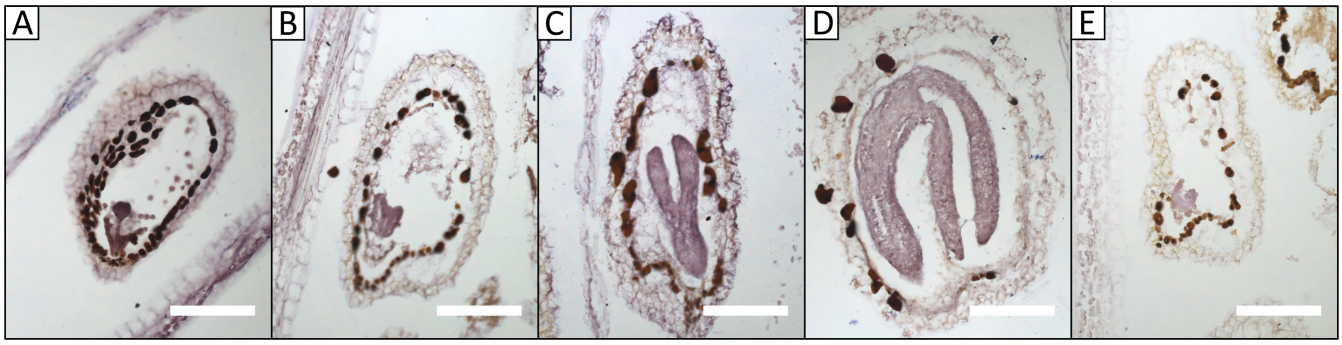


Figure 27: *In situ* hybridization against *FRB1* mRNA. Signal is revealed by a purple coloration. The dark brown circles are background from the endothelium. (A) Globular, (B) Heart, (C) Torpedo, (D) Mature (E) Negative control. Scale bar: 200µm

4.2.3 *FRB1* expression is not altered in *gso1 gso2, ale1-4* and *zou-4* mutant backgrounds

We have found it difficult to generate high quality *frb1-2* RNA samples from developing seeds in order to show conclusively whether other pathway components show altered expression levels in *frb1-2* mutants. However, consultation of RNA-seq data available in the laboratory shows that *FRB1* expression levels in developing seeds

are not altered in *gso1 gso2, ale1-4* and *zou-4* mutant backgrounds (Xing et al., 2013; Creff et al in revision). Thus *FRB1* expression is likely not affected by GSO1/GSO2 signalling.

4.2.4 FRB1 act in the same genetic pathway as ALE1 and GSO1 GSO2

To understand the basis for the clear phenotypic similarities between *frb1, ale1 and gso1 gso2* mutants, we generated multiple mutant combinations to uncover their genetic relationships. We were able to obtain *frb1-2 ale1-4* double mutants, as well as *frb1-2 gso1 gso2* triple mutants. We performed the very same analyses on this mutants as the carried out with *frb1-2* single mutants. The seed shape phenotype of both *frb1-2 ale1-4* double and *frb1-2 gso1 gso2* triple mutants is identical to that in *frb1* single and *gso1 gso2* double mutants (Figure 24E-F). When developing seeds from *frb1-2 ale1-4* double and *frb1-2 gso1 gso2* triple mutants were subjected to clearing and microscopc analysis we also found identical embryo bending phenotypes to those observed in *frb1* single and *gso1 gso2* double mutants (Figure 25). Finally, both qualitative and quantitative analysis of cuticle permeability in *frb1-2 ale1-4* double and *frb1-2 gso1 gso2* triple mutants gave very similar results to those obtained in *frb1* single and *gso1 gso2* double mutants (Figure 26). Our results support the hypothesis that FRB1, ALE1, GSO1 and GSO2 act in the same signalling pathway controlling embryonic cuticle integrity. Additionally, we investigated the phenotype of the *frb1 cricrs-1* double mutant and did not find any phenotypic differences compared to the *frb1-2* single mutant *(*Figure 24 and Figure 26).

4.2.1 FRB1, like GSO1 and GSO2, is required for the release of sheath material onto the embryo surface.

As demonstrated in the first results chapter of my thesis, cuticular integrity shows an apparent correlation with the deposition of the embryo sheath surrounding the embryo. Notably, I demonstrated that in *gso1 gso2* mutants, this deposition is altered. In these mutants sheath material (labelled with the JIM12 and LM1 antibodies) is produced apparently normally in the endosperm surrounding the developing embryo, but this material, which in wild-type seeds coats the embryo surface, is apparently not released from endosperm cells in *gso1 gso2* mutants, leaving the embryo apparently devoid of its sheath. We therefore tested for the presence/absence of the JIM12 signal around the embryo of *frb1-2* mutants (Figure 28). We found that like the seeds and

cuticle permeability phenotypes of this mutant, the immunohistochemical phenotype of *frb1-2* mutant seeds is identical to that of *gso1 gso2* seeds, with the presence of a JIM12 epitope clearly visible in the embryo-surrounding endosperm, but a total absence of this epitope around the developing embryo. This result appears to further confirm the genetic interaction between GSO1, GSO2 and FRB1, with strikingly similar phenotypes in all the seed phenotypes investigated.

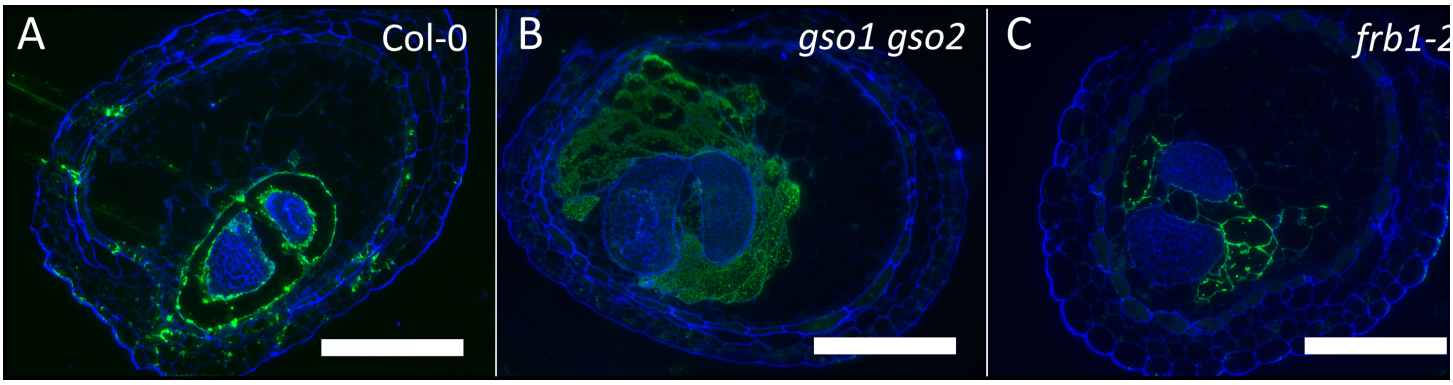


Figure 28: JIM12 antibody immunolocalizations used to reveal the embryo sheath. Transverse sections o seeds allow the vizualisation of the two cotyledons. (A) Col-0,(B) *gso1 gso2* (C) *frb1-2*. Scale bar: 100µm

4.2.1 *FRB1* is not involved in Casparian strip formation.

Because of the remarkable similarity between the seed phenotypes of *frb1* and *gso1 gso2* mutants we tested whether FRB1 might also be involved in Casparian strip formation. We therefore tested for Casparian strip permeability in *frb1-2* mutants (Figure 29), using WT as a negative control and *gso1 gso2* as a positive control. We used a simple PI permeability test previously published by the group of Professor Geldner (Naseer et al., 2012). The test is based on the fact that in roots with an intact Casparian strips, exogenously applied PI cannot enter the stele via the apoplastic pathway. However if Casparian strip integrity is disrupted, PI will enter and stain the stele of the root. After 10 minute of PI treatment treatment, we found, as expected, that the intact Casparian strip in wild-type seedlings does not let PI passing through, leading to a dark stele (Figure 29). In contrast defects in the Casparian strip lead to the penetration of PI into the stele of *gso1* gso2 mutant roots (Figure 29). However, we found that the *frb1-2* mutant has no coloration inside the stele after treatment,

suggesting that the Casparian strip of this mutant is entirely functional (Figure 29). Thus, FRB1 does not have a function in maintaining Casparian strip integrity.

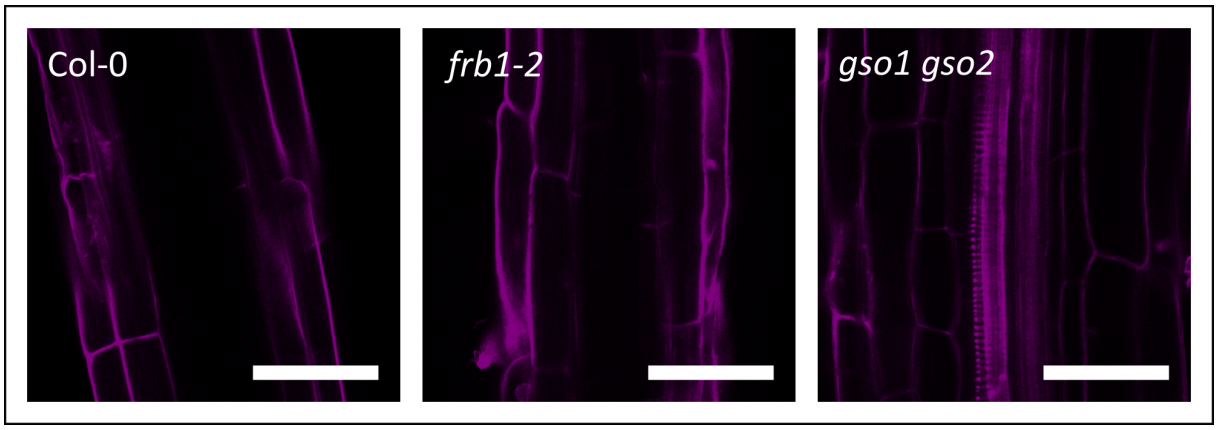


Figure 29: Confocal images of roots stained with external Propidium Iodide (PI) treatment. This represents an assay for Casparian strip integrity. Col-0, *frb1-2* and *gso1 gso2* are presented. Col-0 is a negative control, i.e. the Casparian strip is intact, and *gso1 gso2* is a positive control with a permeable Casparian strip that allows penetration of PI into the stele. A similar phenotype is not observed in *frb1-2*

4.3 DISCUSSION

Phenotypic and genetic evidence provided here strongly support the involvment of FRB1 in the same signalling pathway as GSO1, GSO2 and ALE1 in the regulation of the integrity of the embryonic cuticle. However, the precise function of this protein clearly remains to be determined.

FRB1 belongs to the GT-65 family of glycosyl transferases (Hansen et al., 2009; Neumetzler et al., 2012). Based on this fact, its role almost certainly involves the addition of sugar moieties to protein substrates. The fact the the transcription of *FRB1* is not regulated in response to GSO1/GSO2 mediated signalling suggests a relatively "upstream" role for FRB1 in the signalling pathway. Based on this, it is tempting to speculate that FRB1 could be involved in the production and/or modification of the GSO1/GSO2 ligand. However an important observation argues againt this hypothesis. Our preliminary expression data suggest that FRB1 is more strongly expressed in the embryo than in the endosperm. Since functional FRB1 protein has been shown to

localised in the secretory system (Neumetzler et al., 2012), it seems unlikely that it would have a non cell autonomous action.

Given the remarkably strong similarity between the phenotypes of *frb1* and *gso1 gso2* mutants, another possibility is that FRB1 acts to facilitate the activity of the GSO1 and GSO2 RLKs. The idea that plant receptor kinases could be post-translationally modified by glycosylation is not new. For example, N-glycosylation of RLKs involved in the perception of Pathogen Associated Molecular Patterns (PAMPs) has been proven to be essential to mediate plant immunity (Häweker et al., 2010). In addition, as mentioned in the discussion, EGF-like repeats found in several plant RLKs are known to by glycosylated in animals (Verger et al., 2016). In our case of study, GSO1 and GSO2 might therefore require FRB1 mediated O-Glycosylation/Fucosylation to ensure their function. The similar expression patterns of *GSO1, GSO2* and *FRB1*, the identical phenotypes of *frb1-2* and *gso1 gso2* mutants, and the absence of phenotypic additivity between these mutants all support this hypothesis. However it is impossible at this point to distinguish between the possibility that GSO1 and GSO2 are direct targets of glycosylation by FRB1, and the alternative idea that GSO1 and GSO2 could associate with glycosylated co-receptors. The observation that FRB1 is not required in Casparian strip formation is particularly intriguing in this context. It is possible that modifications of the GSO1 and GSO2 ectodomains might be specific to the seed. This might tend to suggest that these receptors perceive different ligands to those perceived in the root by GSO1, a possibility which we cannot exclude based on the data available to date.

Although the idea that FRB1 could be involved in receptor decoration appears to agree with our observations, we cannot currently exclude the the possibility that FRB1 mediates the decoration of other molecules including signalling peptides, which are known to make common use of glycosylation (Canut et al., 2016). It is certainly not possible, based on our in situ hybridisation results, to state categorically that *FRB1* is not expressed in the endosperm. In this scenario, FRB1 could also be implicated in decorating the sheath epitope-containing protein, with glycosylation necessary for secretion but not for generating the JIM12/LM1 epitope.

To start to resolve the mystery of FRB1 function, it will be imperative to undertake experiments aimed at understanding the spatial requirement for FRB1 activity. To this

end we have generated constructs which will allow expression of *FRB1* specifically in the embryo epidermis (under the promoter of the auxin efflux carrier-encoding gene *PIN1*) or in the endosperm (under the *RGP3* promoter) of developing seeds. The ability of these constructions to complement the *frb1-2* mutant will be assessed.

In an unexpected twist to the story of FRB1, very recently (in the last month of my thesis) mutants defective in a gene named *TWISTED- SEED1 (TWS1),* which encodes a unique, small ER-localised protein of unknown function, have been shown to have a similar, if not identical phenotypes to those shown by *frb1* mutants (Fiume et al., 2016). Intriguingly, the *FRB1(AT5G01100)* and *TWS (AT5G01075)* genes are separated by only two other annotated genes at the extreme distal end of chromosome 5. Unsurprisingly given their close association, both genes fall within the interval deleted in the previously characteristed *msss* mutant, explaining why the *msss* mutants could not be complemented by reintroduction of the *FRB1* gene alone. The fact that the seed phenotype in *tws, frb1* and *msss* mutants appear to be indistinguishable suggests that *FRB1* and *TWS* likely act in the same pathway. Understanding the functional link between the activity of TWS and FRB1, and other components in the embryonic cuticle integrity pathway will likely provide novel and exciting information on the molecular regulation of RLK-mediated signalling in plants.

5 GENERAL DISCUSSION

5.1 General considerations on the embryonic cuticle & sheath

The general focus on my PhD has been on the molecular mechanisms leading to the formation of a functional embryonic surface. Ignoring the genetic actors, which will be further discussed in the second part of this chapter, I will first focus on how my results have changed our view both of the structure of the embryonic surface, and ofg how separation of this surface from surrounding tissues is achieved.

5.1.1 The embryonic cuticle and its biogenesis

Figure Prior to this work, we had very little idea of when and how the embryonic cuticle arose on the embryo surface. TEM analysis has now revealed that embryonic cuticle deposition initiates in a patchy manner, rather than through the deposition of a continuous layer of cutin, which thickens during seed development. No cuticle is found at the 4-8-cell stage, and patches start to be deposited at the globular

stage, to finally produce a continuous structure from the late globular-heart stage onwards (Figure 30). After this stage in wild-type embryo development we never observed breaks in the embryonic cuticle. Interestingly, this "patchy" initiation of cuticle deposition is reminiscent of observations made during the deposition of the cuticle in insect larvae (Andersen, 1979), hinting at broadly conserved cuticle deposition mechanisms across kingdoms. One possible explanation for this patchiness is that each patch represents the contents of only one, or a limited number of secretory vesicles. If the lateral diffusion of cuticle components within the epidermal cell wall were relatively restricted, this could lead to an initially patchy deposition of cuticle components. It would be interesting to develop methods of observing cuticle deposition in real-time, although whether this is possible in the context of the early developing embryo is doubtful.

In mutants specifically affected in the embryonic cuticle integrity pathway discussed in my thesis, ultrastuctural evidence shows defects in cuticle continuity rather than in production. In agreement with this interpretation, cuticle load analysis (Creff et al, in revision) shows no significant differences between WT and mutants in this pathway. This suggests that at least two distinct mechanisms are involved in producing a continuous embryo surrounding cuticle layer. One is the biosynthesis and secretion of the cuticular material, which likely occurs in a similar way in embryo protoderm cells as in protodermal and epidermal cells in the aerial parts of the post-germination plant. The initiation of cuticle biosynthesis is likely to be directly linked to the acquisition of embryonic epidermis integrity in the apical regions of the very early embryo. The second mechanism, specifically studied in my thesis, appears to be involved in assuring the integrity of the cuticle, thus ensuring the filling of holes present in the nascent cuticle. We propose that this pathway acts throughout embryogenesis, likely also playing a role in filling defects and breaks that could arise during embryo expansion. Molecular evidence supporting this hypothesis will be discussed in the second part of this chapter.

5.1.2 The embryo in need of external cues?

The conclusion that an integrity checking mechanism is at play during zygotic embryo development, but is apparently not necessary during post-embryonic development is very intriguing. Why is such a mechanism necessary? Experiments

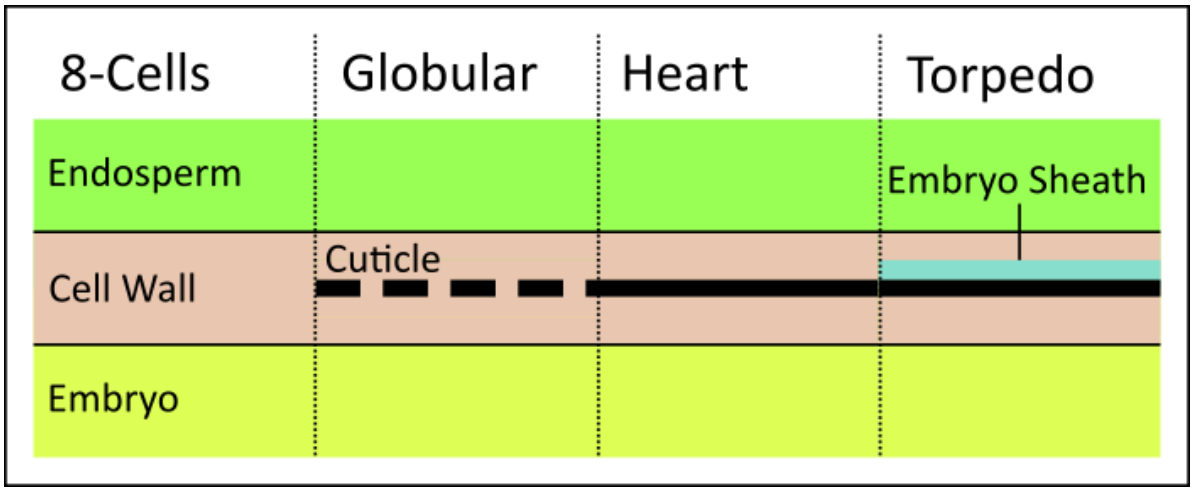


Figure 30: Developmental timing of the embryonic surface formation. Cuticle and embryo sheath deposition are represented.

conducted on Carrot somatic embryos may give some useful insights regarding embryonic cuticle formation. The experiments performed in this study were focused on the comparison of somatic embryogenesis on solid media versus somatic embryogenesis in liquid media (Dobrowolska et al., 2012). The relevant observation made in this study was that when in contact with air, somatic embryos developed a detectable cuticle, while in liquid media, embryos developed without an obvious cuticle. One interpretation of these results could be that exposition to the air (potentially inducing oxidative stress) might act as a strong signal "locating" the embryonic surface, and, importantly, alerting the embryo to breaks in the cuticle. In contrast, embryos in liquid media might not perceive this stress and leading to the production of embryos lacking a defined cuticle. Intuitively, the seed context is very similar to the liquid media context, the embryo being surrounded by the endosperm. In such case, the embryo may need the provision and perception of an informative signal from the outside to define its boundaries, and to verify whether its cuticle is intact.

5.1.3 The physiological function of the embryonic cuticle

The presence of a pathway monitoring cuticle integrity suggests that the embryo requires an intact cuticle to assure its normal development. Why should this be? One of the most evident answers is to prepare the embryo for germination, at which point the young seedling may suddenly and brutally encounter desiccating conditions. The importance of an intact cuticle at germination is underlined by the fact that several

mutants in the pathway we have been studying are affectively non-viable when sown directly on soil (Xing et al., 2013). Thus an intact cuticle must be formed during embryogenesis to allow the embryo go through this harsh transition. Germination of these mutants in humidity-saturated environment such as MS media petri dishes allows for a rather normal germination rate.

If seedling protection were the only function of the cuticle, then its formation as an intact barrier from the heart stage onwards would be excessively early. It seems likely therefore that the cuticle plays other roles during early seed development. One possibility is that the cuticle might selectively control the movement of nutrients within the developing seed. The cuticle has been characterized as a selective hydrophobic barrier (Schreiber, 2005). Thus, the cuticle might help in discriminating debris from useful nutrients originating from endosperm elimination. However this level of selectivity seems unlikely. In addition our experiments with the movement of apoplastically localized fluorescent proteins, suggests that the embryo cuticle is surprisingly "tight" from an early stage. Another possibility is therefore that the embryonic cuticle, rather than regulating nutrient uptake, could prevent the leakage of metabolites, which are thought to be principally taken up by the embryo though its suspensor and root pole (although this has not been conclusively shown) out of its apical regions and into the endosperm. To test this it would be interesting to measure metabolite leakage from integrity mutants and wild-type embryos over time.

A further potentially important role of the embryonic cuticle, which is perhaps better supported by both our results and the literature, could be to prevent embryo damage by endosperm cell wall modifying enzymes. Such enzymes have recently been shown to be involved in endosperm elimination through endosperm softening/cell separation coupled with embryo growth (Fourquin et al., 2016). If such cell wall softening enzymes made their way from the endosperm to the embryo, embryo cell adhesion defects would be an expected consequence. Both published (Neumetzler et al., 2012; Yang et al., 2008) and preliminary data not shown in this manuscript, suggest that such defects are observed in the embryos of all cuticle integrity mutants. In addition, it is important to note that in wild-type embryos, cuticle integrity is achieved at, or just before the precise developmental time-point when these enzymes start to be expressed in the endosperm (Denay et al., 2014; Fourquin et al., 2016).

The second part of chapter 2 addresses partially the question of the function of the cuticle as a protein diffusion barrier, showing an absence of movement of CRS-

mCitrine proteins from the endosperm into the wild-type embryo apoplastic space, while embryos from mutants such as *gso1 gso2* and *ale1-4* clearly show signal within their walls. This indicates that the cuticle acts as a barrier to proteins with a size larger than about 38kDa and that defects in the structure impair this function. Most cell wall modifying enzymes fall within this size category. In the future, investigating the diffusion of small hydrophilic fluorescent dyes and FP multimers *in vivo* should lead to better characterization of the cuticle diffusion selectivity in both wild-type and mutant plants.

Although the idea that the cuticle protects the embryo from endosperm-specific cell wall modifying enzymes is very seductive, uncoupling the effect of such enzymes from biophysical defects induced by the lack of an intact cuticle may prove to be challenging. TEM analysis of embryo cell walls and/or the use of further immunofluorescence studies of the accumulation of cell wall epitopes may be informative in this context.

Finally, and importantly, it has been proposed in the literature that the formation of the embryonic cuticle plays an important role in mediating the separation of the embryo from surrounding endosperm tissues (Tsuwamoto et al., 2008; Xing et al., 2013). However, in the course of my thesis we have described the deposition of an additional extra-cuticular structure we called the "embryo sheath". This structure surrounds the cuticle from heart stage onwards, and we show that it plays a critical role in embryo-endosperm separation. This throws into question the precise role of the embryonic cuticle in mediating embryo/endosperm separation.

5.1.4 Does the embryonic cuticle or the embryo sheath mediate proper separation of the embryo and the endosperm?

The embryo and the endosperm originate from the egg-cell and the central-cell of the female gametophye, and at fertilization they therefore share a common cell-wall. The deposition of the cuticle, and later of the embryo sheath, appear to be essential in allowing a proper separation of the two tissues, setting up a physical boundary between the two. However, our results suggest that the formation of an intact cuticle and the

embryo sheath appear to be integrally related. An important question is how to separate the functions of these two structures. A key first step will be to analyse whether embryonic sheath formation and endosperm/embryo separation occur normally in mutants with defective cuticle biosynthesis. This is, however, challenging. For example, analysis of double *gpat4 gpat8* mutant embryos (Li et al., 2007) show that these embryos, which are defective in cutin biosynthesis have dramatically reduced cutin levels in embryonic cotyledons (Creff et al., in revision). However, phenotypic analysis of mutant seedlings shows that their cuticle is less permeable to toluidine blue than that of *ale1* mutants. These mutants show few seed shape defects suggesting that embryo/endosperm separation is relatively normal. However even if sheath deposition in this background is normal, its lack of cuticle permeability will make drawing conclusions different. It appears probable that mutants totally unable to produce cuticle, are embryo lethal due to the tight link between the presence of a cuticle and epidermal cell fate maintenance (Delude et al., 2016; Javelle et al., 2011).

Conversely, it would be interesting to identify mutants in which embryo sheath production is lost in the absence of cuticle biogenesis defects. It is possible that *crs* mutants represent such a situation, however further studies of CRS function (below) will be needed to clarify this.

5.1.5 What is the function of the embryo sheath?

The embryo sheath surrounds the embryo, starting at the heart stage, and persists until embryo maturity. It is composed of extra-cuticular material and appears translucent to electrons in TEM experiments on Osmium tetroxide stained samples, arguing against either a high concentration of proteins or of lipids in this structure. The presence of glycoproteins in the sheath is strongly supported by its immune-reactivity to JIM12 and LM1 in immunolabelling experiments. The absence of an intact sheath is, in our experiments, unambiguously associated with defects in embryo/endosperm separation. One of the main functions we can attribute to the embryo sheath is therefore to facilitate embryo growth by allowing a smooth displacement of the growing embryo within the endosperm tissues. CRS, is a member of the STIG1-like peptide family, of which other members have been shown to be necessary for PT growth (Tang et al., 2004). Obvious parallels exist between embryo and PT growth in terms of tissue

invasion. However further ultrastructural investigation of the embryo sheath in WT and mutant conditions may reveal more about its function.

Finally, and critically, we will need to carry out ultrastructural analyses of *crs* mutants. These experiments have not been completed due to time constraints, but they will be critical in determining when and what kind of embryonic surface defects arise in *crs* mutants.

5.2 The molecular mechanisms behind embryonic surface formation: A genetic dissection

The control of embryonic cuticle identity involves inter-tissue signaling between the endosperm and the embryo. The combination of previously identified genes and proteins, with those newly characterized in my thesis, provides a complex picture that can be very difficult to comprehend. In this section, I will therefore review each member of the extended pathway, their genetic interactions and the spatio-temporal specificities of their expression. My aim is to produce a working model of potential molecular signaling mechanisms regulating embryonic surface formation and cuticle integrity maintenance, taking into account all the genetic and molecular evidence available to date.

5.2.1 Summary of the genetic actors involved: Spatio-temporal expression profiles and functions.

In this section we review the identities and functions of the various genes involved in the signaling pathway. We will review these genes based on the spatial localization of their expression. Consistent with its function in transmitting information between the endosperm and the embryo to ensure the formation of the embryonic surface, we find genes specifically expressed in the endosperm and others in the embryo.

5.2.2 In the endosperm.

5.2.2.1 ZOU: a bHLH transcription factor

ZOU encodes a bHLH transcription factor with two partially separable functions (Xing et al., 2013; Yang et al., 2008). Firstly, ZOU, together with its bHLH partner ICE1 acts as an important regulator of endosperm breakdown (Denay et al., 2014; Fourquin et al., 2016). Mutants in both *ZOU* and *ICE1* have a persistent endosperm. Recent research has shown that this function is likely mediated by the regulation of the expression of endosperm-specific cell wall modifying proteins that cause cell wall softening/cell separation in the endosperm from the heart stage of development onwards. Secondly, and possibly more importantly in the context of this thesis, ZOU is essential for the formation of the embryonic surface (Xing et al., 2013; Yang et al., 2008; Creff et al., in revision), notably through the activation (probably indirect) of the expression of both *CRS* and *ALE1*. Loss of *ZOU* function not only leads to the production of a discontinuous embryo cuticle, but also to a complete lack of production of the embryo sheath, likely explaining the severe adhesion observed between the embryo and endosperm in *zou* mutants.

In terms of expression, activity of the *ZOU* promoter is detected in the endosperm directly after fertilization. However, functional ZOU:GFP protein fusions driven from the same promoter are not detected until the heart stage in development (Yang et al., 2008). This discrepancy could be due to post-translational control, although the mechanism has not yet been elucidated. Consistent with the idea that active ZOU protein is not present before the heart stage, expression of the direct ZOU target *RGP3 (Denay et al., 2014),* and of several other targets of ZOU which may or may not be direct (Fourquin et al., 2016), also initiates at the heart stage of seed development (Denay et al., 2014). The transcriptional control activity of the ZOU/ICE1 complex therefore initiates with the onset of endosperm breakdown. ZOU expression is only ever detected in the developing endosperm, and has never been detected in other tissues, even under the influence of a range of biotic and biotic stresses (unpublished data).

5.2.2.2 ALE1: a subtilisin-like serine protease.

ALE1 was the first gene to be characterized with a function in embryonic cuticle formation (Tanaka et al., 2001; Xing et al., 2013). The *ale1* mutant phenotype is relatively mild compared to that of other mutants such as *gso1 gso2* mutants, suggesting the involvement of one or more other genes in mediating embryo surface formation.

TEM experiments corroborate this result, showing that *ale1* mutants present defects in the cuticle at the heart stage that appear to be repaired by later stages. This contrasts with the situation in *gso1 gso2* mutants where persistent holes are observed throughout embryo development. Although *ale1* mutants clearly show cuticle defects, defects in sheath formation are not clear, with most of the embryonic surface covered normally in sheath material.

Interestingly, although *ALE1* expression appears fully dependent on ZOU function from the heart stage onwards, *ALE1* is also expressed in the endosperm of *zou* mutants during early development (Xing et al., 2013). This suggests the involvement of another TF in mediating early *ALE1* transcription. Furthermore, experiments involving complementation of *zou* mutants by expressing *ALE1* independently of ZOU function in the endosperm, only partially restored the cuticle phenotype, hinting again at the involvement of other effectors (Xing et al., 2013). To date, the substrate of ALE1 has not been identified. Again *ALE1* expression appears to be completely restricted to the developing endosperm.

5.2.2.3 CRS: a secreted peptide

The main part of my PhD has been focused on the characterization of CRS function. CRS is a STIG1-related peptide with a unique proline-rich C-terminal domain, which is expressed specifically from the heart stage of seed development onwards, with expression in the seed strictly dependent on ZOU function and confined in the embryo-surrounding zone of the endosperm. Mutants harbor a slightly defective cuticle, as well as strong embryo endosperm separation defects associated with an apparently almost complete lack of embryo-sheath formation. Preliminary results obtained a few days before submission of this manuscript show that *crs ale1* double mutant have an additive/synergistic phenotype, with almost all seeds harboring a *gso1 gso2* like phenotype. Taken together, these results suggest that ALE1 and CRS act in parallel to control embryonic surface formation, even though both are under the (indirect) transcriptional control of ZOU from the heart stage of development onwards.

5.2.2.4 TPST: a peptide modifier

TPST was originally characterized as a key regulator of root development with a biochemical function in the tyrosyl-sulfation of secreted peptides, allowing them to be fully functional and bind their matching receptors (Amano et al., 2007b; Komori et al., 2009; Zhou et al., 2010). We started to investigate the function of this protein very recently, when our collaborator identified it as involved in the tyrosyl-sulfation of a peptide (PEP-X) involved in binding to GSO1 during Casparian strip formation. TPST appears to be broadly expressed during seed development, with a preferential expression in the early micropylar endosperm. Functionally, TPST is indeed required for embryonic cuticle integrity, with a strong cuticle integrity and seed shape phenotype observed in mutants. It is interesting to note that CRS contains tyrosine residues that could be targeted for sulfation by TPST.

5.2.2.5 PEP-X and PEP-X': two potential signaling peptides ?

PEP-X has been shown to be important ligands interacting with GSO1 during Casparian strip formation by our collaborator. Our preliminary expression analysis revealed that the PEP-X-encoding gene is expressed early during seed development, although apparently not at early stages, nor at high levels, and not specifically in the embryo-surrounding endosperm as might have been expected. *PEP-X'*, encoding a related peptide is apparently not expressed in the seed. The functional importance of PEP-X and PEP-X' in seeds has yet to be tested with the investigation of mutants and/or with overexpression approaches in seeds. Interestingly however, both PEP-X and PEP-X' are strong candidates as targets of ALE1 or other related proteins, as they both contain canonical subtilisin-like serine protease cleavage sites in their protein sequences.

5.2.3 In the embryo.

5.2.3.1 GSO1 and GSO2: The Receptor-Like Kinases

GSO1 and GSO2 are two redundantly acting LRR-RLKs known to regulate embryonic cuticle formation, and acting within the same genetic pathway as ALE1 and ZOU (Tsuwamoto et al., 2008; Xing et al., 2013). The phenotype of double mutants is one of the strongest observed among all the mutants described in this thesis. Mutants are conditional embryo lethal, requiring germination on agar plates to survive the first

stages of seedling establishment. Seeds are systematically misshapen due to adhesion of the developing embryo to the surrounding endosperm/testa. Structurally, TEM analysis has revealed holes in the cuticle of mutant embryos, which persist throughout seed development. Together with their expression patterns in the embryonic epidermis (Creff et al., in revision), these findings make GSO1 and GSO2 the main candidates for the perception of endosperm derived peptides required for cuticle integrity monitoring during seed development.

The role of GSO1 and GSO2 in embryo sheath formation is intriguing. Consistent with their expression in the embryo, these proteins are not required for the production of sheath material in the endosperm, but they are absolutely required for its deposition on the embryonic surface, hinting that GSO1/GSO2 mediated signaling may feed back to the embryo/endosperm interface to mediate this deposition.

5.2.3.2 FRB1: The putative O-fucosyl-transferase

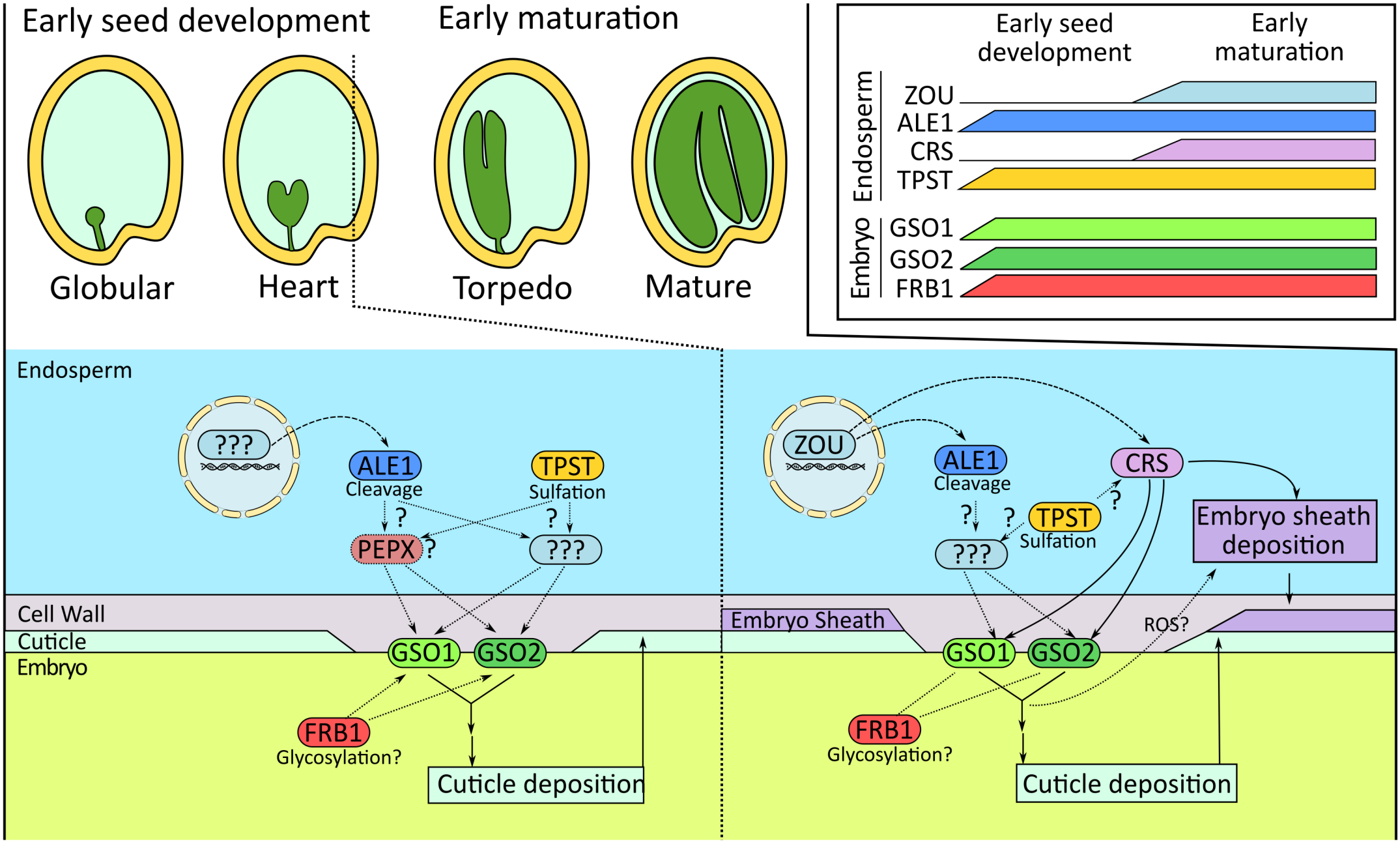
FRB1 in expressed broadly in the embryo throughout the whole of seed development. Mutants lacking *FRB1* function have a phenotype that is strikingly similar to that of the *gso1 gso2* mutant and the triple *frb1 gso1 gso2* mutant does not show phenotypic additivity, placing FRB1, GSO1 and GSO2 in the same signaling pathway during seed development. Consistent with this, *frb1-2 ale1-2* double mutants also show no phenotypic additivity. At the level of sheath formation, *frb1-2* mutants also show exactly the same deposition defect as seen in *gso1 gso2* double mutants. Based on these, and published results (Neumetzler et al., 2012), and despite the fact that it is tempting to propose that FRB1 could be involved in decorating a putative ligand, our current preferred hypothesis is that FRB1 functions by fucosylating or glycosylating either the GSO1 and GSO2 receptors, or co-receptors which are required for GSO1 and GSO2 function in the seed. However the identities of these putative co-receptors (if they exist) has not yet been established. Experiments are currently underway to monitor the behavior of GSO1::VENUS protein fusions in wild-type plants and in the *frb1-2* background in order to explore these hypotheses further.

5.2.4 Embryonic surface formation: A model

Figure 31 presents a state of knowledge model of the pathways involved in embryonic surface formation in Arabidopsis, placing the various proteins involved in their spatio-temporal context, as well highlighting their confirmed and/or potential interactions with other members of the pathway. The main posit of this model is that signaling via GSO1 and GSO2 functions to fill holes/mend damage in the cuticle during embryo development. Such holes result at first from a non-continuous deposition of the cuticle during early seed development, and then hypothetically because of embryo growth-induced rupture of the cuticle. In addition, adhesions between the embryo epidermis and endosperm are prevented by an extra-cuticular embryo structure the sheath, the deposition of which is GSO1 GSO2 dependent and tightly linked to cuticle integrity. The production and deposition of the sheath has therefore been integrated into this model.

We propose that GSO1 and GSO2, whose expression is maintained during the whole of seed development at the embryo epidermis, and whose functionality is likely to depend on the activity of FRB1, serve as sensors of cuticle integrity. If there is a functional cuticle (apoplastic diffusion barrier) between the plasma membrane of the embryonic epidermis and the endosperm, GSO1 and GSO2 will not perceive diffusing signals from the endosperm, and therefore will not be activated. However, if holes are present in the cuticle, they will then be able to perceive apoplastically located signals, originating from the endosperm. Thus we propose that the production of ligands perceived by GSO1 and GSO2, and their post-translational modification, occurs in the endosperm.

Figure 31: (Right) Cartoon representation of the different genetic component involved in the formation of an intact embryonic surface. Temporal (two phases) and spatial (three compartments) are represented. The expression of each gene along seed development in represented in the top right corner. Plain arrows indicate validated genetic interaction. Dotted arrowed indicates potential interaction.


Early seed development
Early maturation
Globular
Heart
Torpedo
Mature
Early seed development
Early maturation
Endosperm
ZOU
ALE1
CRS
TPST
Embryo
GSO1
GSO2
FRB1
Endosperm
???
ALE1
Cleavage
TPST
Sulfation
PEPX
Cell Wall
Cuticle
Embryo
GSO1
GSO2
FRB1
Glycosylation?
Cuticle deposition
ZOU
ALE1
Cleavage
CRS
TPST
Sulfation
Embryo sheath deposition
Embryo Sheath
ROS?
GSO1
GSO2
FRB1
Glycosylation?
Cuticle deposition

Concerning the ligand required for embryo cuticle integrity monitoring during early embryo development, we have not identified the peptide required, although PEP-X is a possible candidate based on its known binding to GSO1 during Casparian strip formation and its sulfation by TPST, which together with ALE we show to be involved in embryonic cuticle integrity maintenance. Whether these modifiers could both act on the same peptide should be revealed by ongoing genetic analysis. Given that the "hole filling" function of GSO1 and GSO2 requires a high degree of spatial resolution, their function must at least partially depend on localized responses at the cytoplasmic level, providing spatial cues about where to deposit cuticular material, and presumably regulating vesicular transport. The mechanisms underlying this process remain to be elucidated

From the heart stage onwards, GSO1 and GSO2 are also involved in triggering the deposition of the embryo sheath on the embryo surface. How this is achieved remains very unclear, especially since sheath material, unlike cuticle material, appears to be produced in the endosperm. Preliminary genetic data support the idea that CRS (required for sheath production) and GSO1 and GSO2 (required for sheath deposition) act in the same signaling pathway, but that ALE1, which acts in the same pathway as GSO1 and GSO2 with respect to cuticle formation, but does not play an obvious role in either sheath production or deposition, acts in a parallel pathway to CRS. CRS is therefore likely not to be a substrate of ALE1. Thus we hypothesize that sheath production and cuticle integrity maintenance may have functional links, but could be effectively independent functions of GSO1 and GSO2-mediated signaling. Again, how this apparent duality in GSO1/GSO2 mediated function is achieved remains to be studied.

Signaling by several RLKs, including the PEPRs (which are closely related to GSO1 and GSO2) causes bursts of extracellular ROS production by stimulating the activity of respiratory burst oxidase homologs (RBOH proteins) (Flury et al., 2013). One possible mechanism via which GSO1/GSO2 mediated signaling could trigger sheath deposition is therefore that ROS is produced by GSO1/GSO2 activity at the embryo surface during early development and could stimulate changes in embryo surrounding endosperm cells leading to the release and deposition of the sheath. Although this hypothesis is interesting, attempts to visualize ROS production in living Arabidopsis seeds have so far failed to give satisfactory results. Whether the CRS

protein itself can actually stimulate the process of sheath deposition, by acting as a ligand for GSO1 and GSO2, in addition to being involved in the process of sheath production, is also not clear, and will be addressed by ongoing studies aimed at functionally dissecting the CRS peptide. These studies should also help us to answer the burning question of whether the CRS peptide itself plays a structural role in the embryo sheath and whether it could, in fact, form the epitope detected by JIM12 and LM1 in the embryo sheath.

In conclusion, the work produced in this thesis has contributed to our understanding of the control of embryonic surface formation in Arabidopsis. We have identified several novel players in this process, and are currently completing analyses aiming to clarify and pinpoint the basis for genetic interactions between these components. Ultimately biochemical analyses (for example ligand/receptor binding studies, studies of ALE1 substrate specificities, and studies of ligand and receptor post-translational modifications) will be necessary to conclusively prove how these different signaling molecules interact. It will also be interesting in the long term to start to investigate to what extent the mechanisms that we have uncovered in Arabidopsis are conserved in other plant species. We have proposed (Moussu et al., 2013; San-Bento et al., 2014) that the signaling pathways studied here evolved as mechanisms to overcome developmental difficulties encountered by developing embryos due to the concomitant development of the embryo and endosperm in the angiosperms. Studying to what extent are endosperm/embryo signaling pathways are conserved in legumes for example, where germination is hypogeal (and not epigeal as in Arabidopsis), and where the formation of a robust cuticle on cotyledon surfaces is potentially less important, could help us to start to understand how theses signaling pathways have been recruited to the seed during angiosperm evolution.

6 MATERIALS AND METHODS

In this section I present the supplementary materials and protocols used in the experiments performed in Chapter 2.3, Chapter 3 and Chapter 4.

6.1 Mutant Materials

All the mutants used in my thesis are in the Columbia-0 (Col-0) background.

Table 3: Mutants used in the studies.

Mutant	Reference	Provenance
ale1-4	(Xing et al., 2013)	T-DNA (SAIL)
gso1-1 gso2-1	(Tsuwamoto et al., 2008)	T-DNA (SALK)
zou-4	(Yang et al., 2008)	T-DNA (SAIL)
frb1-2	(Neumetzler et al., 2012)	T-DNA (SALK)
tpst1-1	(Komori et al., 2009)	T-DNA (SALK)
sgn2	Collaborator, non published	SNP
crs-1	This study	T-DNA (GABI)
cricrs-1	This study	CRISPR
cricrs-2	This study	CRISPR

6.2 Plant Growth conditions

To produce soil-grown plants seeds were surface strerilized with chlorine gas, were sown on Murashige and Skoog (MS) agar plates with 0.5% sucrose, were stratified in the dark for 2-3 days at 4C and were then transferred to soil under standard long-day conditions (21°C, 16h light/8h dark).

6.3 Crosses

Crosses between genotypes were performed over 2 days. The first day, unopened flower buds from the acceptor plant (female) were emasculated. On the morning of the following day, anthers from the donor plant (male) were gently brushed against the acceptor stigma to deposit the donor pollen. Mature siliques containing F1 seeds were harvested in small paper bags. Selection for double or triple mutants involved sowing F1 seeds and genotyping the F1 plants to check for the heterozygous

character of the required alleles. Double heterozygous F1 plants were selected to generate F2 seeds. F2 seeds were sown and plantlets were genotyped to identify the desired double or triple mutants in the segregating population.

6.4 Genomic DNA extraction

For genotyping purposes, genomic DNA was extracted using the following protocol. Small pieces of leaf were harvested into 96 collection tube plaques in which each tube contained two small metal beads. Samples were frozen with liquid nitrogen and ground with a TissueLyser. The powder was suspended in 300 µL CTAB extraction buffer (100mM Tris, 1,4M NaCl, 20mM EDTA, H20) at 60°C for 20 minutes. Upon cooling, 300 µL of chloroform was added to each tube and the plaque was vortexed for 30 seconds. Each plaque was subjected to centrifugation at 4000 rpm at 4°C for 20 min. The upper phase was transferred into new collection tubes and 200µL of isopropanol was added. Samples were mixed by inversion and the centrifugation step was repeated. The supernatant was discarded from the resulting pellets of DNA. The pellets were rinsed with 70% Ethanol and the centrifugation step was repeated. The ethanol wash was carefully removed, pellets were air-dried and pellets were then resuspended in 50 microlitres of TE (10mM Tris, 1mM EDTA)+RNAase (10µg/mL) by vortexing.

6.5 Genotyping

Genotyping of T-DNA lines were performed using 2 microlitre aliquots of the DNA isolated above. In the case of T-DNA alleles, two couples of primers were used, one couple to amplify the WT allele, and the other couple to amplify the T-DNA containing allele, making use of a T-DNA border specific primer. Genotyping of CRISPR alleles is presented in chapter 2.

The primers used for genotyping T-DNA alleles in this thesis are presented in Table 2:

6.6 Agrobacterium & Plant transformation

T-DNA bearing construct were transformed in *A. tumefaciens* strain C58 by the means of electroporation. Cells were plated onto YEB-Agar medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgCl_2$ and 1% Agar) and incubated for two days at 28°C. the resulting bacterial film was suspended in 150 mL of transformation media (MgCl2 10mM ; sucrose 5% with silwet 0.03% added extemporaneously). Buds and flowers from healthy plants were dipped ion this mixture for 10 seconds and then placed in sealed bags for 24 hrs. They were then transferred back into growth chambers and allowed to set seed.

6.7 Seed sterilization and stratification

Seeds were sterilized using chlorine gas. Seeds were places small aliquots (approx. 200) in open 2.0mL tubes in a sealed box. Before closing, Chlorine gas was generated by diluting 3mL 37% HCl in 100mL of bleach. Seeds were sterilized for up to 3 hrs before sowing on MS media. In order to achieve a synchronous germination, plated seeds were stratified at 4°C for 48h.

Mutant	Allele	Primer 1	Primer2	Primer name	Primer sequence
ale1-4	WT	ale1-genoF	ale1-genoR	ale1-genoR	TTGTAATATTTGGTATTATGGTGTGAG
	Mutant	ale1-genoF	LB3 sail	ale1-genoF	TGTAGTTCACAATCTTATCAATCTGG
frb1-2	WT	frb1-RP	frb1-LP	frb1-RP	TTCGTTGTTTCAATTCATCCC
	Mutant	frb1-RP	LBb1-3 salk	frb1-LP	TGTTTCCAGTCGAAAATGTCC
gso1	WT	GS1R	GS1F	GS1R	GTTACCTAAACTGGCGGGAAG
	Mutant	GS1R	LBb1-3 salk	GS1F	CTCGGCTCCCTCGTTAATATC
gso2	WT	GS2F	GS2R	GS2F	ATCGGTGACATGAAAAGCTTG
	Mutant	GS2F	LBb1-3 salk	GS2R	TCCCCATACTGTAAACATCGC
crs-1	WT	Crs-1RP	Crs-1LP	Crs-1RP	ACCAACAAAATTTGCTATGCG
	Mutant	Crs-1RP	Gabi 8049	Crs-1LP	TCAGTATCTCACCACGTCACG
tpst-1	WT	TPST_RP	TPST_LP	TPST_RP	TGTTGTGTGGTTAAGGCTGTG
	Mutant	TPST_RP	LBb1-3 salk	TPST_LP	GGCTTTTGAATTTCTGCACAC
zou-4	WT	zou GenoF	Zou GenoR	zou GenoF	ATTTGCATTCAGAGAATGGTCTAG
	Mutant	Zou5'	Gabi 8049	Zou GenoR	CTGATTGGTAATAAACAGAATGACG
				Zou5'	ATGACTAATGCTCAAGAGTTGG
				Gabi 8049	ATATTGACCATCATACTCATTGC
				LBb1-3 salk	ATTTTGCCGATTTCGGAAC

Table1: Primers used for genotyping

6.8 Toluidine Blue qualitative assay on etiolated cotyledons

Seeds were sterilized and plated on ½MS plates with 0,5% sucrose and Phytagel (Sigma) at pH 5.8 and stratified for 48h at 4°C in the dark. Plates were placed in the light for 6h in a growth cabinet to induce germination. Etiolation was achieved by double-wrapping the plates in aluminium foil (for complete darkness) in the growth cabinet for 4 days. Etiolated seedlings were then treated with toluidine blue (m/v 0,05%) tween 20 (v/v 0,1%) in Millipore H_2O for 2 min, then rinsed directly on plates at least 10 times with tap water until the residual water was no longer showing blue coloration. Finally, treated seedling cotyledons were observed under a binocular microscope (Leica) equipped with a camera (Axiocam Zeiss).

6.9 Toluidine Blue quantitative assay

Seeds were plated on Phytagel media as described above but were not grown in darkness. Seedlings were grown for 10 days in the growth cabinet prior treatment. Plants were treated with Toluidine Blue solution for 2 min and rinsed with tap water. For each genotype considered, 20x2 cotyledons were harvested into 1mL of 80% Ethanol in 1,5 Eppendorf tubes. Three technical repetitions were performed for each genotype. After 2hrs in the ethanol, both the chlorophyll and the toluidine blue have leached fully into the solution. Absorbance of the resulting solution was measured at 626nm and 430nm. The ratio A626/A430 was used as relative quantification of toluidine blue penetrance, using the chlorophyll to eliminate any cotyledon-size/number effects.

6.10 Western Blotting experiments

Staged siliques were quickly frozen in liquid nitrogen, ground, and total proteins were extracted in buffer containing 50 mM Tris pH 7.5, 200 mM NaCl, 1mM EDTA pH 8, 10% glycerol, 0.1% tween 20, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1x protease inhibitor cocktail P9599 (Sigma-Aldrich), and 1x MS-Safe protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Equal amounts of proteins (20µg) were resolved on 12% polyacrylamide gels and transferred onto a nylon membrane (iBlot 2). Ponceau red was used to stain proteins directly on membranes for

1min, then gently rinsed off with distilled water. Membranes were blocked for 15 minutes with a solution of PBS-T (PBS (1%) + Tween 20 (v/v 0,5%)) + 5% milk. Membranes were then incubated with primary antibodies (mouse anti-GFP monoclonal (Roche)) at a 1/1000 dilution overnight at 4°C in PBS-T + milk 0,5% under gentle agitation. Membranes were rinsed for 3 x 5minutes at RT with PBS-T. Incubation with Anti-Mouse HRP secondary antibodies at a 1/5000 dilution was performed for 1h at room temperature. Membranes were then rinsed for 3 x 5min at room temperature with PBS prior revelation. Revelation was performed with ECL prime (GE healthcare) for 2 min following the manufacturer's instructions. Photo-activity was revealed with

6.11 Plasmid generation

Gateway cloning was used to generate vectors suitable for plant transformation.

6.11.1 Plasmids already available

Prior to my PhD, the following vectors were already available in the laboratory: pENTR5'-pRGP3, pENTR5'-pRPS5A, pENTRgene-VENUS-N7, pENTR3'-mCitrine, pENTR3'-Mock and pENTR3'-GFP. I generated the pENTR5'-$pCRS_{1600}$ and pENTR5'-$pCRS_{4000}$ as described in the "Materials and Methods" of the manuscript presented in Chapter 2 Part 1.

6.11.2 BP- cloning generated plasmids

I generated the pENTRgene-CRS (nostop) plasmid using a BP reaction between pDONR221 and a PCR product resulting from the amplification of CRS genomic DNA using the following primers containing attB borders:

CRS_F_ATTB1	GGGGACAACTTTGTACAAAAAAGCAGGCTCAACAATGATGAGCATTAAGCTGACATTG
CRS_NOSTOP_R_ATTB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACCACGTCACGCTTTATCC

6.11.3 LR- cloning generated plasmids:

The following table presents the various entry-vector combinations used to generate constructions described in this thesis.

Expression vector	pENTR5'	pENTRgene	pENTR3'	pDEST
pCRS1600::VN7	pENTR5'-pCRS$_{1600}$	pENTRgene-VN7	pENTR3'-Mock	pBART
pCRS4000::VN7	pENTR5'-pCRS$_{4000}$	pENTRgene-VN7	pENTR3'-Mock	pBART
pRGP3::VN7	pENTR5'-pRGP3	pENTRgene-VN7	pENTR3'-Mock	pBART
pRPS5A::VN7	pENTR5'-pRPS5A	pENTRgene-VN7	pENTR3'-Mock	pBART
pRGP3::CRS-mCit	pENTR5'-pRGP3	pENTRgene-CRS	pENTR3'-mCitrine	pBART
pCRS1600::CRS-mCit	pENTR5'-pCRS$_{1600}$	pENTRgene-CRS	pENTR3'-mCitrine	pBART
pRGP3::CRS-GFP	pENTR5'-pRGP3	pENTRgene-CRS	pENTR3'-GFP	pBART

6.11.4 In situ hybridization against FRB1 mRNA

FRB1 cDNA was cloned by insertion of a PCR product generated from seed cDNA using the following primers into pTOPO ZeroBlunt.

FRB1_F	ATGTCAGTCGGCGTTCCA
FRB1_R_stop	TTATCTCAGAGATTGTGCTCGTA

The subsequent *in situ* hybridization protocol is detailed in Chapter 2 Part 1

6.12 Visualisation of the cuticle using TEM analysis.

For transmission electron microscopy analysis, seeds were removed from staged siliques by removal of the replum tissue with attached seeds. Seeds were high-pressure frozen with a Leica EM-PACT-1 system. For this, three seeds were inserted into a flat copper carrier, fast-frozen, and cryosubstituted into the Leica AFS1 device. The different freeze-substitution steps were as follows: 54 h at −90°C in acetone solution containing 0.2% glutaraldehyde, 1% osmium tetroxide, and 0.1% uranyl acetate. The temperature was then raised in steps of 2°C/h before remaining for 8 hours at -60°C. The temperature was raised again to -30°C for 8h00 before being increased to 4°C. Samples were washed three times for 10 min in 100% acetone before embedding in Spurr's resin. Resin embedding was performed progressively (8 h in 25% Spurr's resin in acetone, 24 h in 50% Spurr's resin in acetone, 24 h in 75% Spurr's resin in acetone, and two times for 12 h in 100% Spurr's resin). Polymerization was performed at 70°C for 18 h. Samples were sectioned (65 nm sections) and imaged at 120 kV using an FEI TEM Tecnai Spirit with 4 k x 4 k eagle ccd.

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8 APPENDIX

As an appendix is an Addenda to San-Bento et al.,2014 published (As a first Author) at the beginning of my PhD in the journal Plant Signalling and Behavior.

Plant Signaling & Behavior 8:12, e27491; December; © 2013 Landes Bioscience

Embryonic cuticle establishment

The great (apoplastic) divide

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The plant cuticle, a dynamic interface between plants and their environment, is formed by the secretion of hydrophobic lipids and waxes into the outer wall of aerial epidermal cells. Cuticle formation is such a ubiquitous feature of epidermal cells, and is of such fundamental importance for plant survival, that identifying and understanding specific developmental roles for this structure has been a major challenge for plant scientists. In recent work, we have tried to understand the functional relationships between a signaling feedback loop required for epidermal cell specification in developing plant embryos, and a seed specific signaling cascade, involving components localized both in the embryo and in the embryo surrounding endosperm, and necessary for embryo cuticle function. Analysis of the strongly synergistic genetic relationships between these 2 independent pathways, combined with mathematical simulations of the behavior of the signaling feedback loop, have allowed us to propose an important, and hitherto unsuspected, role for the embryonic cuticle as an apoplastic diffusion barrier, necessary for preventing the excessive diffusion of developmentally important signaling molecules away from developing embryo into surrounding tissues.

Keywords: endosperm, embryo, cuticle, signaling, epidermis, apoplast, diffusion barrier

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Submitted: 11/20/2013

Accepted: 12/11/2013

Citation: Moussu S, San-Bento R, Galletti R, Creff A, Farcot E, Ingram G. Embryonic cuticle establishment: The great (apoplastic) divide. Plant Signaling & Behavior 2013; 8:e27491; PMID: 24398513; http://dx.doi.org/10.4161/psb.27491

The plant epidermis is a highly specialized cell layer which covers all aerial plant surfaces, and which is characterized by a number of fundamental traits of which one of the most important is the secretion of lipids and waxes into its outer cell wall (reviewed in [1]). The continuous hydrophobic layer thus formed, called the cuticle, has been proposed to fulfil a multitude of different functions, including protecting the plant from uncontrolled water loss[2] and from damage by both biotic and abiotic factors.[3,4] In addition the cuticle plays an important developmental function by preventing adhesion and fusion of developing organs (reviewed in [1,5]). Because the cuticle is a ubiquitous feature of aerial epidermal surfaces, and is essential for plant survival, it has been very difficult to functionally separate cuticle production and function from epidermal fate specification in genetic studies.

In this context, the GASSHO1 (GSO1) and GSO2 receptor kinases are of particular interest, as recent studies have shown that they affect cuticle function specifically during embryogenesis.[6,7] Double *gso1 gso2* mutants produce seedlings which are highly permeable to hydrophilic dyes, strongly desiccation sensitive, and which show cotyledon fusion at rates of between 50 and 80%. These mutants can be rescued to produce fertile plants, by culturing seedlings under highly humid conditions. Consistent with the fact that GSO1 and GSO2 are only necessary for cuticle formation in the developing embryo, recent work has shown that they act in the same signaling pathway as 2 endosperm specific proteins, the transcription factor ZHOUPI (ZOU)[8] and the subtilisin serine protease ABNORMAL LEAF SHAPE1 (ALE1)[9], leading to the hypothesis that formation of a functional embryonic cuticle necessitates signals derived from the endosperm in the seed context[7].

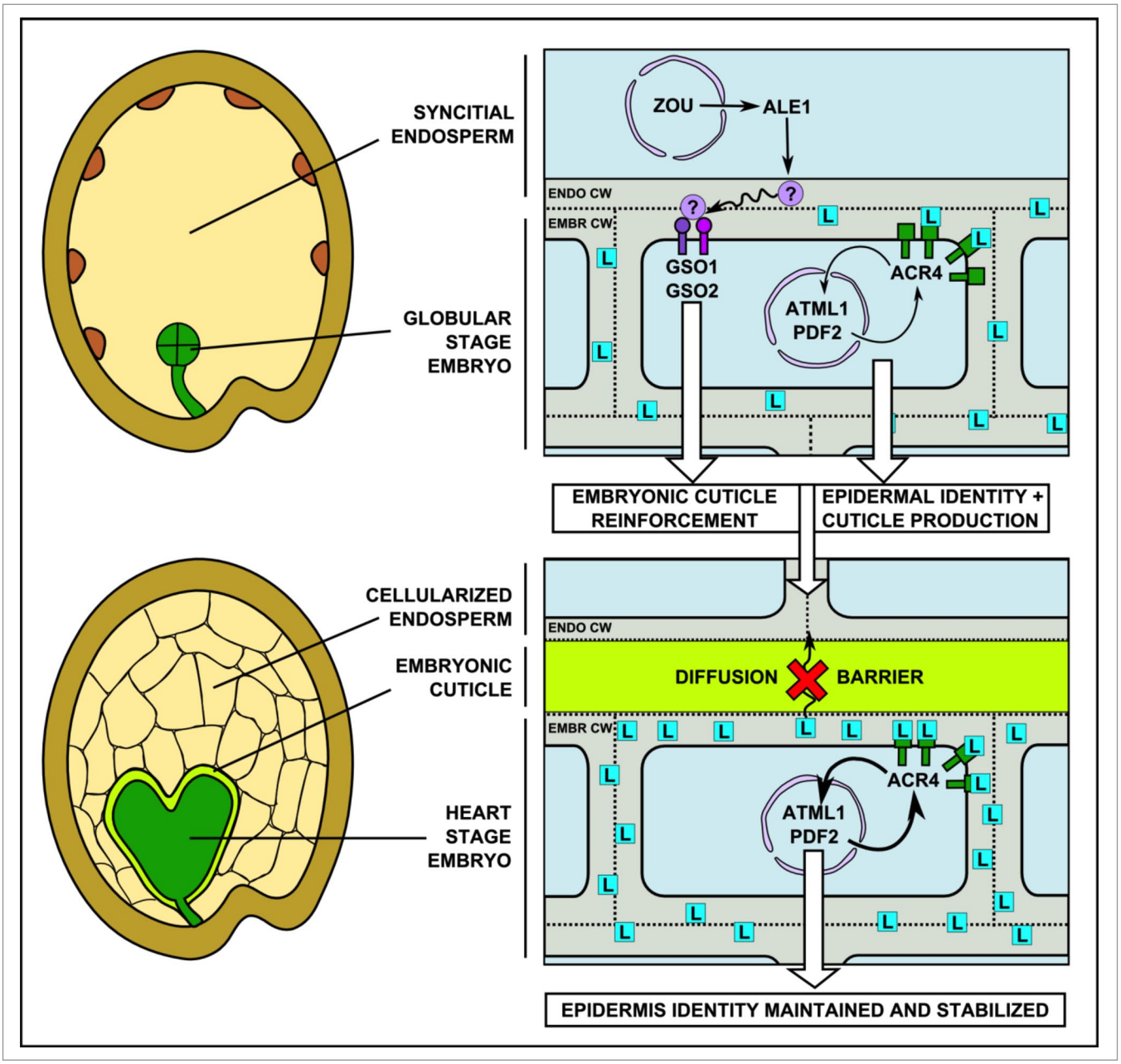

Figure 1. Illustration of a potential mechanism for the observed role for an apoplastic diffusion barrier (the embryonic cuticle) in robust epidermal cell fate establishment during *Arabidopsis* embryogenesis. A signaling feedback loop within the embryo, implicating the receptor kinase ACR4, the hypothetical ACR4 ligand (L) and the transcription factors ATML1 and PDF2, is established during very early embryogenesis and is necessary for the maintenance of epidermal fate in the outer cell layer of the embryo. This maintenance depends upon levels of Ligand L which, in the absence of an apoplastic barrier, is free to move away from the embryo in the apoplast. At around the late globular stage in development, as the endosperm starts to cellularize, a second signaling pathway involving the receptor-like kinases GSO1 and GSO2 and the endosperm-specific subtilisin protease ALE1 (produced under the control of the bHLH transcription factor ZOU), mediates a reinforcement of the embryonic cuticle, effectively sealing off the embryo to permit maintenance of high levels of ligand L within the embryo and thus stabilize epidermal cell fate. Solid arrows represent positive regulation which can be either direct or indirect. ENDO CW = Endosperm Cell Wall, EMBR CW = Embryo Cell Wall.

In *Arabidopsis thaliana*, epidermal fate specification during early embryogenesis is controlled by the largely redundant activity of the protoderm specific transcription factors *ARABIDOPSIS THALIANA* MERISTEM L1 (ATML1) and (PROTODERMAL FACTOR 2) PDF2,[10] and it has generally been assumed that these proteins, as has been shown for other members of the HDZIP IV transcription factor family, regulate cuticle biosynthetic processes as well as other epidermal traits.[10,13] In a recent study[14] we have shown that these proteins act together with the receptor kinase *ARABIDOPSIS* CRINKLY4 (ACR4)[15,17] in a feedback loop which is necessary for the maintenance of epidermal identity

during post embryonic growth. In this work[14] we show that ACR4, probably acting together with other receptors[17] is necessary for maintaining the expression levels of *ATML1* and *PDF2*. During post germinative growth, these transcription factors in turn feed-back directly, and negatively, on their own expression and that of *ACR4*, a situation which mathematical modeling suggests would provide robust epidermal cell fate maintenance in the face of fluctuations in signaling input. Interestingly however, results both from our work, and from previous studies,[10] suggest that ATML1 and PDF2, potentially together with other members of the HDZIP IV transcription factor family, provide a net positive rather than negative regulation of their own expression and that of *ACR4* during early embryogenesis. When this scenario was modeled, it gave a bi-stable situation in which a decreased signaling intensity can lead to irreversible loss of epidermal cell fate.[14] Such a bi-stable situation in globular embryos is consistent with the observed rapid loss of epidermal identity markers, including *ATML1* transcripts, in the central cells of the dermatogen stage embryo after periclinal divisions in the octant embryo,[18] reviewed in.[1]

Based on these results, 2 different signaling pathways directly impact epidermal development during embryogenesis: the ZOU/GSO1/GSO2/ALE1 pathway, and the ACR4/ATML1/PDF2 feedback loop (**Fig. 1**). Several studies,[8,17] have shown that *acr4* mutants have strong synergistic interactions (leading to loss of epidermal fate and embryo lethality) with *zou*, *ale1* and *gso1 gso2* mutants, suggesting that the 2 signaling pathways are distinct. We therefore tested the hypothesis that the 2 pathways converge to regulate the expression of *ATML1* and *PDF2*. Surprisingly, contrary to what had previously been proposed in the literature, we showed that the ZOU/GSO1/GSO2/ALE1 pathway in fact controls embryonic cuticle properties independently of the activities of ATML1 and PDF2 .

Although novel, this finding leaves the strong synergistic interaction observed between the GSO1/GSO2/ALE1 pathway, and the ACR4/ATML1/PDF2 feedback loop unexplained. Although it has been shown that most "between pathway" mutant combinations (for example *acr4 ale1, gso1 gso2 acr4* or *gso1 gso2 pdf2)* are embryo lethal,[14,17] a possible clue to the question resided in the double *pdf2 ale1* mutants. Single mutants in either gene produce normal looking plants and seedlings with extremely subtle defects in cuticle permeability. In contrast, about 20% of double mutant embryos arrest early in embryo development showing serious epidermal disorganization, while the rest produced abnormal seedlings which were highly permeable to hydrophilic dyes, and showed cotyledon stunting and notching. Despite these defects, if transferred to soil under humid conditions, these seedlings gave rise to normal looking plants. This mutant combination thus reveals a developmental threshold which occurs at around the globular stage in embryogenesis, and at which epidermal identity appears to be either stabilized to allow subsequent development, or irretrievably lost. Interpreted in the light of our mathematical models, it therefore appears that even weak defects in cuticle reinforcement at this specific stage are enough to flip the bistable switch to the "no epidermis" state in the outer cell layer of young embryos in which the ACR4/ATML1/PDF2 feedback loop establishing epidermal identity is compromised.

In order to understand why embryonic epidermis formation is particularly sensitive to cuticular perturbations, it is necessary to consider its developmental context. We have previously proposed that the specific need for cuticle reinforcement in zygotic embryos could be engendered by the fact that they develop surrounded by the developing endosperm, which is not thought to be cuticularized.[7,19] However, the developmental role of this reinforcement early in embryogenesis has remained unclear. Our study may shed light on this issue, since the activity of the ACR4/ATML1/PDF2 feedback loop in our mathematical models is assumed to involve an ACR4-binding, apoplastically located, ligand which to date remains unidentified. Because this feedback loop is active both during and after embryogenesis, it is reasonable to assume that the ACR4 ligand must be produced within the embryo and accumulate within the embryonic apoplast. We propose then, that the stochastic loss of epidermal identity in a background in which both the ACR4/ATML1/PDF2 feedback loop and cuticle formation are compromised, could reflect a role for the embryonic cuticle in preventing the diffusion of apoplastic signaling molecules, including the ACR4 ligand, out of the developing embryo, and into apoplast of the surrounding endosperm. Thus, by cutting apoplastic bridges between the embryo proper and the endosperm, the cuticle could play a critical role in concentrating the ACR4 ligand within the embryonic tissues, and maintaining ACR4 signaling activity at high enough levels to permit the maintenance of epidermal cell fate specification in developing embryos, even in backgrounds where the signaling loop is compromised (**Fig. 1**)

A logical prediction from this model is that loss of embryonic cuticle biosynthesis alone should critically compromise the maintenance of epidermal identity. Cuticle defects in *ale1–4* or *gso1 gso2* double mutants do not appear to significantly destabilize an uncompromised ACR4/ATML1/PDF2 feedback loop. Interestingly, however, loss of function alleles in the Acetyl-Coenzyme A Carboxylase encoding *ACC1* gene, which has recently been shown to play a non-redundant role predominantly in the biosynthesis of cuticular waxes in *Arabidopsis*,[20] show embryo developmental defects which are startlingly similar to defects reported for double mutants between certain alleles of *ATML1* and *PDF2*,[10] including lack of cotyledon initiation and disruption of cell organization in apical regions of the embryo.[21] However, a loss of epidermal identity in these mutants remains to be proven.

In summary, although further studies will be required to confirm our hypothesis, including quantitative expression analysis of epidermal markers specifically during the early stages of embryogenesis in a variety of backgrounds, our results have permitted us to put forward one of the first propositions for a concrete molecular mechanism underlying the developmental role of an apoplastic diffusion barrier in plants.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Moussu S is supported by a grant from the Rhône Alpes Region (France). San-Bento R was supported by a Marie Curie ITN network (SIREN). This work also forms part of an ANR (France) Chaire D'Excellence awarded to Ingram G and supporting Creff A and Galletti R.

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