CREATE THE SCENE AND WATCH THE SHOW UNFOLD: FOLLOWING VEGETATIVE TO EMBRYONIC DEVELOPMENTAL TRANSITIONS BY OVER-EXPRESSING LEC2 IN LEAVES OF *ARABIDOPSIS THALIANA*

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ABSTRACT

During seed development, the lytic vacuole (LV) is replaced by a protein storage vacuole (PSV) which specializes in accumulating seed storage proteins (SSPs). As seed protein reserves are mobilized upon germination, the PSV is once again replaced by the LV which takes on different roles in vegetative tissues. Cellular events occurring during these developmental transitions are not well understood, particularly, the transition between vacuole types. This research investigates whether PSVs can exist in leaves. To study vacuole transitions in leaves, an *Arabidopsis thaliana* line over-expressing the LEAFY COTYLEDON2 (LEC2) transcription factor was used. LEC2 is a master regulator of embryogenesis responsible for creating a cellular environment that promotes embryogenic development. Over-expression of LEC2 causes vegetative tissues to change their developmental fate to an embryonic state. LEC2 alters the leaf phenotype at the subcellular level; chloroplasts de-differentiated and contained more starch. The cytoplasm becomes filled with oil bodies, which are typically seed organelles. The large LV was replaced by small-sized vacuoles that accumulated protein deposits. Since LEC2 is responsible for activating the synthesis of SSPs during seed development, SSP accumulation was investigated in leaves. The major *Arabidopsis* SSP families were shown to accumulate within small sized vacuoles in leaf cells. By exploiting the developmental and tissue specific localization of two tonoplast intrinsic protein (TIP) isoforms, the small vacuoles were identified as PSVs. A time course following the cellular alterations and accumulation of seed proteins in leaves after induction of LEC2 activity with dexamethasone (DEX) revealed the appearance of embryonic characteristics as early as 4 days on DEX and became more prominent over time. Additionally, a dynamic view of the transition between vacuole types was observed using TIP isoforms fused to fluorescent markers. The morphology of leaf vacuoles was altered to resemble an amalgamation of a LV and PSV. Results suggest that as the LV transitions to a PSV, the tonoplast remodels before the large vacuole is replaced by smaller PSVs. The formation of PSVs in leaves in response to LEC2 over-expression is a novel approach to study vacuoles and will lead to a better understanding of their basic biology.

KEYWORDS

Arabidopsis thaliana, protein storage vacuole, LEC2, leaf, seed storage protein, tonoplast intrinsic protein, developmental transition, chloroplast, oil body

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35S	Cauliflower mosaic virus 35S promoter
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
AP	aspartic protease
ATP	adenosine triphosphate
BP	band pass
BSA	bovine serum albumin
ctVSS	C-terminal vacuolar sorting signal
d	day
DEX	dexamethasone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
ER	endoplasmic reticulum
GA	gibberellic acid
GFP	green fluorescent protein
GR	glucocorticoid receptor
GUS	β-glucuronidase
HPF-FS	high pressure freezing and freeze substitution
HRP	horseradish peroxidase
IAA	indoleacetic acid
LEC	LEAFY COTYLEDON
LP	long pass
LV	lytic vacuole
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
MVB	multivesicular body
NA	numerical aperture
NPIR	Asn, Pro, Ile, Arg

NR	neutral red
PAC	precursor accumulating vesicles
PAGE	polyacrylamide gel electrophoresis
PB	protein body
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
PSV	protein storage vacuole
psVSS	physical structure vacuolar sorting sequence
PTFE	polytetrafluoroethylene
PVC	prevacuolar compartment
PVDF	polyvinylidene fluoride
PVPP	polyvinylpolypyrrolidone
rER	rough endoplasmic reticulum
RFP	red fluorescent protein
RMR	receptor homology-transmembrane-RING H2 domain protein
RNA	ribonucleic acid
RT	room temperature
SAV	senescence-associated vacuole
SDS	sodium dodecyl sulfate
SE	somatic embryogenesis
SSP	seed storage protein
ssVSS	sequence specific vacuolar sorting signal
TAG	triacylglycerol
Taq polymerase	*Thermus aquaticus* DNA polymerase
TBO	toluidine blue-O
TEM	transmission electron microscopy
TGN	*trans* Golgi network
TIP	tonoplast intrinsic protein
UA	uranyl acetate
UV	ultraviolet
VPE	vacuolar processing enzyme
VSP	vegetative storage protein

VSR	vacuolar sorting receptor
VSS	vacuolar sorting signal
wk	week
YFP	yellow fluorescent protein

All numerical units included in this thesis are standard SI units. In addition, several units outside the SI, but that are accepted for use with the SI, are used in the text.

CHAPTER 1

INTRODUCTION

1.1 Embryogenesis in flowering plants

1.1.1 Angiosperms seize the day

One of the most important reproductive adaptations allowing plants to colonize land is the evolution of seeds (Steeves, 1983). The ability to make seeds is not universal among plants and seed-bearing plants are represented today by two major groups: the angiosperms (flowering plants) and four gymnosperm groups (cycads, ginkos, gnetae and conifers) (Linkies et al., 2010; Harada, 2001). Angiosperms differ from gymnosperms in their more complex seed development. In flowering plants, ovules are enclosed and protected in the ovary, the female gametophyte is drastically reduced in size and they undergo double fertilization leading to the formation of the endosperm (Linkies et al., 2010; Steeves, 1983). These reproductive adaptations have contributed to the relative success of Angiosperms over other seed plants in colonizing terrestrial biomes. Today flowering plants account for almost 90% of all plants on Earth (Crepet and Niklas, 2009). Angiosperms are separated into two classes that diverged early during flowering plant evolution, monocots and dicots. These classes are named for the number of cotyledons, or seed leaves, that first emerge upon germination (Crepet, 2000).

1.1.2 Embryogenesis in *Arabidopsis thaliana*

In flowering dicots such as *Arabidopsis thaliana*, seed formation begins with double fertilization of the multicellular female embryo sac. This involves the delivery of two sperm cells into the ovule. One sperm fuses with a haploid egg cell to produce the zygote and a second sperm fuses with a diploid central cell to give rise to the endosperm which will serve a nutritive role. Embryonic development proceeds inside the protective maternal tissue of the ovule which becomes the seed coat that will surround the embryo and endosperm (Dubreucq et al., 2010; Western et al., 2000; West and Harada, 1993).

In *Arabidopsis*, the endosperm begins to develop before the embryo. The triploid endosperm develops in two stages. The central cell proliferates to form a syncytium of

nuclei which then becomes cellularized and differentiated. The endosperm imports nutrients from maternal tissue and synthesizes storage reserves. During the maturation phase of embryo development, the endosperm is absorbed by the embryo and reduced to a peripheral layer surrounding the embryo (Huh et al., 2008; Otegui et al., 2002).

Embryogenesis describes the development of the single-cell zygote into an embryo and consists of two phases: morphogenesis and maturation. The morphogenesis phase establishes the basic body plan of the embryo. Through a series of precise cell divisions, the polarity of the embryo is determined, the major tissues and the major organ systems are formed (Harada, 2001). The *Arabidopsis* embryo passes through sequential morphological stages. During the globular stage, the protoderm which gives rise to the epidermis, becomes detectable and a suspensor is formed. The suspensor is a transient structure that functions in nutrient transfer to the embryo (Goldberg et al., 1994). The embryo transition from globular to heart stage marks the appearance of plant organs. Increasing cell divisions cause the formation of lobes on the two cotyledons, or seed leaves, to become distinct and cause the axis to elongate, giving the embryo a heart-shaped appearance. The axis represents the precursor to the other major plant organs; shoot apical meristem, hypocotyl, root and root apical meristem. Two other major tissues are recognized at the heart stage; the procambium which becomes the vascular tissue and the ground meristem which gives rise to the parenchyma (De Smet et al., 2010; West and Harada, 1993).

The maturation phase is characterized by activities that prepare the embryo for developmental arrest and germination. The most distinctive activity that takes place during maturation is the high level accumulation of storage reserves. Upon germination, these reserves serve as a nutrient source to sustain the growing seedling until it becomes autotrophic (Harada, 2001). The beginning of the maturation phase is marked by the cessation of cell division and the embryo subsequently goes through a period of cell expansion and differentiation. At an early stage of maturation, the embryo accumulates storage reserves in the form of starch. During mid-maturation, starch levels decrease while lipids and proteins accumulate. In late-maturation, synthesis of storage reserves ceases while the embryo acquires the ability to withstand desiccation; seed water content

declines while a low level of synthesis and accumulation of sugars continues. At the end of the maturation stage, the mature desiccated embryo is filled with nutrient reserves and lies metabolically quiescent inside a protective maternally-derived seed coat. The embryo remains dormant until conditions are favorable to resume its life cycle (Baud et al., 2008; Mansfield and Briarty, 1992).

1.1.3 The maturation phase is unique to seed plants

In lower plants, there is no separation between morphogenesis and the beginning of post-embryonic development (West and Harada, 1993). The maturation phase was integrated into embryogenesis and has enabled plants to make seeds (Braybrook et al., 2006; Vicente-Carbajosa and Carbonero, 2005). The ability to make seeds has provided many advantages for seed plants and is largely responsible for the success of angiosperms. One of the most important advantages is the ability to pause their life cycle. This pause is unique to seed plants, allowing embryos to withstand long periods of time until conditions are suitable to ensure survival of the seedling (Kroj et al., 2003; Harada, 2001; Steeves, 1983). Thus, the processes that take place during the maturation phase make the seed an efficient organ to disperse and establish offspring, which largely accounts for the success of seed plants. Despite the dependence of seed-bearing plants on the maturation phase to generate viable seeds, it is not a compulsory step in the plant life cycle. In several species, immature embryos excised from the ovule can continue to grow (Santos-Mendoza et al., 2008; Braybrook et al., 2006; Vicente-Carbajosa and Carbonero, 2005).

1.2 LEC2 shares the power: a short introduction to the control of embryogenesis by LEC2 and its interactions within the intricate seed regulatory network

1.2.1 Key transcriptional regulators of embryogenesis

During the evolution of seeds, the maturation phase was incorporated into the plant life cycle. For this to happen, regulatory programs that initiate and terminate the maturation

phase have had to evolve within the seed developmental program. The maturation phase can be further divided into overlapping gene expression programs that accomplish different activities such as the synthesis of storage reserves, acquisition of desiccation tolerance or the establishment of dormancy (Zhang and Ogas, 2009; Braybrook and Harada, 2008). Surveys of gene expression patterns in *Arabidopsis* reveal a large number of genes that are specifically expressed in seeds but the role of these genes in controlling seed development are unknown (Le et al., 2010; Spencer et al., 2007). However, important regulators of maturation were identified through the analysis of mutants impaired in seed formation and by over-expression of individual seed genes in vegetative tissues (Santos-Mendoza et al., 2008; Vicente-Carbajosa and Carbonero, 2005). Four transcription factors were identified as key regulators of seed gene expression: *LEAFY COTYLEDON1* (*LEC1*; West et al., 1994), *LEC2* (Stone et al., 2001), *FUSCA3* (*FUS3*; Bäumlein et al., 1994; Keith et al., 1994) and *ABSCISIC ACID INSENSITIVE3* (*ABI3*; Nambara et al., 1995; Parcy et al., 1994; Giraudat et al., 1992). These transcription factors act to initiate and control seed maturation and to prevent germination. Thus, they determine a state of competence under which maturation gene expression programs occur (Santos-Mendoza et al., 2008; Vicente-Carbajosa and Carbonero, 2005).

1.2.2 LEC and ABI3 transcription factors function in distinct but overlapping ways to control embryo development

LEC genes are composed of three loci: *LEC1*, *LEC2* and *FUS3*. These genes operate over a wide range of seed gene expression programs and they act earlier than *ABI3* during the maturation phase. Because of this, *LEC* genes are often referred to as master regulators of embryogenesis while *ABI3* is not always included in this group (Zhang and Ogas, 2009; Santos-Mendoza et al., 2008; Vicente-Carbajosa and Carbonero, 2005; Harada, 2001). In early embryogenesis, LEC transcription factors act during the morphogenesis phase to specify cotyledon identity and to maintain embryonic cell fate. The name LEAFY COTYLEDON was given to a mutant phenotype that all LEC transcription factors share: cotyledons acquire leaf traits. That is to say that the surface of the seed leaves develop

trichomes which are characteristic features of leaves in *Arabidopsis* (Casson and Lindsey, 2006; Goldberg et al., 1994). In addition, *LEC* genes control the development of suspensor cells, which is important for nutrient acquisition for the embryo (Harada, 2001; Lotan et al., 1998). In late embryogenesis, during the maturation stage, ABI3 becomes involved with the LEC transcription factors in initiating and maintaining the maturation phase (Zhang and Ogas, 2009; Santos-Mendoza et al., 2008; Harada, 2001).

LEC and ABI3 transcription factors coordinate the maturation phase. Therefore, mutations in any of the four regulators cause severe defects in major activities that occur during the maturation stage and shift embryo development toward germination (Vicente-Carbajosa and Carbonero, 2005). Many mutant phenotypes are shared among the four regulators, albeit to different degrees of severity; storage reserve accumulation is reduced, embryos are less tolerant to desiccation and they germinate precociously (Stone et al., 2001; Nambara et al., 1995; Bäumlein et al., 1994; Keith et al., 1994; West et al., 1994). In addition, some mutant phenotypes are distinct. During the maturation phase the embryo accumulates chlorophyll and turns green and then the chlorophyll is broken down as the seed desiccates. Mutations in *abi3* prevent chlorophyll breakdown (Parcy et al., 1997). FUS3 is named after the purple-red color of *fusca* mutant embryos. The same phenotype is observed, but to a lesser extent, in *lec1* and *lec2* mutants. This color is caused by the accumulation of anthocyanins, which are pigments that are characteristic of vegetative tissues (To et al., 2006; Keith et al., 1994). Although loss-of-function mutations in *LEC* and *ABI3* produce similar phenotypes, they are not identical which suggests that they act in a partially redundant manner (Santos-Mendoza et al., 2008; Kagaya et al., 2005; Harada, 2001). Their distinctive functions are thought to arise in part from differences in temporal and spatial patterns of expression (Santos-Mendoza et al., 2008; Suzuki and McCarty, 2008; Kroj et al., 2003).

1.2.3 LEC2 structure and mechanism of action

As the LEC2 transcription factor is demonstrated to produce similar phenotypes to the other three transcriptional regulators, it should be no surprise that LEC2 also shares a

similar structure and mechanism of action to some of its counterparts. Characterization of LEC and ABI3 proteins revealed that they are transcriptional activators (Zhang and Ogas, 2009). LEC2, along with FUS3 and ABI3, are transcription factors that belong to the plant-specific B3 family (Kagaya et al., 2005; Stone et al., 2001; Luerssen et al., 1998), however LEC1 belongs to a different class of transcription factors (Lotan et al., 1998). All members of the B3 family possess a B3 region, a sequence-specific DNA binding domain (Swaminathan et al., 2008). The B3 domain recognizes RY sequence motifs present in the promoters of many seed genes and activates their expression. The RY motif has CATGCATG as the core sequence (Braybrook et al., 2006; Reidt et al., 2000).

1.2.4 A piece of the puzzle: LEC2 and the regulation of the maturation phase

Embryogenesis is regulated by complex interactions between genetic, metabolic and physiological controls which are poorly understood. The nature and origin of the molecular mechanisms that end morphogenesis and control entrance into the maturation phase remain to be elucidated. However, a considerable number of mechanisms have been revealed that regulate the maturation phase (Santos-Mendoza et al., 2008; Gutierrez et al., 2007). LEC2 is intimately involved in this regulation and therefore the description of LEC2 actions can serve to highlight both the recognized roles of LEC2 and the complexity of the regulatory network.

LEC and ABI3 transcription factors interact among themselves in a hierarchical manner to form a complex regulatory network to establish seed maturation. By over-expressing individual transcription factors in single, double or triple mutant backgrounds, or by using a combination of these methods, interactions among LEC2 and the other transcriptional regulators have been discovered. LEC1 is thought to function upstream of LEC2, FUS3 and ABI3 while LEC2 acts upstream from ABI3 and FUS3. Furthermore, LEC2 regulates ABI3 and FUS3 expression and causes a feedback loop acting on LEC1 expression (Junker et al., 2010; Stone et al., 2008; To et al., 2006; Kroj et al., 2003).

The seed maturation process is indirectly regulated by LEC and ABI3 regulators through secondary transcription factors which activate their specific transcriptional program. These secondary transcriptional regulators remain largely unidentified and are not well understood (Gutierrez et al., 2007) with the exception of the activation of *WRINKLED1* (*WRI1*) by LEC2. Activation of *WRI1* is necessary for the up-regulation of fatty acid biosynthesis during the maturation phase (Santos-Mendoza et al., 2008; Baud et al., 2007).

Very little is known about the interactions between the four transcription factors and their target genes with the exception of seed storage protein (SSP) genes (Gutierrez et al., 2007). During the maturation stage, SSP genes are highly expressed and are tightly regulated by transcriptional control (Verdier and Thompson, 2008; Kroj et al., 2003). Ectopic expression of LEC2 was demonstrated to promote SSP mRNA expression in vegetative tissues (Braybrook et al., 2006; Santos-Mendoza et al., 2005). Moreover, Kroj et al. (2003) demonstrated the activation of SSP expression by direct binding of the RY motif in the SSP promoter through the LEC2 B3 domain.

Plant growth regulators provide important physiological control of embryogenesis and they have been demonstrated to interact with transcriptional regulators. The ratio of abscisic acid (ABA) to gibberellic acid (GA) regulates seed maturation, germination and seedling growth. A high ABA:GA ratio exists during the maturation phase but upon germination, the ratio is reversed (Braybrook and Harada, 2008). Thus, ABA promotes maturation and dormancy and suppresses germination whereas GA promotes germination and cell division (Gazzarrini et al., 2004; Bäumlein et al., 1994). LEC and ABI3 transcription factors help to establish and respond to the high ABA:GA ratio that is characteristic of the maturation phase (Braybrook and Harada, 2008). For example, LEC2 indirectly represses GA levels by activating *AGAMOUS-LIKE15* which encodes a transcription factor that activates *GA2ox6*, a gene whose product is involved in GA degradation (Braybrook and Harada, 2008; Braybrook et al., 2006). In addition, auxin biosynthesis is positively regulated by LEC2 through its direct activation of auxin biosynthetic genes (Stone et al., 2008; Braybrook et al., 2006). Auxin has been demonstrated to be essential to coordinate events during the morphogenesis phase of

embryogenesis and to initiate somatic embryogenesis (Ledwoń and Gaj, 2009; Jenik and Barton, 2005; Goldberg et al., 1994).

Metabolite concentration gradients provide signals for the onset of the seed maturation phase and its regulation (Gutierrez et al., 2007). The most recognized signaling pathways involve sugar, however these pathways are not well understood (Gibson, 2005). Sugar metabolism is a dynamic process that is altered dramatically, depending on the sugar exporting (source) and sugar importing (sink) tissues and organs. Although sucrose is the major photosynthetic product and transport sugar in plants, signaling can rely on the action of its hexose breakdown products such as glucose and fructose (Gutierrez et al., 2007; Rolland et al., 2006). A key component in sugar sensing and signaling in plants is hexokinase (Karve et al., 2008). This enzyme plays a dual role in catalyzing the phosphorylation of glucose for incorporation into metabolic pathways as well as acting as a glucose sensor. Through characterization of *hexokinase1* mutants, hexokinase-mediated glucose signaling was shown to promote or repress growth by altering gene expression and sensitivity to plant growth regulators (Smeekens et al., 2010; Ramon et al., 2008; Rolland et al., 2006). Sugar signaling is an important regulatory mechanism involved in seed maturation (Weber et al., 2010; Gutierrez et al., 2007). Indeed, the availability of soluble sugar was found to enhance the expression of LEC2 and several of its target genes involved in storage reserve synthesis (Tsukagoshi et al., 2007). In addition, in developing *Arabidopsis* embryos, LEC2 was demonstrated to control the sink strength of the embryo, carbon partitioning toward lipid, protein and carbohydrate reserves and the rate of starch synthesis and degradation in developing seeds through unknown mechanisms (Angeles-Núñez and Tiessen, 2011).

1.3 Storage reserve accumulation in *Arabidopsis* seeds

1.3.1 Storage reserve accumulation in *Arabidopsis*

One of the most distinctive activities occurring during the maturation phase is the high level of storage reserve accumulation. Carbohydrates, lipids and proteins are synthesized

in different proportions and accumulated in different locations, depending on the species (Baud et al., 2008; 2002). In *Arabidopsis*, lipids and proteins are highly accumulated in the endosperm and embryo but as the embryo matures, the endosperm is reduced as it transfers most of its nutrients to the growing embryo (Baud et al., 2008). Within *Arabidopsis* embryos, the majority of storage reserves are accumulated in the cotyledons (Mansfield and Briarty, 1992). Upon germination, storage reserves are mobilized and degraded to provide the embryo with nutrients during the short period before it switches from a largely heterotrophic to an autotrophic lifestyle (Penfield et al., 2006; Mansfield and Briarty, 1996). Thus, the cotyledons initially function as storage organs but turn into photosynthetic organs after germination (West et al., 1994).

In *Arabidopsis*, storage compounds contribute up to 90% of the seed dry weight with seeds usually accumulating 30-40% each of oils and proteins (Baud et al., 2008). Seed proteins provide a rich source of nitrogen and sulfur for the germinating embryo (Shewry et al., 1995) while lipids provide a carbon and energy source (Baud et al., 2002). In addition, a small proportion of storage reserves are composed of carbohydrates, which account for only 2% of the seed dry weight (Baud et al., 2008).

1.3.2 Seed storage proteins

Plants synthesize and accumulate several different types of storage proteins in their seeds. The seed proteins are classified into four groups based on their extraction and solubility properties: albumins (water), globulins (saline), prolamines (alcohol) and glutelins (acid) (Shewry et al., 1995). The major SSPs that accumulate in *Arabidopsis* seeds are the 12S globulins (cruciferins; Pang et al., 1988) and 2S albumins (napin or arabidin; Fujiwara et al., 2002) which are encoded by small multi-gene families (Fujiwara et al., 2002). In *Arabidopsis*, the 12S globulins are encoded by four genes that are arranged into three subfamilies; *CRUCIFERIN A* (*CRA1* and *CRA2*), *CRUCIFERIN B* (*CRB*) and *CRUCIFERIN C* (*CRC*) (Sjödahl et al., 1991; Pang et al., 1988). The 2S albumins are represented by five genes; *2S1* albumin (*At2S1*) - *2S5* albumin (*At2S5*) (da Silva Conceição and Krebbers, 1994; Krebbers et al., 1988). SSP genes are expressed

exclusively during the maturation stage of embryogenesis but the individual genes within families differ in their expression levels, tissue specificity and temporal expression (Fujiwara et al., 2002; Scarafoni et al., 2001; da Silva Conceição et al., 1994; Guerche et al., 1990).

The synthesis of SSPs is dependant on sugars and amino acids acquired from maternal tissues. Thus, SSP accumulation is influenced by the metabolic status and nutritional conditions of the plant (Fujiwara et al., 2002). Each 12S globulin gene synthesizes a precursor protein which is cleaved into an alpha and a beta subunit that are connected by a disulfide bond. The mature 12S globulin contains six subunit pairs assembled into hexamers through non-covalent interactions (Tai et al., 1999; Shewry et al., 1995). The 2S albumins are more extensively processed. Each precursor protein is cleaved at four specific sites releasing three peptide fragments. The mature protein is composed of a small and a large subunit connected by two disulfide bridges (D'Hondt et al., 1993; Krebbers et al., 1988).

Vacuolar processing enzymes (VPEs) are key enzymes involved in proteolytic processing of both 12S and 2S precursors to produce their mature forms. VPEs belong to a family of cysteine proteinases. *Arabidopsis* has four VPE homologs which are expressed in seeds (*βVPE* and *δVPE*) or seed and vegetative tissues (*αVPE* and *γVPE*). All VPEs are expressed during seed development but differ slightly in the timing of expression. The enzyme most essential for SSP processing in seeds is βVPE and it has a similar expression profile to SSP genes. In addition to VPEs, processing of 2S albumins also involves an aspartic proteinase (AP) which functions by trimming the subunits produced by VPE activity. Both VPE and AP enzymes cleave peptide bonds of their protein substrates at specific amino acid sites (Gruis et al., 2004; Shimada et al., 2003b). Proper proteolytic processing of SSPs is an important factor that affects their storage ability within specialized storage organelles called protein storage vacuoles (PSVs) (Scarafoni et al., 2001).

1.3.3 Lipids and oleosins

Arabidopsis belongs to the Brassicacea which represents many oilseed crops. The major lipids accumulated in *Arabidopsis* seeds are triacylglycerols (TAGs). TAGs are composed of three fatty acyl chains esterified to a glycerol-3-phosphate backbone. The synthesis of TAGs involves the co-operation of plastids and the endoplasmic reticulum (ER). Fatty acid synthesis takes place in plastids as a series of reactions to produce long chain fatty acids. The fatty acid chains are activated by coenzymeA on the outer membrane of the chloroplast envelope and exported to the ER. Upon delivery to the ER, the fats are assembled into TAGs. Enzymes within the ER lumen catalyze the esterification of three fatty acyl chains to a glycerol-3-phosphate molecule. The accumulating TAGs are then packaged into specialized storage organelles called oil bodies (Baud et al., 2008).

Oleosins are a class of SSPs that are highly expressed during the maturation phase. These proteins are vital for the stabilization of oil bodies which are specialized organelles that accumulate TAGs (Fujiwara et al., 2002). *Arabidopsis* has 16 oleosin genes divided into three groups according to their tissue specificity; five genes are expressed in maturing seeds (designated *S1*-*S5*), eight genes are expressed in anther tissues (*T1*-*T8*) and three genes are expressed in seeds and microspores (*SM1*-*SM3*). However, oleosin transcripts are not detected in leaves, stems or roots (Kim et al., 2002). Oleosin gene expression is not only tissue specific but is also temporally regulated. The expression pattern of seed oleosin genes is similar to that of SSP genes (Huang, 1996) and storage oil synthesis (Beaudoin and Napier, 2000; Huang, 1996).

Oleosins are synthesized on the rough endoplasmic reticulum (rER) as small amphipathic proteins with three distinct structural domains; a highly conserved central hydrophobic region flanked by two terminal hydrophilic domains (Huang, 1992). Oleosin does not have a recognizable signal peptide at the N-terminal directing it to the ER lumen. Instead, sequences within the hydrophobic regions target the protein to the ER. The nascent protein is guided to the ER by a signal recognition particle pathway (Hsieh and Huang, 2004; Abell et al., 2002). Once at the ER, the hydrophilic N-terminal residues

interact with phospholipids on the cytoplasmic surface of the ER membrane while the hydrophobic region inserts into the ER membrane. The hydrophobic domain is exceptionally long and has a proline knot motif composed of one serine and three proline residues at its center allowing the hydrophobic region to bend into a hairpin loop within the ER membrane. The hydrophobic region is thought to prevent the translocation of the C-terminal domain across the ER membrane (Abell et al., 2002). Consequently, the hydrophilic residues at each terminal remain at the cytosolic surface of the ER membrane. Thus, the overall architecture of the protein resembles a thumb tack embedded in the ER membrane (van der Schoot et al., 2011; Abell et al., 2004).

1.3.4 Starch and carbohydrates

Carbohydrates do not make up a significant proportion of the total storage reserves in *Arabidopsis* seeds. During the early stage of maturation before proteins and lipids are synthesized, starch is transiently accumulated. However, starch reserves are largely depleted by the end of maturation. Instead, *Arabidopsis* seeds accumulate sugars in the form of sucrose, raffinose and stachylose during mid- to late-maturation. In the dry seed, these sugars only account for 2% of the seed weight (Baud et al., 2008; 2002; Mansfield and Briarty, 1992). Two theories have been proposed to explain the low sugar accumulation in late-maturation. One interpretation is that sucrose is quickly available as an energy source to support embryo growth before TAG degradation begins at germination. An alternate explanation is that sucrose and the other two oligosaccharides participate in preparing the embryo for desiccation by protecting membranes from desiccation damage (Baud et al., 2002).

1.4 Seed germination: a transition from embryonic to vegetative development

1.4.1 Termination of the seed maturation program

The end of the maturation phase represents the transition from an embryonic to a vegetative state. To allow germination and seedling development to occur, maturation phase gene expression must come to an end. Repression of maturation-specific gene expression involves two mechanisms; factors that directly repress specific maturation genes and factors that repress the key LEC and ABI3 regulatory proteins (Bouyer et al., 2011; Verdier and Thompson, 2008). Both mechanisms typically achieve seed gene repression through the modification of chromatin structure however these mechanisms are not well understood (Zhang and Ogas, 2009; Tang et al., 2008; Gutierrez et al., 2007). Several factors have been identified that repress seed maturation genes by covalent and non-covalent modification of histone proteins and DNA [HISTONE DEACETYLASE6 and HISTONE DEACETYLASE19 (Tanaka et al., 2008), FERTILIZATION INDEPENDENT ENDOSPERM (Bouyer et al., 2011), PICKLE (Aichinger et al., 2009; Zhang et al., 2008) and BRAHMA (Tang et al., 2008)]. Seed gene repression can also be achieved through transcriptional repression by *ARABIDOPSIS* 6B-INTERACTING PROTEIN 1-LIKE 1 (ASIL1) (Gao et al., 2009) and VP1/ABI3-LIKE1 (VAL1) and VAL2 (Suzuki and McCarty, 2008; Suzuki et al., 2007; Tsukagoshi et al., 2007).

1.4.2 Mobilization of storage reserves

Once the seed receives sufficient cues for germination, the embryo resumes growth. During early seedling development, storage reserves are used to promote seedling establishment until chloroplast and root development are complete and photosynthesis can begin (Penfield et al., 2008). The seed imbibes water, cells expand and the seed coat ruptures. Radicle cells divide and elongate causing the radicle to emerge from the seed coat. The radicle continues to elongate into the substrate and root hairs form shortly afterwards. Subsequently, cotyledons emerge from the seed coat, the hypocotyl elongates and positions the unfolding cotyledons over the seed coat. The cotyledons expand and

they begin to green. At the subcellular level, storage vacuoles transition to lytic vacuoles (LVs) as proteins are mobilized. At the same time, oil bodies are degraded. Concomitant with cotyledon greening, proplastids multiply and differentiate to give rise to chloroplasts (Mansfield and Briarty, 1996; Huang, 1992).

At early germination, the embryo is not yet equipped to be self-sufficient so it relies heavily on heterotrophic growth. Lipid and protein reserves are first metabolized in the layer of endosperm cells. Later, seedling reserves are first mobilized in the radicle and hypocotyl (Kaneko and Keegstra, 1996; Mansfield and Briarty, 1996). During mobilization of storage reserves, many hydrolytic enzymes are required to degrade storage reserves. Proteases hydrolyse SSPs to amino acids, which become incorporated into newly synthesized proteins. Phytate, a storage form of phosphorus and minerals in the storage vacuoles, is hydrolysed by phytase and the solubilized minerals are released. Triglycerides are hydrolysed by lipases and the fatty acids are used to produce sugars and ATP (Bethke et al., 1998). As reserves are depleted from the cotyledons to support embryo growth, the cotyledons are transformed into photosynthetic organs which will eventually allow the seedling to convert to autotrophic growth. The transition of cotyledons from a storage to a photosynthetic tissue occurs 48-60 hr after imbibition (Mansfield and Briarty, 1996).

1.4.3 Storage protein reserves are accumulated in leaves

Vegetative tissues transiently accumulate storage proteins in vacuoles to serve as a supply of nutrients for growth and development. When nutrients are plentiful, plants assimilate them into vegetative storage proteins (VSPs) to build up a reserve of nutrients. These proteins are an important source of carbon, sulfur and especially nitrogen. When required by the plant, VSPs are degraded to release amino acids that will be redistributed for other metabolic purposes (Rennenberg et al., 2010; Staswick, 1994).

All proteins sequester amino acids which are later released as proteins turn over. Thus, all plant proteins can be considered as storage reserves because they can provide a

nutritional need. But what sets VSPs apart from other proteins? Unlike VSPs, most proteins are not abundantly accumulated in vegetative tissues (Staswick, 1994). An exception is Rubisco, the most abundant protein on Earth. Rubisco can contribute up to 50% of the soluble leaf protein and 20-30% of the total leaf nitrogen (Feller et al., 2008). Many consider Rubisco to be a storage protein but others argue that it is not a true VSP because it is required for other functions rather than storage. To be specific, the biosynthesis and degradation of Rubisco are regulated according to the need for its metabolic function and not for storage purposes (Rennenberg et al., 2010; Staswick, 1994). However many proteins that are classified as VSPs have alternate roles. The VSPs in soybean leaves are acid phosphatases (Berger et al., 1995), sweet potato tuber sporamin is a trypsin inhibitor (Yeh et al., 1997) and potato tuber patatin is a lipid hydrolase (Andrews et al., 1988).

VSPs were first described in soybeans and are well-characterized in this species (Wittenbach, 1983). Two soybean VSPs were discovered, VSPα and VSPβ. They are glycosylated polypeptides that are ~ 80% identical and have no sequence similarity with known SSPs. These proteins exist as homo- and heterodimers and accumulate in vacuoles of cells associated with the vascular system in stems, leaves, flowers and pods. Soybean VSPs can accumulate to as much as 50% of the total leaf protein but levels can decline to 1%. Their expression is enhanced by wounding, high nitrogen nutrition, drought stress, and the plant growth regulator jamonic acid (Utsugi et al., 1998; Berger, 1995; Staswick, 1994). Two genes homologous to soybean VSPs were identified in *Arabidopsis* (Berger et al., 1995). *Arabidopsis* VSPs are induced by similar stimuli and act as acid phosphatases but they primarily accumulate in flowers. However Liu et al. (2005) raised the question of whether they should be classified as VSPs because they have not been demonstrated to function as storage proteins. Thus, VSPs are recognized to exist in many plant species but identification and characterization of these proteins seems to be made difficult because many have alternate functions.

1.5 A portrayal of three prominent organelles involved in embryonic and vegetative growth and development

1.5.1 Vacuoles: more than empty cell spaces

Vacuoles are one of the most recognized organelles that exist in plant cells, but surprisingly, one of the least understood (Zheng and Staehelin, 2011). When plant cells were first observed with a microscope, the most prominent feature was described as 'an empty cell space devoid of cytoplasmic matter' and was therefore given the name 'vacuole' (Marty, 1999). Since that time, much progress has been made in our understanding of vacuoles. Today, the plant vacuole is known to be essential for the existence of plant cells. Vacuoles are defined as a multi-functional organelle bound by a single membrane, called a tonoplast. Over the plant life cycle and through different developmental stages, a vacuole will drastically alter its shape and assume diverse functions to respond to the changing needs of the cell (Rojo et al., 2001; Marty, 1999).

Vacuoles are involved in many different cellular processes such as protein storage and degradation and they are important for maintaining a structural role. To accommodate these seemingly unrelated functions, different types of vacuoles exist (Jolliffe et al., 2005). Two major vacuole types are recognized in the literature; PSVs and LVs.

1.5.1.1 Lytic vacuoles

The central LV is the largest and most instantly recognizable organelle in a vegetative plant cell. It can account for up to 90% of the total cell volume (Jolliffe et al., 2005). Thus, LVs occupy most of the cell volume and squeeze the cytoplasm and other organelles between the tonoplast and plasma membrane (Becker, 2007). LVs are present in young seedlings shortly after germination and generally exist throughout vegetative growth.

The LV lumen is filled with water and numerous hydrolytic enzymes and is maintained at a low pH. The tonoplast plays a major role in maintaining this luminal environment. The tonoplast is a selective membrane that contains 39 specific channels, transporters and pumps that mediate the transport of substances between the cytoplasm and vacuole (Jacquinod et al., 2007; Müntz, 2007; Carter et al., 2004). To maintain a luminal pH of 5.5, vacuolar ATPase (V-ATPase) and pyrophosphatase (V-PPase) catalyze ATP-dependant proton transfer across the tonoplast which also creates an energy gradient to transport ions (Krebs et al., 2010; Beyenbach and Wieczorek, 2006; Dettmer et al., 2006; Maeshima et al., 1994). The movement of ions across the membrane is facilitated by specific ion channels. The movement of water across the tonoplast is facilitated by water channels called aquaporins (Maurel et al., 2009). There are also transporters that mediate the movement of inorganic cations (Mg^{+2} or Ca^{+2} ATPases) and organic compounds (ABC transporters) (Müntz, 2007). Within the vacuole lumen, numerous hydrolytic enzymes are present such as proteases, glycosidases, lipases, nucleases and peroxidases (Carter et al., 2004).

The LV participates in diverse physical and metabolic functions that are critical for the survival of a plant. A significant role for LVs is to allow the cell to increase its size without expending too much energy. Plants have evolved a unique cell architecture whereby their vacuoles account for most of the cell volume. This permits cellular growth at lower costs because vacuoles largely consist of water and have a low density of organic compounds to synthesize (Zouhar and Rojo, 2009; Taiz, 1992). Likely one of the most universal functions of the LV is its role in maintaining turgor pressure which determines the rigidity of the cell and is important for growth and mechanical stability of the plant (Müntz, 2007; Staehelin and Newcomb, 2000).

In addition to physical functions, LVs play important metabolic roles in storing a large variety of compounds such as vacuolar storage proteins (Staswick, 1994), toxins (Riechers et al., 2010), salt (Krebs et al., 2010), heavy metals (Song et al., 2010), pigments (Zhang et al., 2006; Reuveni et al., 2001) and defense compounds (Zhao and Dixon, 2009).

The harsh environment of the LV lumen allows it to play a fundamental role in the degradation of cytoplasmic materials from small molecules to organelles. Autophagy is a conserved mechanism in eukaryotes whereby the cell contents are transferred to the LV to be digested and recycled, typically in a non-selective manner. Generally, a basal level of autophagy functions constitutively for turnover of unwanted cellular components however it can be induced to a high level during plant development or in times of cell stress (Bassham, 2009; 2007; Nakatogawa et al., 2009; Bassham et al., 2006; Thompson and Vierstra, 2005).

1.5.1.2 Protein storage vacuoles

At first glance, it is hard to believe that PSVs and LVs are related. PSVs differ from LVs in many characteristics such as size, shape, number, structure, luminal contents and occurrence. However, they share some features such as their ability to maintain a suitable environment for storage and degradation of compounds and may have similar protein trafficking mechanisms. In the literature, PSVs are also referred to as protein bodies however this terminology is being used less to avoid confusion with ER-derived protein bodies (Vitale and Raikhel, 1999). The PSV is unique to plants (Wang et al., 2011b): it is found exclusively in seed and young meristematic plant cells (Zheng and Staehelin, 2011; Olbrich et al., 2007). In seeds, PSVs begin to appear during the maturation phase of embryo development as an organelle specializing in accumulating storage proteins (Mansfield and Briarty, 1992). Whereas LVs typically occupy most of the cell space, PSVs are much smaller in size and range ~ 1.5-8 μm in diameter (Gillespie et al., 2005). Therefore, in seeds, PSVs are numerous and are usually positioned close to the nucleus in the center of the cell (Shimada et al., 2008). In meristematic cells, the existence of PSVs is thought to be due to the persistence of seed-specific signals in the meristem (Olbrich et al., 2007).

PSVs have a more complex internal organization than their fluid-filled relatives. PSVs are compartmentalized organelles whose tonoplast may only differ slightly from the LV tonoplast in its composition and abundance of proteins (Jauh et al., 1998). The

level of compartmentalization differs depending on the species studied. For example, in tobacco and tomato seeds, PSVs are divided into three domains; a matrix that contains soluble proteins, a crystalloid composed of soluble proteins arranged in a lattice structure and globoid cavities that contain phytic acid crystals. However, in *Arabidopsis* and other *Brassica* species, there are only two PSV compartments: the matrix and the globoid (Gillespie et al., 2005; Scarafoni et al., 2001; Jiang et al., 2000). The morphology of PSV compartments not only varies between species but also varies within different cells and tissues of the embryo and with the type of storage product (Scarafoni et al., 2001; Mansfield and Briarty, 1992). Globoid cavities are inclusions within seed PSVs that contain stored minerals in the form of phytic acid crystals. Phytic acid is a mixed salt composed of a highly phosphorylated myo-inositol backbone. Besides binding phosphorus, phytate chelates divalent cations such as potassium, calcium, iron and magnesium. Globoids are responsible for storing up to 90% of the total seed phosphorus content which is broken down during germination to release phosphorus and other minerals for the growing embryo (Zheng and Staehelin, 2011; Raboy, 2007; Otegui et al., 2002). Crystalloids accumulate soluble proteins, integral membrane proteins and lipids. The lattice structure of the crystalloid is thought to be due to the arrangement of soluble proteins between parallel arrays of membranes (Jiang et al., 2001; 2000). The matrix is the major site of soluble seed protein accumulation and also accumulates some phytate (Zheng and Staehelin, 2011). In species that have both PSV crystalloid and matrix subdivisions, the distribution of soluble proteins between the two regions is determined by the surface properties of the storage protein (Scarafoni et al., 2001; Herman and Larkins, 1999).

The defining feature of PSVs is their ability to store seed proteins. In fact, the appearance of storage vacuoles in embryonic cells coincides with storage reserve accumulation during the maturation phase (Mansfield and Briarty, 1992). Lesser recognized roles of seed storage vacuoles are the storage of phosphorus and minerals, protective compounds and proteolytic enzymes. Seed storage vacuole globoid inclusions are the main sites of mineral accumulation in embryonic tissues (Otegui et al., 2002). Proteolytic enzymes such as cysteine or aspartic proteinases are stored in the PSV matrix alongside their target proteins however several mechanisms are in place to prevent their

premature degradation (Gruis et al., 2004). PSVs also accumulate defense compounds such as lectins (De Hoff et al., 2009) and chitinase (Sanmartín et al., 2007) which are thought to be used to deter seed predators. Like their LV counterparts, PSVs are also involved in autophagy. For example, the PSVs of wheat (Herman and Larkins, 1999) and maize (Reyes et al., 2011) sequester SSP-filled, ER-derived protein bodies by autophagy (Bassham et al., 2006). Upon germination, the PSV quickly switches from a storage to a degradative function. SSPs and stored minerals are broken down by PSV resident enzymes to make them available to the seedling (Gallardo et al., 2001; Mansfield and Briarty, 1996).

1.5.1.3 Protein trafficking to vacuoles

1.5.1.3.1 The secretory system

Vacuolar proteins may take several alternate routes to reach the vacuole and for the majority of proteins, the ER serves as the starting point of the journey (Ibl and Stoger, 2011). Many proteins destined for the vacuole travel through the secretory pathway via the endomembrane system to arrive at their destination. Therefore, the main route taken by secretory proteins begins in the ER and passes through the *cis*- *medial*- and *trans*-Golgi complex to the *trans* Golgi network (TGN). Prevacuolar compartments (PVCs) named multivesicular bodies (MVBs) arise from the TGN and eventually fuse to vacuoles and deliver their contents (Scheuring et al., 2011; Miao et al., 2008). Vacuoles represent the intracellular endpoint of the secretory system. Alternatively, proteins can be directed to the plasma membrane to be secreted to the cell surface. In the course of their journey through the secretory pathway, proteins are sorted to their specific destinations by sorting signals (Jolliffe et al., 2005; Vitale and Hinz, 2005; Raikhel and Chrispeels, 2000; Vitale and Raikhel, 1999).

1.5.1.3.2 Vacuole sorting signals

Soluble proteins bound for the vacuole must possess sorting signals to direct them through the secretory system. Vacuolar proteins are synthesized with an N-terminal signal peptide which directs its translocation into the ER. Once in the ER lumen, the signal peptide is removed. Vacuolar proteins must also possess a vacuole sorting signal (VSS) which guides them to the vacuole. VSSs are generally grouped into three categories; sequence specific vacuolar sorting signals (ssVSS), C-terminal vacuolar sorting signals (ctVSS) and physical structure vacuolar sorting sequences (psVSS) (Vitale and Hinz, 2005; Vitale and Raikhel, 1999). Most of these signals are situated within propeptide sequences that are cleaved during the maturation of the protein (Vitale and Hinz, 2005). ssVSSs can be found in any position within the precursor protein and they contain a characteristic tetrapeptide motif; Asn, Pro, Ile, Arg (NPIR) (Jolliffe et al., 2005; Carter et al., 2004). It is generally accepted that ssVSS signals direct the protein to LVs (Sohn et al., 2007). ctVSSs are situated within C-terminal propeptides of the precursor protein. They lack a consensus sequence but are usually rich in hydrophobic residues and are thought to trigger aggregation-based sorting of proteins (see section 1.5.1.3.4) (Craddock et al., 2008; Jolliffe et al., 2005; Neuhaus and Rogers, 1998). Proteins carrying psVSSs are thought to possess sorting sequences distributed along internal stretches of the protein which become exposed at the surface once the protein folds into a higher order structure. These sorting signals are also thought to be associated with aggregation-based sorting of proteins (Hinz et al., 2007; Neuhaus and Rogers, 1998). Proteins possessing ctVSS and psVSS signals and which may also have an ssVSS signal, are directed to the PSV (Sohn et al., 2007).

Overall, an easily identifiable, general consensus sequence for vacuole sorting has yet to be identified. Relatively few VSSs have been characterized from a limited number of soluble proteins (Zouhar and Rojo, 2009; Jolliffe et al., 2005; Vitale and Raikhel, 1999). In a proteomic study of *Arabidopsis* LVs, aleurain (a cysteine protease) and several other uncharacterized proteins were identified to possess an ssVSS while several vacuolar peroxidases and a few lectins were identified to have a ctVSS (Carter et al., 2004). Although a large number of putative VSSs have been identified from various

species, a very limited number of *Arabidopsis* VSSs have been actually demonstrated to transport proteins to the vacuole (Fuji et al., 2007). For example, an ssVSS of the cysteine protease aleurain (Ahmed et al., 2000) and an uncharacterized C-terminal signal on a 12S globulin (Shimada et al., 2003a) were shown to mediate transport of the proteins to the LV and PSV, respectively. Surprisingly, VSSs for *Arabidopsis* 12S globulins and 2S albumins have not yet been identified (Craddock et al., 2008; Shimada et al., 2003a). Regardless, *Arabidopsis* is capable of recognizing VSSs because the expression of foreign proteins harboring ctVSSs and ssVSSs are correctly sorted to the vacuole (Park et al., 2005; Vitale and Hinz, 2005). For instance, a commonly used LV lumen marker possessing an NPIR-like ssVSS motif is aleurain (Hinz et al., 2007). Typical PSV lumen protein markers harboring a ctVSS are tobacco chitinase, bean phaseolin and barley lectin (Park et al., 2007; Vitale and Hinz, 2005). Of the total pool of vacuolar proteins in *Arabidopsis*, only a small number have been shown to carry VSSs. Thus, there is speculation that more complex mechanisms exist to direct proteins to the vacuole (Carter et al., 2004).

1.5.1.3.3 Vacuole sorting receptors

VSSs are recognized by receptors in the Golgi network which help direct them along the secretory pathway toward the vacuole. Two plant-specific families of vacuolar sorting receptors have been identified; vacuolar sorting receptors (VSRs) and receptor homology-transmembrane-RING H2 domain proteins (RMRs) (Zouhar et al., 2010; Zouhar and Rojo, 2009). In *Arabidopsis*, the VSR family is composed of seven members (*AtVSR1*-*AtVSR7*). Through loss-of-function mutation analysis *VSR1* was shown to be the major seed isoform and was identified to be most closely related to homologs that were previously characterized in other plant species (Fuji et al., 2007; Shimada et al., 2003a). Thus, the *VSR1* isoform is best characterized. The RMR family is composed of five members in *Arabidopsis* and two have so far been characterized (*AtRMR1* and *AtRMR2*) (Wang et al., 2011a). *AtRMR1* is expressed in all tissues regardless of the developmental stage (Scabone et al., 2011) but *AtRMR2* is more highly expressed in seeds than *AtRMR1*

(Hinz et al., 2007). Both VSR and RMR receptors are transmembrane proteins with two major binding domains; one domain for binding vacuolar proteins within the lumen of the Golgi and a second domain in the cytoplasm that would be recognized by proteins that would assemble the receptor and its ligand into vesicles destined for the vacuole (Neuhaus and Rogers, 1998). Their luminal domains are more-or-less structurally similar but their cytoplasmic domains are not conserved; VSRs have a short cytoplasmic tail that can recruit clathrin adaptor proteins, which plays a major role in the formation of vesicles, but RMRs have a long tail with a RING-H2 zinc finger motif whose function is unknown (Wang et al., 2011a; Zouhar and Rojo, 2009; Fuji et al., 2007; Hinz et al., 2007).

Not long ago, a "one vacuole, one pathway" model was accepted whereby proproteins carrying ssVSSs would interact exclusively with VSR1 and travel to the LV while ctVSSs would only interact with RMR and be directed to the PSV (Jolliffe et al., 2005). However, this model is now outdated because exceptions were discovered to exist (Lousa et al., 2012). Loss-of-function of *vsr1* caused SSPs to missort to the apoplast in seeds, suggesting that VSR1 also functions as a receptor for SSP trafficking to the PSV (Shimada et al., 2003a). Similarly, expression of ctVSS and ssVSS reporters in *vsr1* mutant seeds caused secretion of both reporters (Craddock et al., 2008). Additionally, conflicting observations were made by different groups. Otegui et al. (2006) showed that VSR1 associated with SSPs in the Golgi but Hinz et al. (2007) observed a separation of VSR1 and SSPs in the Golgi. A recent genetic analysis of VSR and RMR gene families demonstrated that VSR1, VSR3 and VSR4 act redundantly as key sorting receptors for storage cargo en route to both PSVs and LVs. However, RMR receptors did not play a significant role in sorting cargo to both vacuole types (Zouhar et al., 2010). Studies on RMR receptors have consistently demonstrated that they are associated with aggregation-based sorting of SSPs to the PSV and do not associate with ssVSS proteins (Wang et al., 2011a; Hinz et al., 2007; Park et al., 2007; 2005). However, the transmembrane and cytosolic domains of the RMR1 receptor have been demonstrated to deliver fluorescent reporter proteins to both PSV and LV lumens in embryos or roots and leaves, respectively (Scabone et al., 2011). A theory proposed to explain the roles of RMR and VSR receptors is that they function as co-receptors for SSPs. RMRs may be associated

with storage protein aggregation while VSRs function by targeting the aggregates to vesicles destined for vacuoles (Zouhar and Rojo, 2009; Rojo and Denecke, 2008).

Generally, receptors must be recycled after they have trafficked their ligands to promote proper membrane flow (Park et al., 2007). VSR1 is observed to traffic from the *trans*-Golgi cisternae and TGN to the PVCs and is recycled between the two compartments (Rojo and Denecke, 2008; Hinz et al., 2007; Otegui et al., 2006). Recycling of VSR1 is dependent on pH differences between the endomembrane compartments (Lousa et al., 2012). Along the secretory pathway, there is a decreasing luminal pH and recycling of VSR1 is dependant on this pH difference between endomembrane compartments. VSR1 has highest affinity for its ligand at pH 6, which is close to the Golgi lumen pH of ~ 6.5 and is observed to release its ligand in the PVC and recycle back to the Golgi. VSR1 affinity for its ligand is known to decrease by 50% at pH 5 or 7.5 (Vitale and Raikhel, 1999). Although the pH of PSVs (pH 4.9-6.1) and LVs (pH ~ 5.5) have been estimated, the pH of the TGN or the MVB has yet to be measured (Scheuring et al., 2011; Otegui et al., 2006; Müntz, 1998). In contrast, RMR receptors do not recycle back to the Golgi (Scabone et al., 2011). RMR receptors are observed to interact with their ligands at the *cis*- and *medial*- Golgi cisternae and traffic to the MVB where they become internalized into the luminal contents and subsequently delivered to the PSV (Hinz et al., 2007; Park et al., 2007; 2005). Within PSVs, RMRs are localized to the crystalloid and matrix (Gillespie et al., 2005; Jiang et al., 2000). Since RMR does not recycle, it is proposed to maintain efficient sorting by interacting with an aggregate of proteins, therefore only a limited number of RMR receptors are required to sort a large amount of proteins to the vacuole (Wang et al., 2011a).

1.5.1.3.4 Aggregation-based sorting

Aggregation plays an important role in sorting a number of vacuolar seed proteins such as 12S globulins and 2S albumins (Otegui et al., 2006; Robinson et al., 2005). This mechanism encourages condensation of proteins at the margins of Golgi cisternae which in turn promotes their spatial segregation from other secretory or resident proteins (Hinz

et al., 2007; Park et al., 2007). Hydrophobic sorting sequences, such as ctVSSs and psVSSs, are proposed to promote aggregation-based sorting. These hydrophobic sequences cause neighboring proteins to interact with one another and lead to aggregation. Aggregation is facilitated by factors which enhance the condensation of proteins such as low pH, proteins that encourage aggregation and mechanisms that attach aggregates to the membrane of the compartment (Ibl and Stoger, 2011; Robinson et al., 2005). RMR receptors are involved in aggregation-based sorting of SSPs to the PSV and may fulfill some of these roles (Wang et al., 2011a; Hinz et al., 2007; Park et al., 2007).

1.5.1.3.5 Vesicular trafficking to vacuoles

Protein sorting to the vacuole is a complex process that is slowly being elucidated but there is still much room for discovery (Wang et al., 2011b; Zouhar and Rojo, 2009; Sanmartín et al., 2007; Sohn et al., 2007). The transport of soluble proteins is best characterized whereas the rules for sorting membrane proteins are not so well established (Rojo and Denecke, 2008). Seed proteins accumulate to high levels in PSVs during the maturation phase of seed development and thus represent a convenient system in which to study protein trafficking. Consequently, much knowledge has been obtained about their trafficking pathways (Fujiwara et al., 2008). Several protein sorting pathways to vacuoles have been described in plants and the route that a protein takes can depend on several factors including the species, tissue or developmental stage (Ibl and Stoger, 2010; Vitale and Hinz, 2005).

Most vacuolar proteins begin their journey through the secretory system in the ER (Ibl and Stoger, 2011). Within the ER lumen, some vacuolar proproteins aggregate and are sequestered into precursor-accumulating (PAC) vesicles. PAC vesicles travel directly to the PSV, bypassing the Golgi. PAC vesicles are approx. 200-400 nm in diameter, have electron dense cores surrounded by an electron translucent layer and are sometimes surrounded by ribosomes. PAC vesicles were first discovered to transport SSP precursors from the rER to the PSV in pumpkin seeds (Hara-Nishimura et al., 1998). These vesicles

deliver their contents to the PSV either by an autophagic mechanism or by membrane fusion (Ibl and Stoger, 2011).

A second mechanism for ER to PSV transport is via protein bodies (PBs). This pathway is used predominantly in monocots such as wheat, barley and maize (Herman and Larkins, 1999). In monocots, prolamins are the major SSP family accumulated in seeds but globulins are also accumulated to a lower level (Bethke et al., 1998). In the ER lumen, SSPs aggregate and are accumulated in PB accretions inside the ER. Alternatively, PBs are observed to bud off from the ER and accumulate in the cytoplasm or can be sequestered into PSVs, by a mechanism that resembles autophagy (Ibl and Stoger, 2011; Herman and Larkins, 1999). In maize aleurone cells (the outermost layer of the endosperm in monocots), PBs accumulate SSPs and are delivered to the PSV by an autophagy-like mechanism (Reyes et al., 2011).

Most soluble vacuolar proteins travel from the ER to the Golgi where they are sorted and assembled into different vesicles for delivery to the vacuole (Zouhar and Rojo, 2009). Many SSPs are sorted into dense vesicles by aggregation. SSPs are transported from the ER to the Golgi where they begin to aggregate along the periphery of the *cis*-cisternae (Wang et al., 2011a). These aggregates progress throughout the Golgi stack as the cisternae mature. Upon reaching the *cis*- or *medial*- Golgi cisternae (Hinz et al., 2007) or the *trans*-Golgi or TGN (Otegui et al., 2006), the protein aggregates bud off to produce electron dense vesicles, named for their appearance under the transmission electron microscope (TEM). Dense vesicles are about 120 nm in diameter and contain an electron dense core but no protein coat (Hinz et al., 2007). In contrast, other proteins that don't appear to sort by aggregation, such as SSP processing proteases, are excluded from the cisternal buds possibly as a consequence of the aggregation of SSPs and the narrow structure of the cisternal buds. At the TGN, these SSP processing proteases are recruited into smaller vesicles (30-40 nm in diameter) that may be coated with clathrin (Otegui et al., 2006; Vitale and Hinz, 2005). Dense vesicles carrying SSPs and the clathrin coated vesicles carrying proteases subsequently fuse to form PVCs called MVBs (Otegui et al., 2006).

MVBs are membrane-bound intermediate organelles that mediate protein trafficking from the Golgi or the TGN to the lytic or storage vacuoles (Miao et al., 2008). Recently, the MVB was determined to be derived from the TGN (Scheuring et al., 2011). One of the main functions of MVBs is to invaginate membrane domains containing membrane proteins destined for the vacuole, which gives rise to their characteristic internal vesicle morphology (Otegui et al., 2006; Jiang et al., 2002). Within the MVB, proteolytic processing of SSP precursors begins. Although the pH of MVBs has not been measured, it is proposed that the lumen gradually acidifies as the organelle matures. The fact that proteases, which are activated under acidic conditions, were observed to process SSPs within the MVB lumen supports this hypothesis (Otegui et al., 2006). The MVB then fuses with the PSV tonoplast to deliver its contents (Scheuring et al., 2011).

1.5.1.4 Storage protein deposition in storage vacuoles

In *Arabidopsis*, 12S globulin and 2S albumin storage proteins and their processing enzymes, VPE and AP, are stored alongside each other within the PSV matrix in anticipation for protein reserve mobilization during germination. What prevents storage protein degradation in the PSV until germination? One rationale is that limited proteolytic processing of SSP proprotein precursors leads to conformational changes that alter their physical properties to allow dense packaging and stable storage (Gruis et al., 2004; Scarafoni et al., 2001). Proteolytic processing begins in the MVB but it is unclear how much processing takes place before transfer to PSVs (Otegui et al., 2006). However, once proteins reach the PSV, limited proteolysis of proproteins results in a conformational change into higher order structures. These mature SSPs become resistant to further proteolysis and favor protein deposition (Müntz, 2007). In addition, under the acidic conditions of the PSV lumen, properly processed storage proteins are considerably less soluble and tend to aggregate, which makes them more resistant to proteases and thus less prone to degradation. At the same time, the PSV luminal pH may modulate the activity of proteases (He et al., 2007). The pH of the PSV lumen decreases from 6.1 to 4.9 during storage protein deposition. This increase in acidity may affect the activity of proteolytic

enzymes; VPE is highly active between pH 5-6 and AP at pH 3-4 (Otegui et al., 2006; Gruis et al., 2004). Therefore, two factors appear to act together to allow stable storage of SSPs and their processing enzymes in PSVs: protein conformation and pH. However, the conformation of SSPs is not only important for stable storage but it also affects their accumulation pattern and packaging in the PSV, dehydration and rehydration properties and affects their solubility during protein mobilization after germination (Scarafoni et al., 2001).

1.5.1.5 Characterization of lytic and storage vacuoles

Although PSVs and LVs are morphologically distinct, they can be further distinguished by pH differences and by the expression of particular membrane and lumen proteins (Frigerio et al., 2008). Both LVs and PSVs maintain an acidic lumen but they differ slightly in their level of acidity; PSVs range between pH 4.9-6.1 while LVs have been estimated to have a pH of about 5.5 (Otegui et al., 2006; Müntz, 1998). Thus, vacuoles can be characterized by pH-sensitive dyes and fluorescent probes such as neutral red (Dubrovsky et al., 2006), acridine orange (He et al., 2007) and lysosensor yellow/blue DND-160 (Otegui et al., 2006).

A small number of soluble proteins have been shown to exclusively reside in the lumen of a particular vacuole type. Most of these proteins were characterized to possess specific VSSs that would direct the protein to the specific vacuole (Robinson et al., 2005; Vitale and Hinz, 2005). For example, the cysteine protease aleurain carries an ssVSS (NPIR) which targets the protein to the LV (Ahmed et al., 2000). In contrast, phaseolin, the major storage protein of common bean, carries a ctVSS sequence (Ala, Phe, Val, Tyr [AFVY]) which targets the protein to PSVs (Frigerio et al., 1998). When the gene encoding the protein, or only the VSS, is fused to a reporter and expressed in similar tissues of a foreign plant species, such as *Arabidopsis*, these vacuolar proteins or their VSSs accumulate in the same vacuole type as the native plant (Hunter et al., 2007). Thus, these proteins or their VSSs serve as practical tools to help elucidate their mechanism of transport to vacuoles (Miao et al., 2008) or to distinguish between vacuole types (Hunter

et al., 2007; Paris et al., 1996). In addition, the *Arabidopsis* vacuole proteome has been characterized for both LVs (Carter et al., 2004) and PSVs (Le et al., 2010) and has led to the identification of several new vacuole marker candidates.

Vacuoles tend to possess the same complement of integral membrane proteins in their tonoplasts but the existence of one type of membrane protein has been shown to differ between vacuole types (Hunter et al., 2007; Jauh et al., 1998). Tonoplast intrinsic proteins (TIPs) belong to a family of transmembrane proteins that function as aquaporins which form channels to transport water and small molecules across the tonoplast (Maurel et al., 2009; Johanson et al., 2001). Studies using aquaporin inhibitors such as mercury have demonstrated that their main functions are osmoregulation and control of water flow which leads to control of cell enlargement during growth (Beebo et al., 2009). *Arabidopsis* has ten TIP isoforms that are divided into five subgroups (*AtTIP1*- *AtTIP5*). The TIP isoforms are very similar and differ only in their C-terminal cytoplasmic tails, thus providing a means to distinguish the isoforms using antibodies against that region (Johanson et al., 2001; Jauh et al., 1999). By over-expressing fluorescent reporter fusions of all TIP isoforms under control of their native promoters, TIP expression patterns were determined to be developmentally and spatially controlled (Gattolin et al., 2011; 2009; Beebo et al., 2009). Two TIP isoforms (TIP3;1 and TIP3;2) are strictly expressed in the embryo during seed maturation and early germination and both localize to the PSV tonoplast (Gattolin et al., 2011). The expression of TIP3;1 (also called alpha-TIP) was compared with TIP1;1 (also called gamma-TIP), an isoform highly expressed in LV tonoplasts, and was found to have minimal overlap in the timing of their expression. During *Arabidopsis* seed development, TIP3;1 is highly expressed in embryos where it localizes to the PSV tonoplast. During germination, TIP3;1 expression declines and is gradually replaced by TIP1;1 expression in the tonoplast of the developing LV. Thus, concurrent with the replacement of TIP isoforms is the transition of vacuole types during germination (Gattolin et al., 2009; Hunter et al., 2007). Therefore TIP1;1 and TIP3;1 localization patterns have been exploited to determine the identity of LVs and PSVs, respectively (Frigerio et al., 2008; Jauh et al., 1999).

1.5.1.6 Multiple vacuoles in plant cells: the debate

Plant cells are unique in that they have two functionally distinct vacuole types, LVs and PSVs (Becker, 2007). This has raised questions about whether the two vacuoles co-exist in cells. One of the first proponents to argue for the co-existence of separate vacuoles in cells were Paris et al. (1996). By immunolabeling pea, barley and tobacco root tips with a combination of antibodies against TIP1;1 (LV), TIP3;1 (PSV), lectin (PSV) and aleurain (LV) antigens, they discovered two separate vacuole compartments in the same cell, one labeled with TIP3;1 (PSV) and one labeled with TIP1;1 (LV). They concluded that two vacuole types existed as separate compartments which later fuse to form the LV. This idea was accepted by the scientific community because it provided a convenient explanation for the diverse sorting signals and routes that proteins take to arrive at the two vacuoles (Frigerio et al., 2008; Vitale and Hinz, 2005; Vitale and Raikhel, 1999). Separate vacuole types were also discovered in leaf protoplasts from tobacco, common bean and *Arabidopsis* but only when TIP3;1 and TIP1;1 isoforms were constitutively expressed. Consequently, the authors proposed that PSVs were present in vegetative cells such as leaves (Park et al., 2004). Recently, however, two groups have challenged the multiple vacuole theory. Hunter et al. (2007) constitutively expressed a combination of fluorescently labeled TIP fusions as well as PSV and LV lumen markers in various *Arabidopsis* tissue types and discovered that the TIP3;1 and TIP1;1 isoforms were targeted to the same vacuole, regardless of the tissue type. However, when they expressed TIP3;1 and TIP1;1 under control of their native promoters, they realized that TIP3;1 was exclusively expressed in seeds while TIP1;1 expression was specific for vegetative tissues. They inferred from their results that TIP isoform distribution is tissue- and development-specific rather than organelle-specific. Olbrich et al. (2007) reinvestigated the work of Paris et al. (1996) by studying the distribution of TIPs in barley and pea roots and, in contrast to the previous results, found that root meristematic cells do not harbor separate PSV and LV compartments. Instead, their results pointed to a single vacuole type with mixed PSV (TIP3;1) and LV (TIP1;1) characteristics in the root meristem which would take on more LV characteristics (TIP1;1) as the cell matured. These conflicting results fueled a heated debate as to whether multiple vacuoles with different functions co-exist in cells (Frigerio, 2008; Robinson, 2008; Rogers, 2008).

Today, the prevailing view is that most cells contain only one vacuole type, however, both vacuoles can co-exist in cells but it is usually short-lived (Frigerio et al., 2008). A model example of vacuole co-existence is the ice plant (*Mesembryanthemum crystallinum*) which contains two separate vacuoles during periods of salt stress; one vacuole functions to accumulate salt while the second is acidic and stores photosynthate (Frigerio, 2008). In another example, senescing tissues of soybean and *Arabidopsis* accumulate small senescence-associated vacuoles (SAVs) in the same cells as LVs. SAVs have a higher cysteine protease activity and a lower pH than the LVs and also lack TIP1;1 (Otegui et al., 2005). Multiple vacuoles have also been observed during developmental transitions but vacuoles co-exist only for short time periods (Frigerio et al., 2008; Hoh et al., 1995).

The multi-vacuole debate was brought about largely by a lack of understanding about TIP expression patterns. Over the years, several authors on both sides of the debate contributed a great deal toward our present understanding of these factors. Since the melting point of the multi-vacuole debate, a detailed map of the TIP isoform expression patterns in *Arabidopsis* has been made to better understand the distribution pattern of these vacuole markers (Gattolin et al., 2011; 2010; 2009). However, some differences in TIP expression patterns are still being reported (Bolte et al., 2011).

1.5.1.7 Developmental transitions and biogenesis of vacuoles

Vacuoles are dynamic organelles that are capable of transforming in both form and function to suit the needs of the cell (Marty, 1999). This is most obvious during vegetative to embryonic developmental transitions. How do such morphologically distinct vacuoles replace each other in the cell? It is now accepted that most cells contain only one vacuole type (Frigerio et al., 2008), therefore the existing vacuole must somehow be replaced by the other. There are two models to explain the development of vacuoles in plant cells; they can form *de novo* or by remodeling the pre-existing vacuole (Müntz, 2007).

1.5.1.7.1 LV to PSV transition

During embryogenesis, a large LV forms in the fertilized zygote. The zygote then divides to produce a vacuolated basal cell (which will form the suspensor) and a non-vacuolated apical cell (which will give rise to the embryo). As the embryo continues to divide, LVs develop in all cells (Zouhar and Rojo, 2009). During the maturation phase, LVs are replaced by PSVs to provide a refuge to store protein and mineral reserves (Mansfield and Briarty, 1991). There are some obstacles that interfere with studying LV to PSV transitions. Seed tissues are not easy to study because they can be difficult to handle, especially *Arabidopsis* which has such small seeds (Ibl and Stoger, 2011; Girke et al., 2000). In addition, PSVs develop in a short time period (Mansfield and Briarty, 1992).

1.5.1.7.2 PSV to LV transition

During seed germination, the seed imbibes water and storage reserves are mobilized to provide nutrients and energy for the growing embryo. Once most of the reserves have been mobilized, the PSV is replaced by a LV which has the ability to take up water and increase in size to create turgor and support cell growth and expansion (Mansfield and Briarty, 1996). In *Arabidopsis*, it takes approximately 3.5 d for PSVs to transition into LVs (Hunter et al., 2007). An alternate approach to study PSV to LV transitions in seeds is to study root cells. PSVs are present in the radicle as it emerges from the seed coat. As the root elongates, meristematic regions of the root tip retain PSVs while PSVs transition to LVs in the distal regions of elongating roots (Gattolin et al., 2011; Zheng and Staehelin, 2011; Olbrich et al., 2007).

1.5.1.7.3 Vacuole remodeling

One means by which vacuoles may replace each other in a cell is by remodeling or reprogramming the vacuole that is already present in the cell. Several studies support this hypothesis. During *Arabidopsis* seedling development, Mansfield and Briarty (1996)

observed multiple PSVs fusing together to form the LV after the mobilization of most protein reserves. Olbrich et al. (2007) observed the formation of a single vacuole in barley and pea root tip cells. Close to the root tip, cells contain PSVs labeled by TIP3;1. As root cells differentiate, the enlarging vacuole becomes a PSV-LV hybrid labeled by TIP3;1 and TIP1;1 that contains storage proteins. The hybrid vacuole then gradually differentiates into a LV with increasing TIP1;1 and decreasing TIP3;1 labeling. Similarly, Zheng and Staehelin (2011) discovered that PSVs in tobacco root tips were transformed into LV. Using a combination of TEM procedures to carefully study the PSV to LV transition, they showed that vacuole transformation involved unique spatial and temporal changes in vacuole architecture that were highly tissue-specific. In addition, within some cell types, the transformation involved autophagosome formation and engulfment by the developing LVs.

1.5.1.7.4 *De novo* vacuole formation

An alternative hypothesis to explain how different vacuole types replace each other involves the independent generation of a vacuole within a cell that already has a pre-existing vacuole. A key study to support this theory was conducted by Hoh et al. (1995). Using TEM techniques, the transition from LV to PSV was studied in cotyledons during pea seed development. The authors observed the development of a tubular PSV structure which overtook the pre-existing LV. A second example backing this idea is the demonstration that vacuoles can be regenerated from evacuolated protoplasts. Using autophagy inhibitors, the authors demonstrated that an autophagy-like mechanism is involved in vacuole biogenesis (Yano et al., 2007). If the *de novo* theory holds true, then where does the membrane for new vacuole come from? If an autophagy-like mechanism is involved in vacuole biogenesis, perhaps the vacuole membrane is generated by a process similar to autophagosome formation. In autophagosome formation, the first step is the formation of an isolation membrane which occurs in the cytoplasm close to the vacuole. This process involves recruitment of several autophagy-related (ATG) proteins which assemble in a coordinate manner to form a cup-shaped membrane structure which

elongates and eventually engulfs material to be taken to the vacuole (Nakatogawa et al., 2009; Mizushima, 2007). Vacuole tonoplasts are also proposed to originate from the ER or from the Golgi apparatus (Neuhaus and Rogers, 1998). Several mutants defective in vacuole formation have been identified (Zouhar and Rojo, 2009). Most are characterized to be involved in mediating membrane fusion. An essential gene involved in vacuole biogenesis has been identified as *VACUOLELESS1* (*VCL1*) through a mutant screen (Rojo et al., 2001). Loss-of-function *vcl1* embryos were unable to form vacuoles; mutants accumulated large numbers of small vesicles which were unable to fuse to form the vacuole but instead would fuse with the plasma membrane and deliver their vacuolar contents to the apoplasm. Thus, *VCL1* is proposed to be involved in regulating the fusion of prevacuolar vesicles. The lack of vacuoles caused defects in the pattern of cell division and elongation during embryo morphogenesis and eventually led to embryo death.

1.5.2 Oil bodies: now you see them, now you don't

Oil bodies are organelles that specialize in storing neutral oils. They are small organelles ~ 0.5-2.5 μm in diameter and their size varies depending on the plant species (van der Schoot et al., 2011; Lersten et al., 2006). The matrix of oil bodies is filled with TAGs and is surrounded by a phospholipid monolayer. The single membrane is stabilized by proteins; the most abundant and best characterized protein is oleosin but other minor proteins have been identified such as caleosin and steroleosin (Hsieh and Huang, 2004; Jolivet et al., 2004; Frandsen et al., 2001). Oil bodies are most commonly associated with seed tissues for storage of lipids but are also found in anther cells (Hsieh and Huang, 2007; Wu et al., 1997). During seed maturation, oil bodies accumulate TAGs to provide energy and nutrients for the embryo upon germination (Penfield et al., 2006). In *Arabidopsis* seeds, oil bodies make up ~ 60% of the cell volume and accumulate in cells of the embryo and endosperm (Baud et al., 2002). Oil bodies are generally localized along the periphery of cells and surround PSVs (Shimada et al., 2008).

1.5.2.1 Foliar oil bodies

TAGs are storage lipids that are not normally associated with plant leaves however the presence of TAGs has been demonstrated in the leaves of several plant species (Lin and Oliver, 2008). Leaf mesophyll cells from diverse plant species have been reported to contain foliar oil bodies that accumulate TAGs (Pascual-Villalobos and Lopez, 2010; Lersten et al., 2006; Wahlroos et al., 2003; Parker and Murphy, 1981). These oil bodies range from 1-14 μm in diameter, depending on the plant species (Parker and Murphy, 1981). Foliar oil bodies are found accumulating in palisade and spongy mesophyll cells but not in vascular bundle or epidermal cells. Generally, single particles are observed in leaves but occasionally multiple particles are seen. They are thought to play a role in short-term storage of lipids generated by photosynthesis and to help the plant adapt to cold temperatures (Pascual-Villalobos and Lopez, 2010; Lin and Oliver, 2008; Lersten et al., 2006). The occurrence of foliar oil bodies in *Arabidopsis* leaves has not been reported in the literature. However, foliar oil bodies were absent from leaves in a survey of selected species of Brassicaceae, to which family *Arabidopsis* belongs (Lersten et al., 2006).

In the literature, lipid-containing vesicles are given different names with slightly different meanings. In many cases, these names are used interchangeably, which can be confusing. Oleosomes are used instead of oil bodies but are a less popular term, spherosomes describe particles with an unspecified matrix content and lipid bodies refer to a less well-defined oil body (van der Schoot et al., 2011; Huang, 1992). This raises the question of whether foliar oil bodies are oil bodies as defined in seeds. It is generally agreed that the presence of oleosin and the accumulation of TAGs are defining features of oil bodies (van der Schoot et al., 2011; Lersten et al., 2006; Hsieh and Huang, 2004). Vesicles that accumulate leaf lipids were largely identified by histochemical staining and lipid analysis but the presence of oleosin or other structural proteins embedded in the membrane was not confirmed (Pascual-Villalobos and Lopez, 2010; Lersten et al., 2006; Wahlroos et al., 2003; Parker and Murphy, 1981). In addition, oleosin transcripts were not found to be expressed in leaves (Kim et al., 2002). Therefore, it is unclear whether foliar oil bodies should be grouped together with seed oil bodies.

1.5.2.2 Structure and biogenesis of oil bodies

Seed oil bodies have been most extensively studied and were shown to originate from the ER. TAGs are synthesized in the ER lumen and accumulate between the ER phospholipid bilayer (Hsieh and Huang, 2004). At the same time, structural proteins such as oleosin are synthesized and embedded in the ER double membrane (see section 1.3.3). The protein migrates along the ER double membrane and is incorporated into the single membrane of the budding oil bodies. The means by which oleosin is targeted to the oil body is thought to depend on its topology within the ER membrane. The hydrophobic domain embedded in the ER membrane is thought to exist in a constrained state which is relaxed once the protein transitions from the phospholipid bilayer of the ER to a phospholipid monolayer containing TAGs. Within the phospholipid monolayer, the interaction of the oleosin hydrophobic domain with TAGs is favored over the phospholipid bilayer (Abell, 2004; Huang, 1996). Ultimately, oleosins are anchored to the oil body by insertion of their central hydrophobic region into the phospholipid monolayer and TAG matrix while the hydrophilic N- and C- terminal ends interact with the phospholipids present on the surface of the oil body (Huang, 1996). Oil bodies bud off from the ER surrounded by a phospholipid monolayer embedded with oleosins (Huang, 1992). Oil bodies accumulate in the cytoplasm and often small oil bodies will fuse until they reach their characteristic size (van der Schoot et al., 2011). In *Arabidopsis* seeds, oil bodies are ~ 0.5-2 μm in diameter and their size varies depending on factors such as the plant's nutritional status (Frandsen et al., 2001).

1.5.2.3 Function of oleosins

A remarkable feature of oil bodies is that they maintain their small size and resist fusion and aggregation (van der Schoot et al, 2011). Oleosins play an important role in controlling oil body structure and stability. Once oil bodies reach their characteristic size, the entire surface of the mature oil body is covered by oleosins (Huang, 1996; 1992). This protein conformation forms a negatively charged, cage-like barrier over the phospholipid membrane. The barrier stabilizes oil bodies by preventing the fusion of

adjacent oil body membranes through steric hindrance and by preventing the aggregation of oil bodies by electrical repulsion (van der Schoot et al., 2011; Tzen et al., 1992). Oleosins also control the size of oil bodies, favoring a small size with a high surface to volume ratio. The small size is preferred because it facilitates access to lipases and accelerates TAG catabolism during germination. Disruptions in oleosin accumulation, which affect oil body size, have also been shown to retard germination and affect the quantity and composition of synthesized TAGs (Siloto et al., 2006) and to disrupt freezing tolerance in over-wintering seeds (Shimada et al., 2008).

1.5.3 Plastids: a plant cell's best friend

As the name suggests, plastids are the organelles that display the most plasticity in terms of size, shape, structure and function. All plastids are surrounded by two membranes, the inner and outer envelopes and possess an internal membrane system that can vary in structure from rudimentary to highly complex (Vothknecht and Westhoff, 2001; Staehelin and Newcomb, 2000). Plastids are essential for plant survival. In addition to carbon fixation through photosynthesis, they are involved in numerous metabolic processes such as the biosynthesis and accumulation of starch, lipids and amino acids (Baud et al., 2008; Zeeman et al., 2007; Neuhaus and Emes, 2000).

1.5.3.1 Origins

Although plastids carry out an integral role in plant cells, these organelles have a unique origin. Plastids were once free-living photosynthetic prokaryotes, closely related to the cyanobacterium present today. According to the endosymbiotic theory, an early mitochondrion-containing eukaryotic cell engulfed a primitive cyanobacterium between 1.2-1.5 billion years ago (Lopez-Juez and Pyke, 2005; Dyall et al., 2004). The evolution of the plastid from an independent endosymbiont to an organelle involved a reduction in its genome size. Many genes were lost and most of those retained were transferred to the nucleus (Dyall et al., 2004). Plastids still contain their own genome which encodes about

100 proteins but most of the proteins required in plastids are encoded by the nuclear genome (Hsu et al., 2010). It is thought that gene transfer from the photosynthetic endosymbiont to the host cell nucleus occurred by lysis of the endosymbiont or DNA escape during endosymbiont division. Once protein translocation machinery evolved to route gene products from the nucleus back to the endosymbiont, the genetic redundancy led to gene loss and genome reduction of the endosymbiont (Dyall et al., 2004).

1.5.3.2 Plastid biogenesis

Plastid development is closely associated with plant growth and development (Hsu et al., 2010). Plastid biogenesis, that is to say, division and differentiation, is controlled by nuclear and plastid-derived proteins. Thus, plastids rely heavily on the post-translational import of nuclear-encoded plastid proteins. The majority of these proteins are produced as precursors which are directed to the plastid by a transit peptide. The transit peptide is located on the N-terminus of the precursor protein and is cleaved upon entry into the plastid. The majority of proteins are imported through the plastid outer and inner envelopes via the translocon of the outer chloroplast envelope (TOC) and the translocon of the inner chloroplast envelope (TIC) complexes (Inaba and Ito-Inaba, 2010; Lopez-Juez and Pyke, 2005; Dyall et al., 2004).

Plastids are found in every plant cell except for pollen (Neuhaus and Emes, 2000). To maintain their presence in each dividing cell and to multiply their numbers within a cell, plastids must divide. Plastids replicate by division of pre-existing plastids and are passed on from cell to daughter cell using a process similar to bacterial fission (Lopez-Juez and Pyke, 2005). Division involves a constriction in the middle of the plastid formed by contractile rings. As the rings narrow, the internal membranes of the daughter plastids separate. Once the plastids pinch off from the narrow constriction, the daughter envelope membranes reseal (Miyagishima, 2011; Pyke, 2010; 1999).

Plastids are ubiquitous in plant cells however their structures and functions vary depending on the cell type or environment. Plastids have acquired the ability to

differentiate into specialized types to carry out essential or specialized functions in different tissues and cell types. In addition, most plastids are capable of inter-converting under specific developmental or environmental conditions. Thus, plastids can differentiate, de-differentiate and re-differentiate (Inaba and Ito-Inaba, 2010; Staehelin and Newcomb, 2000; Thomson and Whatley, 1980).

The progenitor of all plastids is the proplastid, a small colorless plastid found in meristematic and embryogenic cells (Possingham, 1980). It has a very limited internal membrane system composed of invaginations of the inner envelope. The small size and simplicity of these plastids are thought to allow easy transmission in young, embryonic or undifferentiated cells (Lopez-Juez and Pyke, 2005). During cell differentiation, proplastids differentiate into particular plastid types according to the needs of the cell in which they reside (Pyke, 1999).

In the presence of light, proplastids differentiate into chloroplasts. This event can be followed nicely in grass species. In grasses, leaves generally grow from a basal meristem and therefore a developmental gradient is established whereby the young, meristematic cells at the base of the leaf contain proplastids while the oldest cells at the tip of the blade have fully developed chloroplasts (Vothknecht and Westhoff, 2001). Meristematic cells contain ~ 10-20 proplastids that are ~ 0.2-1 μm in diameter with a very limited internal membrane system (Lopez-Juez and Pyke, 2005). Once leaves begin to develop in the presence of light, proplastids actively divide to ensure their transmission into each dividing plant cell. Proplastids also begin to differentiate; they increase in volume and the inner envelope begins to invaginate into the stroma, which is equivalent to bacterial cytosol (Gutiérrez-Nava et al., 2004). The stroma is the site of carbon fixation in chloroplasts and may harbor starch granules and lipid droplets. Proplastids undertake a more highly variable morphology while the internal membrane system becomes more complex resulting in the presence of flattened disc-shaped thylakoids in the stroma. Thylakoids are photosynthetically active membranes that surround a lumen. They appear individually in the stroma (stroma thylakoids) or are organized into an interlinked compartment enclosing a single lumen (thylakoid stacks or grana). The resulting chloroplast is a large lens-shaped organelle between 5-10 μm in diameter and 3-4 μm in

thickness. In photosynthetic cells, chloroplasts continue to multiply by fission (Pogson and Albrecht, 2011). A typical *Arabidopsis* leaf mesophyll cell contains ~ 120 chloroplasts (Vothknecht and Westhoff, 2001; Staehelin and Newcomb, 2000; Reiter et al., 1994; Whatley, 1977).

Non-photosynthetic types of plastids also exist in plant cells. In seeds germinating in the soil, the embryo develops and grows through a period of darkness. The proplastids of dark-grown seedlings differentiate into etioplasts which have few internal membranes but possess a prolamellar body in the stroma. The prolamellar body consists of a crystalline structure composed of lipids and proteins as well as the precursor of chlorophyll, protochlorophyllide. Once the seedling senses light, etioplasts quickly develop into chloroplasts. During this process, flat membrane sacs emerge from prolamellar stacks that eventually become thylakoids. At the same time, light induces the enzymatic conversion of protochlorophyllide to chlorophyll *a* and *b* in the developing thylakoid membranes (Pogson and Albrecht, 2011). Storage organs such as tubers and seeds and even meristematic cells typically contain amyloplasts which are plastids that specialize in starch storage. Amyloplasts are often spherical in shape, lack chlorophyll and generally store sizeable starch granules in their stroma (Wise, 2007; Thompson and Whatley, 1980).

1.5.3.3 Starch and fatty acid metabolism in plastids: an overview

During embryogenesis and vegetative development, plastids play a central role in starch metabolism and participate in metabolic pathways such as lipid biosynthesis. The ability to be involved in such diverse roles is because plastids can function autotrophically and heterotrophically. Thus, they are equipped to either supply the cell with carbon and energy from photosynthesis or assimilate precursors and energy from the cytoplasm (Neuhaus and Emes, 2000; Pyke, 1999).

Starch is the most important storage carbohydrate in plants and is an important product of photosynthesis. In photosynthesizing tissues, chloroplasts convert light into

sugars. The sugars produced by the Calvin cycle as products of photosynthesis in *Arabidopsis* are partitioned and either exported to the cytosol for sucrose synthesis or retained in the chloroplast for starch synthesis (Zeeman et al., 2007). Sucrose is sent to non-photosynthetic parts of the plant while starch transiently accumulates in the chloroplast stroma. At night, the stored starch is degraded to provide a supply of carbohydrate to support the continued growth of the plant (Zeeman et al., 2007; Smith et al., 2005). During early embryo development in *Arabidopsis*, a significant amount of starch accumulates transiently but very low amounts remain in the mature seed (Baud et al., 2002). Proplastids are present in cells until the late globular stage of embryo morphogenesis and then begin to develop into chloroplasts as embryos enter the maturation stage (Hsu et al., 2010). Starch accumulation coincides with the differentiation of proplastids into chloroplasts (Andriotis et al., 2010; Baud et al., 2002). However, in *Arabidopsis* embryos, starch was determined to be synthesized from imported sucrose rather than photosynthesis (da Silva et al., 1997).

Fatty acids accumulate to high levels during oilseed development in species such as *Arabidopsis* (Baud et al., 2002). During the maturation stage of seed development, *Arabidopsis* accumulates lipids in the form of TAGs, which are esters of glycerol and fatty acids. The pathway towards the synthesis, assembly and accumulation of these neutral lipids involves several organelles. However, the initial site of fatty acid synthesis takes place in plastids (North et al., 2010; Baud et al., 2007). The ability to synthesize fatty acids is an important function found in all plastid types (Neuhaus and Emes, 2000; Kaneko and Keegstra, 1996). In seeds, fatty acid synthesis relies on sucrose imported from maternal tissues (Baud et al., 2008; 2007).

1.6 Research goals and objectives

Over the past ten years, significant advances have been made to our understanding of vacuole biology. Our understanding of the spatial and temporal expression patterns of TIPs has greatly improved (Gattolin et al., 2010; Hunter et al., 2007). Similar progress has been made in elucidating the routes that proteins take to reach the vacuole (Rojo and

Denecke, 2008). Microscopy techniques are improving and expanding, which allows for greater clarity of results (Zheng and Staehelin, 2011; Otegui et al., 2006). As a consequence of these advances, it is now generally accepted that cells harbour one vacuole type, with some exceptions. One exception to this rule occurs during developmental transitions, where the two vacuoles co-exist for a short period of time (Frigerio et al., 2008).

The main question of this work is whether PSVs can exist in leaves. If the consensus among scientists is that vegetative leaf cells harbour LVs, then can PSVs be induced to form in leaves? Cues prompting the vegetative cell to switch to PSV formation are not well understood. Despite the fact that SSPs are the major storage reserves that accumulate in PSVs during the maturation phase, their forced synthesis in vegetative tissues does not promote PSV formation. Over-expression of SSPs in vegetative cells was demonstrated to cause formation of novel PAC-like vesicles in *Arabidopsis* leaves (Hayashi et al., 1999) or SSPs were accumulated in the LV or were secreted (Frigerio et al., 1998). Within a plant, PSVs are abundant in seed tissues and are also localized to meristematic areas in vegetative tissues such as root tips (Olbrich et al., 2007). Thus, for PSVs to exist in leaves, these vegetative tissues must be reprogrammed to become embryonic. Regulation of embryogenesis involves a complex network of regulators. Among these, master regulators represent an important genetic control that acts from top-down to promote an embryogenic environment (Santos-Mendoza et al., 2008). Thus, over-expression of these master regulators should promote embryonic characteristics within vegetative tissues. Indeed, the over-expression of LEC2 is sufficient to activate oil and SSP mRNA expression in vegetative tissues. These events occur during the maturation phase of embryo development, concomitant with PSV development (Braybrook et al., 2006; Santos-Mendoza et al., 2005; Mansfield and Briarty, 1991). Moreover, the formation of protein-containing structures was identified in unfertilized ovules and roots constitutively expressing LEC2 (Stone et al., 2008). However these structures were not further investigated.

By over-expressing LEC2 in vegetative tissues, I hypothesize that this transcription factor will promote embryogenic characteristics and cause SSPs to be synthesized and accumulate in PSVs in leaves. Therefore the objectives of this work were:

1. To characterize the phenotype of plants over-expressing LEC2 at the cellular level. Thus far, the focus has been on studying the effects of LEC2 over-expression at the level of transcription (Braybrook et al., 2006; Santos-Mendoza et al., 2005) and very little work has concentrated on the microphenotype (Stone et al., 2008). Therefore the morphological phenotype will be described for plants over-expressing LEC2 and, more importantly, changes in the phenotype will be examined at the cellular level.

2. To detect and localize SSPs in leaves. LEC2 over-expression is known to promote SSP mRNA expression in vegetative tissues (Braybrook et al., 2006; Santos-Mendoza et al., 2005). The accumulation of SSPs in leaves will be investigated and the subcellular location of the seed proteins will be uncovered.

3. To identify the organelle that accumulates SSPs. In seeds, SSPs accumulate to a high level in PSVs (Baud et al., 2008). When over-expressed in vegetative tissues, SSPs were demonstrated to accumulate in LVs or were secreted (Frigerio et al., 1998) or SSPs accumulated in novel PAC-like vesicles (Hayashi et al., 1999). SSPs are predicted to accumulate in the vacuole since SSP precursors possess vacuolar sorting sequences (Hinz et al., 2007; Hunter et al., 2007; Robinson et al., 2005). The type of vacuole that accumulates SSPs will be characterized.

4. To study the biogenesis of the organelle that accumulates SSPs. The aim of this objective is to follow the progression of embryonic characteristics occurring in leaf cells once LEC2 is over-expressed. The focus rests on the formation of PSVs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Arabidopsis thaliana (L.) Heynh. ecotypes used in this study were Wassilewskija (Ws-0) and Columbia (Col-0). For LEC2 over-expression, transgenic Ws-0 plants containing *35S:LEC2-GR* were used (Stone et al., 2008). To study the localization of TIP isoforms, Col-0 transformed with *TIP3;1:TIP3;1-YFP* and *TIP1;1:TIP1;1-RFP*, hereafter referred to as TIP3-YFP/TIP1-RFP, were used (Gattolin et al., 2011). The transgenic line expressing LEC2 was selected with 6 µg/ml glufosinate (Sigma, MO) and the transgenic line expressing TIP isoforms was selected with 50 µg/ml kanamycin (Sigma) or identified by YFP fluorescence of seeds under a stereromicroscope.

2.2 Plant growth conditions

For soil grown plants, dry seeds were transferred to a 100 X 15 mm Petri dish containing Milli-Q^{TM} water (Millipore, MA). The dish was wrapped in Parafilm (Pechiney Plastic Packaging, IL) and placed at 4°C in the dark for 3-4 d for seed stratification. Seeds were sown in 4 inch plastic pots containing moistened soil. Soil was watered with Nemasys (0.5 g/L; Becker Underwood Ltd., IA), a biological control for fungus gnats, and pots were covered with a plastic dome and placed in a growth chamber set at 21°C with 16 h light and 8 h dark photoperiod. Fluorescent lights (Alto II Plus F32T8 TL841, Philips, Netherlands) delivered ~ 100 µmol m^{-2} sec^{-1} light. Domes were removed over a one-week period and plants were watered with 0.5 g/L 20N-20P-20K fertilizer.

2.3 Generation of LEC2/TIP3-YFP/TIP1-RFP plants

For localization of TIP isoforms during LEC2 over-expression, transgenic plants carrying both the *35S:LEC2-GR* and *TIP3-YFP/TIP1-RFP* constructs were generated by genetic crossing. Genomic DNA was isolated from F1 plants by closing a sterile 1.5 ml microfuge tube over a leaf to punch out a leaf disk. Tissues were homogenized in 400 µl extraction buffer [250 mM NaCl, 25 mM ethylenediamine-tetraacetic acid (EDTA) pH 8,

0.5% (w/v) sodium dodecyl sulfate (SDS) and 200 mM Tris pH 7.5] and DNA was precipitated in ice-cold isopropanol and washed in 70% (v/v) ethanol following a modified procedure of Edwards et al. (1991). Pellets were solubilized in 100 µl of sterile Milli-Q water. Genomic DNA was used as a template to screen for transgenes in F1 plants by PCR. PCR reactions were amplified using Taq DNA polymerase (New England Biolabs, MA) following the manufacturer's instructions. Primers were designed to amplify genes specific to each construct. *BAR* primers were designed to amplify the *BAR* glufosinate-resistance gene present in the *35S:LEC2-GR* construct (Stone et al., 2008). *YFP* primer sequences were designed by Hunter et al. (2007) and *RFP* primers were kindly donated by R. Saberianfar. Primer sequences were as follows:

YFP-F:

5' ATATATATCTCGAGGGATCCAAGGGCGAGGAGCTGTTCAC 3'

YFP-R:

5' ACTGACTACCCGGGTCACCTTGTACAGCTCGTCC 3'

RFP-F:

5' GGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGCCTCCTCCGAGGACGT 3'

RFP-R:

5' GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCGCCGGTGGAGTGGC 3’

BAR-F:

5' TGCACCATCGTCAACCAC 3'

BAR-R:

5' ACAGCGACCACGCTCTTG 3'

An AB 2720 Thermocycler (Applied Biosystems, CA) was used with the following cycling conditions: For *YFP* and *BAR* reactions; 5 min 94°C followed by 30 cycles of 30 sec 94°C, 30 sec 55°C, 1 min 68°C with a final extension of 7 min 68°C. For the *RFP* reaction; 5 min 94°C followed by 30 cycles of 30 sec 94°C, 30 sec 68°C, 1 min 72°C with a final extension of 7 min 72°C. Reactions were size separated on a 1.5% (w/v) agarose gel and visualized with ethidium bromide staining. Seed was collected from plants positive for all 3 genes and hereafter referred to as LEC2/TIP3-YFP/TIP1-RFP.

2.4 Tissue culture and growth conditions

Seeds were sterilized by rinsing with 70% (v/v) ethanol for 10 sec followed by a 20% (v/v) bleach solution (6% NaOCl; Lavo Pro6, QC, Canada) twice for 5 min with gentle shaking. Seeds were then rinsed 4 times with sterile Milli-Q water. Sterile seeds were aseptically transferred to 100 X 20 mm Petri dishes containing germination medium. Germination medium consisted of Murashige and Skoog (MS) salts (M524; Phytotech Labs, KS) supplemented with full strength MS vitamins and 0.4 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose, 6.5 g/L agar (A7921; Sigma) and appropriate antibiotic selection. The pH was adjusted to 5.8 with 0.1 M NaOH. All medium components were purchased from Sigma, unless otherwise specified. Dishes were wrapped with Parafilm and seeds were stratified for 3-4 d at 4°C in the dark. Dishes were subsequently transferred to a growth chamber for seed germination. The growth chamber was set at a 16 h light/8 h dark photoperiod provided by a fluorescent light source (Alto II Plus F32T8 TL841, Philips) with a light intensity of ~ 138 μmol m^{-2} sec^{-1}. The temperature was set at 22°C during the day and 18°C at night.

2.5 LEC2 induction

To induce LEC2 over-expression, sterile 35S:LEC2-GR seeds and control Ws-0 seeds were allowed to germinate and grow for 7 d on germination medium. Seedlings were then aseptically transferred to induction medium in 100 X 20 mm Petri dishes. Induction

medium was composed of MS germination medium supplemented with appropriate antibiotic selection and 30 μM dexamethasone (DEX, D4902; Sigma, MO). DEX was solubilized in dimethyl sulfoxide (DMSO), so control seedlings were transferred to MS medium containing appropriate antibiotics and DMSO. Seedlings were incubated in the growth chamber for 14 d before leaves were collected for experiments. For each induction experiment, 6-10 seedlings were transferred to each dish and there were 3-4 dishes for each treatment. Induction experiments were repeated three times.

To observe the effects of LEC2 over-expression over time, 35S:LEC2-GR or Ws-0 seedlings growing for 7 d on germination medium were transferred to DEX induction medium or DMSO control medium. For each experiment, 8-10 seedlings were transferred to each dish and there were 5-7 dishes for each collection day. Dishes were placed in the growth chamber and at 3-4 d intervals over 21 d, dishes were removed and leaves were harvested from plants. After 14 d of incubation, plants were supplemented with DEX to ensure a continuous exposure to the steroid. A 2 ml solution of 30 μM DEX or DMSO in MS liquid was aseptically transferred to each induction or control dish, respectively. Dishes were gently swirled to distribute the liquid over the surface of the medium. Dishes were left for 10 min to allow the medium to absorb the liquid and were then returned to the growth chamber. The time course experiment was repeated twice.

2.6 Tissue collection

Photographs of plants were taken with a Canon PowerShot S5 IS camera (Canon, Japan). For higher magnification images, a Nikon SMZ1500 (Nikon, Japan) dissecting microscope equipped with a DXM1200 digital camera (Nikon) was used. Leaf tissues were harvested for protein analysis and for chemical fixation for subsequent microscopy work. For LEC2 induced plants, leaves were collected from plants displaying the strongest DEX-induced phenotype; these plants were small, with shortened petioles and small, glabrous and curled leaves. All leaves were carefully excised from LEC2 plants using a Leica MZ8 dissecting microscope (Leica Microsystems, Germany) to ensure that cotyledons were not collected. Control plants grew much larger leaves than LEC2

induced plants, therefore young and old leaves were sampled from all plants within the treatment and pooled. For each treatment, leaves collected from replicate plates were pooled.

2.7 Protein extraction and quantitation

For protein extraction, leaves were collected in pre-weighed 2 ml tubes, each containing three 2.3 mm zirconia/silica beads (BioSpec Products, OK). Tubes were placed in dry ice to keep leaf tissue frozen once harvested. Tubes were weighed and transferred to -80°C for storage. The weight of leaf tissue samples ranged between 0.05-0.5 g. To extract protein from seed, ~ 0.1 g seeds were added to a 2 ml tube containing three beads. Tubes were frozen in liquid nitrogen and transferred to -80°C for storage.

Frozen leaf and seed samples were homogenized to a fine powder using a Mixer Mill MM 300 (Retsch Inc., Germany). Tubes were centrifuged to sediment powdered tissue and immediately placed on ice. For extraction of total soluble protein, the powder was resuspended in cold 100-200 µl protein extraction buffer [2% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM EDTA pH 8, 100 mM sodium L-ascorbate, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 µg/ml leupeptin and 0.05% (v/v) Tween-20 (Sigma) in phosphate-buffered saline (PBS) pH 7] and vortexed for 45 sec. The solution was centrifuged at 4°C for 10 min at 18,000 x *g* and the supernatants collected into fresh tubes. Centrifugation steps were repeated two more times for 5 min each and supernatants were collected for further analysis. Total soluble protein concentration was determined based on the method of Bradford (1976) using the Bio Rad Protein Assay kit (500-0006; Bio Rad, CA) with bovine serum albumin as a standard (BSA, BP1600; Fisher Scientific, NH).

For extraction of total protein, frozen leaf and seed samples were ground as described above. Total protein was extracted using a buffer composed of 2% (w/v) SDS and 20 mM dithiothreitol (DTT) in 50 mM Tris pH 6.8. The solution was centrifuged at 4°C for 10 min at 18,000 x *g* and the supernatants collected into fresh tubes.

Centrifugation steps were repeated two more times for 5 min each and supernatants were collected for further analysis. Total protein was quantified based on the method of Lowry et al. (1951) using the Bio Rad RC DC Protein Assay kit (500-0120; Bio-Rad) with BSA as a standard.

After protein quantitation, protein samples were mixed at a ratio of 1:5 with reducing sample buffer [5% (w/v) SDS, 50% (v/v) glycerol, 100 mM DTT, 0.05% (w/v) phenol red in 0.3 M Tris pH 8.0] and stored at -20°C.

2.8 Western blot analysis

Extracted plant proteins were separated by 16% SDS-PAGE tricine gels and transferred to a Sequi Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) by semi-dry blotting. Membranes were incubated overnight in 5% (w/v) blocking buffer [skim milk powder (Carnation^{TM}, Nestle, Switzerland) in TBS-T buffer (300 mM NaCl, 0.1% (v/v) Tween-20, 20 mM Tris pH 7.5)] to prevent non-specific binding of antibodies. Membranes were then incubated with primary antibodies for 1 h at room temperature (RT) with gentle shaking. Primary antibodies and dilutions were: rabbit-anti-12S globulin 1:50,000 (Shimada et al., 2003a), rabbit-anti-napin 1:500 (Scarafoni et al., 2001), mouse-anti-*Arabidopsis* oleosin D9 1:5,000 (SemBioSys Genetics, AB, Canada), rabbit-anti-TIP3;1 0.2 µg/ml (Jauh et al., 1998) and rabbit-anti-TIP1;1 0.24 µg/ml (Jauh et al., 1998). Membranes were washed twice with TBS-T and twice with 0.5% (w/v) blocking buffer. Primary antibodies were detected with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (170-6516; Bio-Rad) or goat-anti-rabbit IgG (170-6515; Bio-Rad). Membranes were incubated with secondary antibodies for 1 hr at RT. All antibodies were diluted in 0.5% (w/v) blocking buffer. Bands were visualized using Amersham ECL Plus kit (GE Healthcare, UK) as described by the manufacturer and exposed on Curix Ultra UV-G Plus X-ray film (Agfa Healthcare, Belgium).

2.9 Chemical fixation

Leaves were excised from plants and immediately transferred to a filter paper (Whatman Intl., UK) wetted with freshly-prepared fixative in a covered Petri dish on ice. Fixative solution was composed of 2.5% (v/v) glutaraldehyde (16350; EMS, PA) in 4% (w/v) paraformaldehyde (P6148; Sigma) in 0.1 M sodium phosphate buffer pH 7.4. Leaf tissue was carefully cut into 1 mm^2 pieces with a sharp blade and immediately transferred to a glass vial containing 1 ml fixative solution. *Arabidopsis* seeds were cut into small pieces using a sharp razor blade and immediately transferred to 1 ml fixative solution in a glass vial. All vials were kept on ice. After all tissues were collected, fixative was discarded from glass vials and replaced with fresh solution. Vials were capped and stored at 4°C for 2 d. Fixative was replaced once during that time. Tissues were then washed twice with 0.1 M sodium phosphate buffer pH 7.4 and stored at 4°C.

2.10 Infiltration and embedding

Samples were embedded in Spurr's resin (14300; EMS) for routine applications or LR Gold resin (London Resin Co., UK) for immunogold labeling. To embed tissues in Spurr's resin, tissue was washed with 0.1 M sodium phosphate buffer pH 7.4. Tissues were post-fixed to enhance contrast of cellular components by incubating in 2% (w/v) osmium tetroxide (OsO_4, Stevens Metallurgical Corp., NY) for 1.5 h in the dark at RT, washed 3 times with Milli-Q water, incubated in 5% (w/v) uranyl acetate (UA, 22400; EMS) for 3 h in the dark and washed twice with Milli-Q water. Samples were then dehydrated in a graded ethanol series [2 exchanges for 15 min for 20% and 50% followed by one exchange for 20 min for 70, 90 and 95% and three exchanges for 15 min with 100% (v/v)]. Tissues were then infiltrated with Spurr's resin in increasing concentrations [33, 50, 67, 100% (v/v)]. Spurr's was diluted in anhydrous ethanol and at each exchange, tissues were immersed in resin and rotated slowly on a mechanical wheel for 24 h. Sample tissues were transferred to fresh 100% (v/v) Spurr's resin and polymerized in flatbed embedding molds (70900; EMS) at 60°C for 2 d.

To embed in LR Gold resin, tissues were washed once with 0.1 M sodium phosphate buffer pH 7.4 and dehydrated in a graded ethanol series [20, 50, 70, 90, 95% and 3 x 100% (v/v)] for 30 min each. Tissues were infiltrated in 50% (v/v) LR Gold diluted in anhydrous ethanol. All steps were performed on ice. Vials were swirled gently on a rotary wheel at 4°C overnight. The solution was discarded and replaced with pure LR Gold containing 0.1% (w/v) benzil (London Resin Co.) and swirled gently at 4°C overnight. Three additional exchanges in LR Gold with 0.1% (w/v) benzil were performed. Tissues were transferred to fresh LR Gold with 0.1% (w/v) benzil and distributed into gelatin capsules (70104; EMS) or PTFE flatbed molds (10506; Ted Pella, CA) covered with a strip of Aclar film® (10501; Ted Pella). Resin was polymerized at 4°C using a Blak-Ray B-100AP UV lamp (UVP, CA) for 7 d.

2.11 Light microscopy

Specimens embedded in Spurr's resin were cut with an Ultracut E microtome (Reichert-Jung, Leica, Germany) into 2 μm semi-thin sections using a glass knife. Sections were transferred to glass slides and heated at 70°C for 5 hr to adhere the sections. To stain sections with toluidine blue-O (TBO, 198161; Sigma), slides were pre-heated to 70°C and leaf sections were covered with 0.05% (w/v) TBO in 0.1 M sodium phosphate buffer pH 7.2 for 10 min (leaf) or 2 min (seed). The stain was gently rinsed off with Milli-Q water and slides were dried at 70°C. For triple staining with OsO_4, TBO and iodine potassium iodide (IKI), sections prepared from specimens that were post-fixed with 2% (w/v) OsO_4 and 5% (w/v) UA were incubated with 0.05% (w/v) TBO for 10 min at 70°C. TBO was gently rinsed off with Milli-Q water and slides were dried at 70°C. Slides were cooled to RT and stained with IKI (100 mM KI, 10 mM I_2) for 5 min. Stain was gently rinsed off with Milli-Q water and slides were dried at 70°C. Coverslips were mounted over tissue using Permount™ (SP-15; Fisher Scientific, NH), as described by the manufacturer. Digital images were captured with an Axioskop 2 Plus microscope (Zeiss, Germany) using a DXM1200 digital camera (Nikon) or with a Zeiss Axio Imager Z1 microscope (Zeiss) with an AxioCam ICc1 digital camera (Zeiss).

2.12 Transmission electron microscopy

Specimens embedded in Spurr's or LR Gold resin were cut into 60 nm sections using an Ultracut E microtome fitted with a diamond knife (Diatome, Switzerland). Sections were transferred to either copper grids (Cu-G400; EMS) for routine specimen analysis or to nickel grids (Ni-G400; EMS) for immunogold labeling experiments. Before imaging, all specimens were stained for 10 min with 5% (w/v) UA and washed gently with Milli-Q water. This was followed by 1 min with Reynold's lead citrate solution under a low CO_2 atmosphere (Bozzola and Russell, 1992). Tissues were washed gently with Milli-Q water and air-dried. Specimens were examined with a CM-10 transmission electron microscope (Philips) operating at 80 kV and equipped with a digital camera (Advanced Microscopy Techniques, MA).

2.13 Electron immunogold labeling

Specimens embedded in LR Gold resin and sectioned onto nickel grids were blocked with goat normal serum (25596; Aurion, Netherlands) for 30 min at RT. Grids were then incubated for 2 hr at RT with primary antibodies diluted 1:10 with dilution buffer [0.2% (v/v) BSA-c^{TM} (Aurion), 0.05% (v/v) Tween-20, 1% (w/v) BSA in PBS pH 7.4]. Primary antibodies were rabbit-anti-12S globulin (Shimada et al., 2003a), rabbit-anti-napin (Scarafoni et al., 2001), mouse-anti-*Arabidopsis* oleosin D9 (SemBioSys Genetics), rabbit-anti-TIP3;1 (Jauh et al., 1998) and rabbit-anti-TIP1;1 (Jauh et al., 1998). For controls omitting primary antibodies, specimens were incubated with dilution buffer only. Grids were washed with dilution buffer and incubated for 1 hr at RT with secondary antibodies diluted 1:10 with dilution buffer. All secondary antibodies were IgG produced in goats and conjugated to either 10 nm or 15 nm gold particles (Aurion). Grids were washed with dilution buffer and Milli-Q water and were air-dried. Experiments were repeated at least twice for each antibody. Grids were stained with 5% (w/v) UA and Reynold's lead as described above and examined under a transmission electron microscope.

2.14 Fluorescence and confocal microscopy

Seedlings co-expressing *LEC2/TIP3-YFP/TIP1-RFP* were used to observe the subcellular localization of TIP isoforms during LEC2 induction. Seeds were germinated for 7 d and seedlings were induced by DEX as previously described. Each dish contained 10 plants and there were 13 dishes. Germinating seedlings and plants were sampled over time on DEX induction medium. For controls, transgenic *Arabidopsis* seed expressing *35S:TIP3;1-YFP* (Hunter et al.. 2007) were germinated and maintained on half-strength MS medium for 7 d. TIP3;1:TIP3;1-YFP embryos (Hunter et al., 2007) were imbibed with water for several hours and dissected from the seed coat before imaging.

For fluorescence microscopy, whole plants were examined using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems) equipped with a digital camera (Q-imaging, BC, Canada). Specimens were illuminated by a 405 nm UV mercury lamp. To observe YFP fluorescence, a standard GFP filter (ex. BP480/40 nm, em. LP510 nm) was used.

For confocal microscopy, small tissue samples were directly examined or were stained before examination. To fluorescently stain vacuole lumens, tissues were immersed in 20 μM neutral red (NR, N129; Fisher Scientific) for 3 min and washed twice with distilled water. To fluorescently label the plasma membrane, tissues were incubated in 8 nM FM4-64 (Invitrogen, CA) in half-strength MS liquid for 2 min and rinsed with water. All specimens were mounted in Milli-Q water and sandwiched between two coverslips separated by a thin film of petroleum jelly. To determine the subcellular localization of fluorescent stains, PSV and chlorophyll autofluorescence as well as TIP3-YFP and TIP1-RFP proteins, a Leica SP5 confocal laser scanning microscope was used (Leica Microsystems). Imaging was performed using 10X (NA 0.3) air, 40X (NA 1.25) oil, or 63X (NA 1.4) water immersion lenses. For imaging chlorophyll autofluorescence, the specimens were excited with a 633 nm laser and fluorescence was detected at 660-700 nm. PSV autofluorescence was excited at 405 nm and emissions collected at 450-510 nm. For visualization of NR, the stain was excited at 543 nm and emission was collected at 560-605 nm. FM4-64 was excited at 514 nm and detected at 616-645 nm. A

561 nm laser was used to excite RFP and emission was detected at 553-638 nm. For YFP, excitation was with a 514 nm laser and emission collected at 525-583 nm. Simultaneous detection of combinations of fluorophores was performed by combining the settings indicated above in the sequential scanning facility of the microscope as instructed by the manufacturer.

CHAPTER 3

RESULTS

3.1 Plants acquire seed characteristics in response to LEC2 over-expression

3.1.1 LEC2 alters the leaf macrophenotype and promotes the formation of callus and somatic embryo-like structures

A transgenic *Arabidopsis* line was used to study the response of plants to LEC2 over-expression (Stone et al., 2008). In this line, the *LEC2* gene was fused to a gene encoding a glucocorticoid receptor and this fusion was placed under control of a 35S promoter. The glucocorticoid receptor acts as a selective inducer of gene expression, thereby allowing the flexibility to activate LEC2 function when desired by treating with a synthetic steroid, DEX (Gatz and Lenk, 1998; Sablowski and Meyerowitz, 1998). Seeds were stratified and allowed to germinate for 7 d before transfer to medium supplemented with 30 μM DEX or DMSO as a control. By 14 d of incubation on DEX or DMSO, seedlings were photographed and leaves collected for subsequent experiments. While untransformed Ws-0 plants were not affected by either DMSO or DEX (Fig. 3.1A, B, C) and LEC2 plants were not affected by DMSO (Fig. 3.1E, F), plants over-expressing LEC2 were easily distinguished from controls by 14 d on DEX (Fig. 3.1G, H). These plants were much smaller than controls and had smaller leaves and shorter petioles. Leaves had a reduced number of trichomes, were fleshy, curled and had smooth leaf margins. Altogether, leaves from plants over-expressing LEC2 began to show phenotypic characteristics resembling cotyledons (Fig. 3.1D).

Plants over-expressing LEC2, but not control plants, formed callus and somatic embryo-like structures after 14 d on DEX. Callus formation was observed on the hypocotyl and along the root (Fig. 3.2B-D). Callus thickened the root and caused it to appear green-yellow in color. Secondary roots were seen sprouting from the callused areas (Fig. 3.2C). In contrast, LEC2 plants growing on DMSO had thin, white to translucent roots (Fig. 3.2A). Some of the callus was observed to produce green leafy structures along the root (Fig. 3.2D). The callus and leafy structures growing on roots might originate from either somatic embryogenesis or from shoot organogenesis, the difference being the morphogenic pathway activated. In somatic embryogenesis, a bipolar embryo containing shoot and root meristems is formed by actively dividing cells, but it

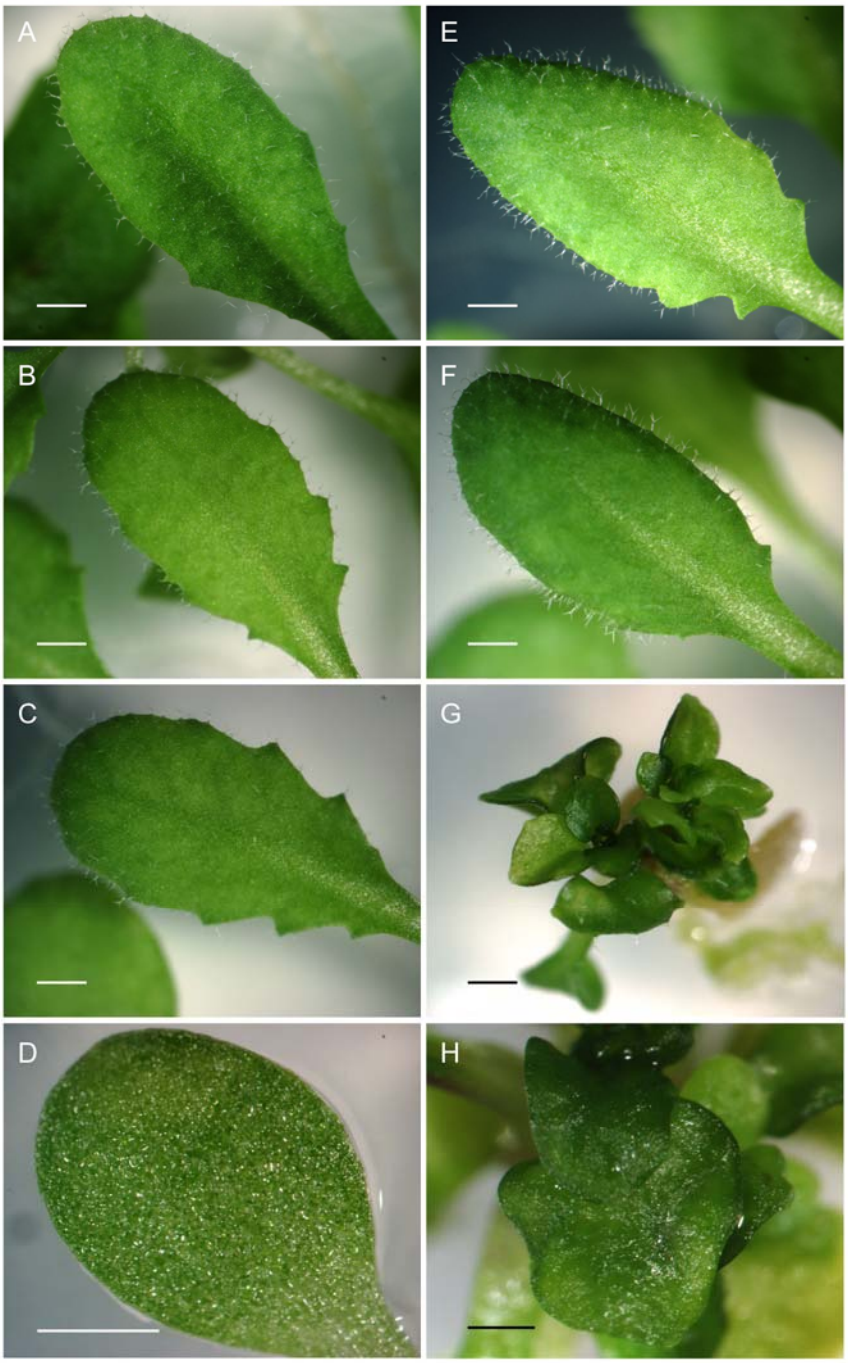

Figure 3.1 LEC2 over-expression alters the leaf macrophenotype to resemble cotyledon-like organs. Seeds were germinated in a growth chamber for 7 d on MS medium then seedlings were transferred to treatments for 14 d. Ws-0 plants incubated on MS medium (A), or MS supplemented with DMSO (B) or 30 µM DEX (C). Ws-0 cotyledon from 7 d-old seedling on MS medium (D). LEC2 plants incubated on MS medium (E), or MS supplemented with DMSO (F) or 30 µM DEX (G-H). Bars = 1 mm (A-F), 0.8 mm (G, H).

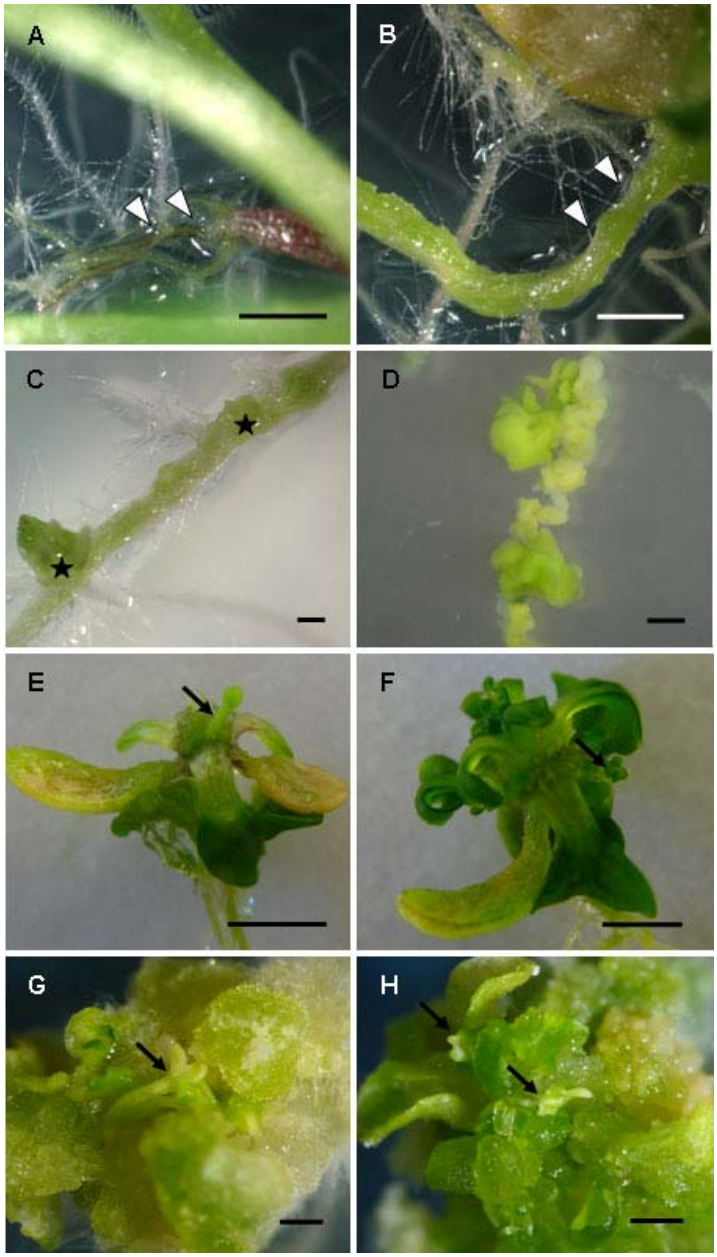

Figure 3.2 LEC2 over-expression promotes callus and somatic embryo-like formation on plants. LEC2 seeds were germinated in a growth chamber for 7 d on MS medium then seedlings were transferred to treatments for 14 d. Roots and the hypocotyl (purple structure) of a LEC2 plant on MS medium supplemented with DMSO (A). Roots growing on DEX (B-D). LEC2 plants exposed to DEX produced somatic embryo-like structures sprouting from the base of petioles (E, F) and many plants formed a mass of callus in which somatic embryos appeared to form (G, H). Arrowheads point to root areas adjacent to the hypocotyl for comparison. Stars indicate organ-like structures forming on roots. Arrows point to germinating somatic embryos. Bars = 1 mm (A, B, D, G, H), 100 µm (C), 2 mm (E, F).

does not form direct vascular connections with the mother plant. In contrast, organogenesis involves the formation of a meristem by a small number of dividing cells. The unipolar organ eventually gives rise to a shoot or a root (Kurczyńska et al., 2007; Ramage and Williams, 2002). Somatic embryo-like structures were observed sprouting from the base of petioles (Fig. 3.2E, F) and from callus (Fig. 3.2G, H). The somatic embryos are pale-yellow to green in color and are usually composed of two rudimentary leaves or cotyledons and an elongated hypocotyl or root. Many plants over-expressing LEC2 produced a mass of callus which seemed to envelop the plants (Fig. 3.2G, H). Leaves and roots of the mother plant were seen protruding from the callus and somatic embryo-like structures and root hairs were also observed.

3.1.2 LEC2 alters the leaf microphenotype

To establish whether changes in leaf phenotype are associated with changes to the leaf cell structure, leaf sections were stained with toluidine blue-O (TBO). TBO is a non-specific polychromatic stain that binds to most cellular components except starch and lipids (Regan and Moffatt, 1990; O'Brien et al., 1964). The stain is commonly used to identify PSVs as the accumulated protein in the matrix is stained blue (Stone et al., 2008; Gazzarrini et al., 2004; Shimada et al., 2003b). The incubation of Ws-0 plants with DEX or LEC2 plants with DMSO did not appear to alter the leaf structure from that of Ws-0 plants (Fig. 3.3A, B). In contrast, TBO staining identified a dramatic alteration in leaf cell structure in LEC2 plants induced by 30 μM DEX (Fig. 3.3C). Cells were arranged much closer together, their shape was altered and the intracellular spaces were densely stained. In wild type leaf sections, the palisade and spongy mesophyll layers are usually distinguished by their shape; palisade cells are tightly packed and elongated while the spongy cells are round and loosely packed (Fig. 3.3B, Fig. 3.4D). The mesophyll layers were not distinguishable in LEC2 induced leaf tissues (Fig. 3.3C). Perhaps the most prominent difference between Ws-0 and LEC2 control plants and plants over-expressing LEC2 was the appearance of the cell contents. In LEC2 cells, vacuoles were drastically reduced in size and the vacuole lumen was lightly stained by TBO (Fig. 3.3C). Overall,

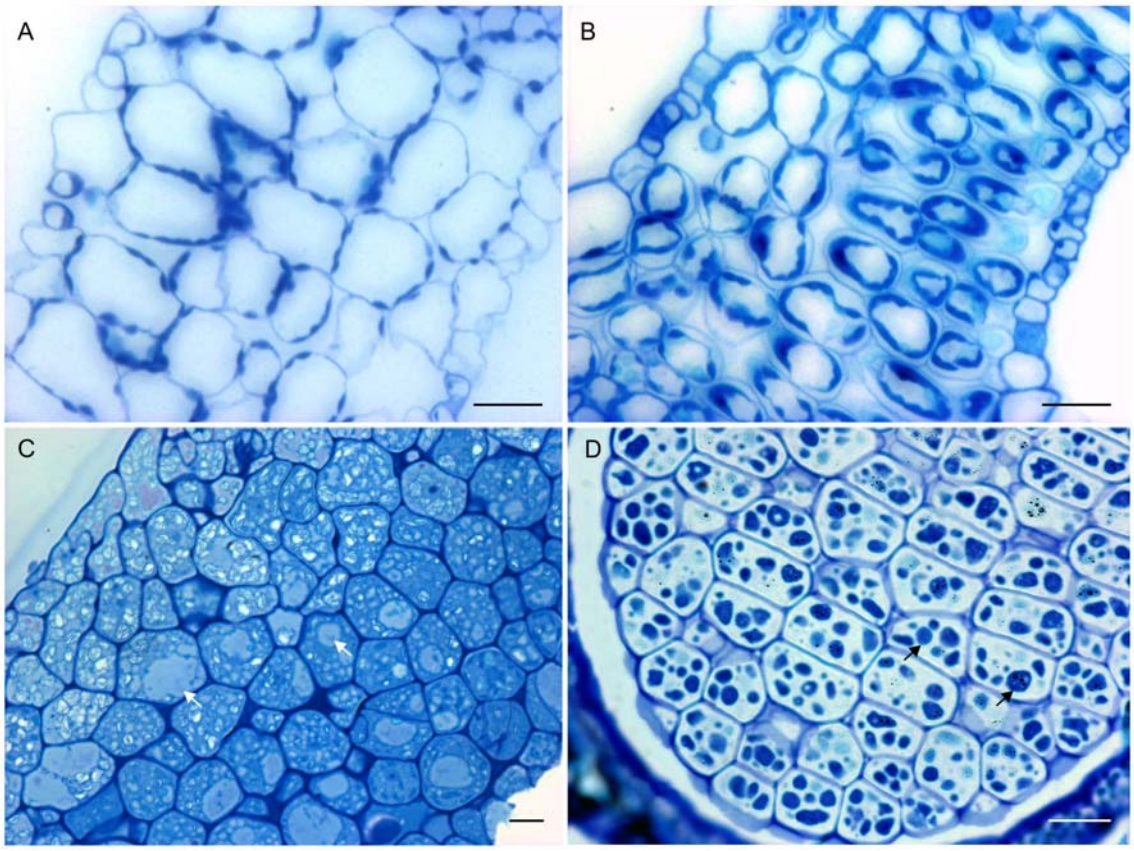

Figure 3.3 LEC2 induces alterations in leaf cell structure causing leaf tissues to resemble seed tissues. Sections of leaf and seed tissues were stained with TBO to reveal cell structure. Leaves were collected from plants incubated on treatments for 14 d. Representative images are shown of Ws-0 leaf sections incubated with DEX (A), LEC2 leaf sections incubated with DMSO (B), LEC2 sections incubated with DEX (C) and a section of a Ws-0 seed (D). TBO does not stain lipids and starch so seed cells show large unstained areas. Arrows point to vacuoles. Bars = 20 µm (A-C), 15 µm (D).

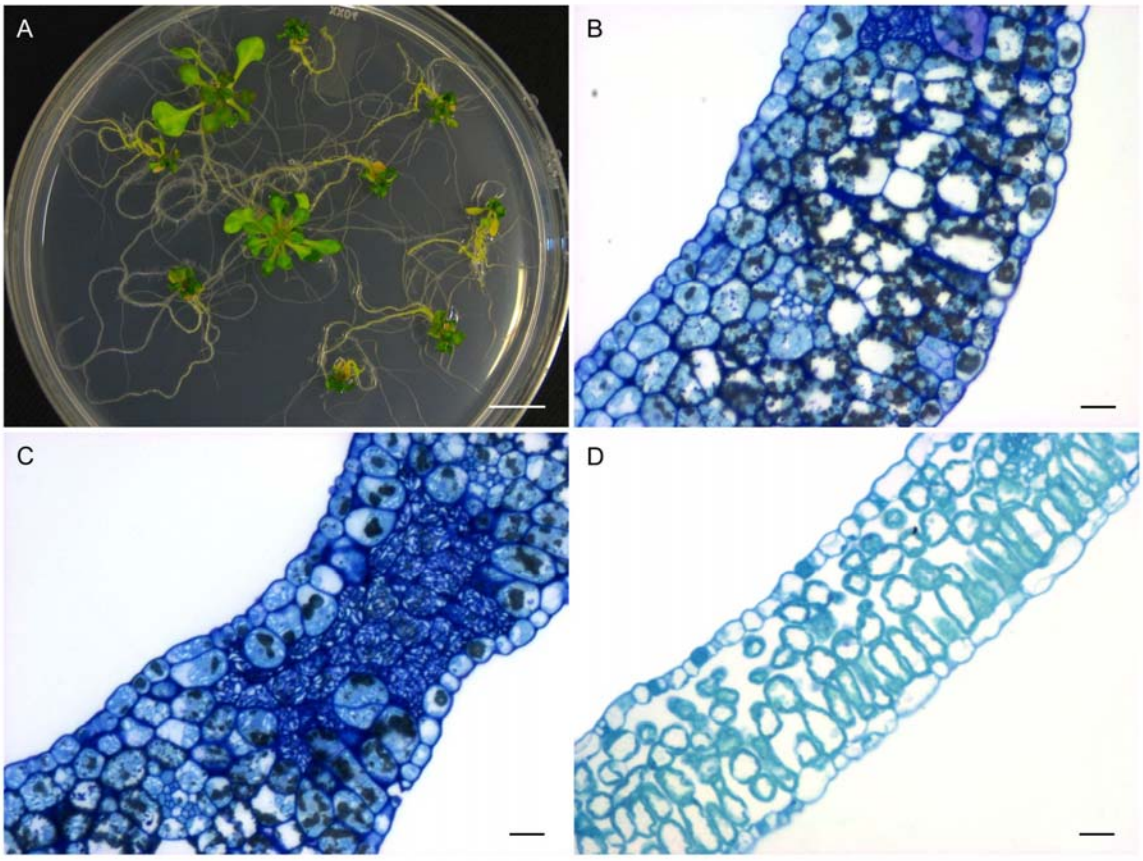

Figure 3.4 Plants over-expressing LEC2 do not reveal a uniform phenotypic response. Seeds were germinated for 7 d on MS medium supplemented with 6 µg/ml glufosinate and seedlings were transferred to MS with 30 µM DEX and 6 µg/ml glufosinate for 14 d. Representative images are shown for plants treated with DEX (A-C) or DMSO (D). A culture dish demonstrating the size difference between LEC2 seedlings treated with DEX (A). Leaf sections stained with TBO (blue) and OsO_4 (black) from plants incubated with DEX (B, C) or DMSO (D). Bar = 1 cm (A), 20 µm (B-D).

the appearance of leaf tissues expressing LEC2 was reminiscent of seed tissues due to the organization and shape of cells. Seed sections stained with TBO revealed compact cells containing heavily stained PSVs. Unstained areas surrounding PSVs are lipid reserves as TBO does not stain lipids (Fig. 3.3D). Thus LEC2 over-expression caused an alteration of the mature leaf phenotype to one that more closely resembles seed tissues.

The alterations in LEC2 macro- and microphenotype did not appear to be a homogenous response. For this work, LEC2 plants exposed to DEX that displayed the characteristic macro- and microphenotypes outlined above are referred to as 'induced'. The response of LEC2 plants to DEX was observed to vary with some plants being strongly induced and others that were weakly induced or not at all. After 14 d exposure to DEX, not all seedlings showed an altered macrophenotype despite being maintained on glufosinate to select for the *35S:LEC2-GR* transgene (Fig. 3.4A). Typically, 10-40% of plants on a plate did not show an induced phenotype. However, when analysed, all plants tested positive for the transgene by PCR. To observe the appearance of cells in leaf sections, tissues were double-stained with TBO and osmium tetroxide (OsO_4). OsO_4 is a lipophilic stain (Bozzola and Russell, 1992) that colors lipids black-grey in combination with TBO. In strongly induced plants, the appearance of mesophyll cells was observed to vary along the section of leaf tissue studied (Fig. 3.4B, C). Leaves had areas of mesophyll cells with more intensely stained cells (Fig. 3.4C) while other areas had cells with large, unstained vacuoles and many oil deposits (Fig. 3.4B). Moreover, leaf thickness appeared to vary; the leaf was constricted in areas harboring cells that were stained more intensely with TBO (Fig. 3.4C). Conversely, leaf sections from LEC2 plants incubated with DMSO were all uniform in thickness and composition, clearly displaying palisade and spongy mesophyll (Fig. 3.4D).

3.1.3 Leaf cell composition is altered to resemble cotyledon cells when LEC2 is over-expressed

To identify the contents of leaf cells over-expressing LEC2, histochemical stains with different binding affinities were used. Three stains were chosen with each having specificity for different cell components. Under the experimental conditions, TBO stained proteins blue-purple, OsO_4 stained lipids a green-brown colour and iodide potassium iodine (IKI) bound to starch to produce a pink-red colour. Leaf starch is primarily composed of amylopectin, a highly branched glucose polymer, combined with a small amount of amylose, a linear glucose polymer (Smith et al., 2005). Upon IKI staining, a purple-black color is generated if starch is largely composed of amylose, while a reddish color is produced if the starch is primarily composed of amylopectin (Hinchman, 1973; Bailey and Whelan, 1961). Thus, the red starch staining in Ws-0 and LEC2 leaves on all treatments in this work suggests that starch granules in *Arabidopsis* leaves are composed primarily of amylopectin (Fig. 3.5).

Leaf tissue from Ws-0 and LEC2 plants without treatment or treated with DMSO, or Ws-0 plants treated with DEX looked alike and therefore a representative image is shown in Fig. 3.5A. Cells observed in all controls had large unstained LVs while the cytoplasm and organelles were stained blue and small red-stained starch granules were commonly observed inside chloroplasts. In cotyledons from 7 d-old seedlings, LVs appeared smaller in size, a higher volume of cytoplasm was present and starch granules were much larger than in leaf cells (Fig. 3.5B). In addition, green-stained, lipid-filled organelles were observed in the cytoplasm of cotyledons. The lipid-filled organelles are most likely oil bodies that accumulate during seed maturation and are metabolized by the growing seedling (Mansfield and Briarty, 1996). Triple staining of LEC2 induced leaf sections showed that their appearance was more similar to cotyledon tissue than leaf tissue (Fig. 3.5C). Compared to control leaf sections, LEC2 induced leaf tissues had an increased volume of cytoplasm that contained abundant lipid-filled vesicles, vacuoles were drastically reduced in size and starch granules were enlarged. Furthermore, within the small vacuoles, TBO-stained deposits were present, suggesting that the vacuoles contain protein deposits or aggregates.

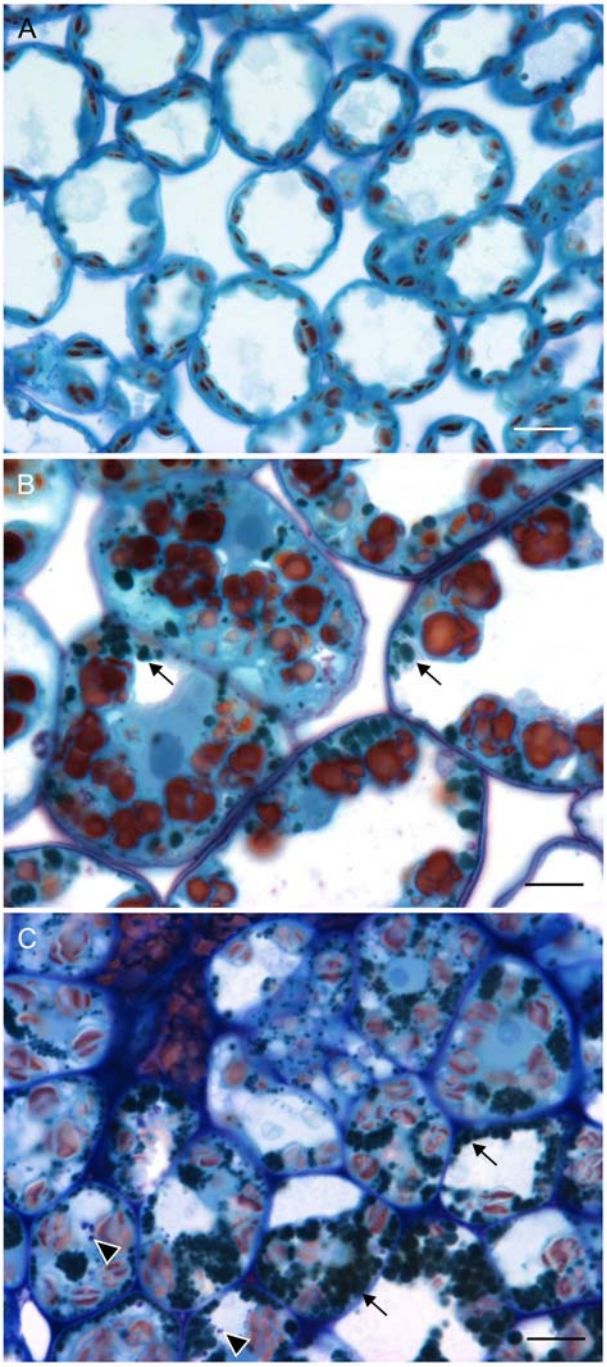

Figure 3.5 The cell composition of leaves from LEC2 induced plants bears a greater resemblance to cotyledons than to leaves from control plants. Leaf sections were stained with TBO, OsO_4 and IKI to distinguish between cell components. Seeds were germinated for 7 d and seedlings were transferred to treatments. At 14 d, leaves were excised from LEC2 plants exposed to DMSO (A) and DEX (C). Cotyledons were taken from 7 d-old LEC2 seedlings before transfer to treatments (B). Arrowheads point to TBO-stained protein deposits. Arrows show lipid-filled vesicles. Bar = 10 µm.

Transmission electron microscopy (TEM) was used to more closely examine the ultrastructure of LEC2 leaves induced by DEX. Leaf sections were post-fixed with OsO_4 to enhance the visualization of cell structures. Under the electron microscope, drastic changes to leaf cell ultrastructure were revealed which correspond to what was observed in triple-stained leaf sections. In control leaf cells, the chloroplasts, cytoplasm and other organelles were pushed to the periphery of the cell by the large LV and it was common to see small starch granules in the chloroplasts (Fig. 3.6A, C). In contrast, LEC2 induced leaf cells were considerably altered. TEM sections, as well as triple-stained sections, showed that the volume of cytoplasm was increased and the LV was reduced in size and appeared to be fragmented into smaller sized vacuoles (Fig. 3.6B, D, E). Novel osmium-stained vesicles were present in the cytoplasm, which were equivalent to the lipid-filled vesicles observed in triple-stained sections (Fig. 3.6B, D, E). Many holes were created in the resin holding the tissue together (Fig. 3.6B). The holes were all associated with the enlarged starch granules present in chloroplasts of LEC2 induced leaves but did not occur in leaf samples from control treatments. The holes are believed to be an artifact created by processing the samples. While sectioning leaf tissues with the microtome, the large starch granules probably shattered and fell out of the resin. Finally, the small-sized vacuoles contained electron dense aggregates (Fig. 3.6B) which correlated with the TBO-stained deposits observed in triple-stained tissues (Fig. 3.6D). Thus the electron dense aggregates may represent protein deposits.

3.2 LEC2 over-expression promotes the appearance of seed organelles, the accumulation of seed proteins and causes leaf organelles to de-differentiate

3.2.1 Oil bodies accumulate in the cytoplasm of LEC2 induced leaf cells

To characterize the lipid-filled vesicles observed upon induction with DEX, electron immunogold labeling was carried out using an antibody against the seed oil body protein,

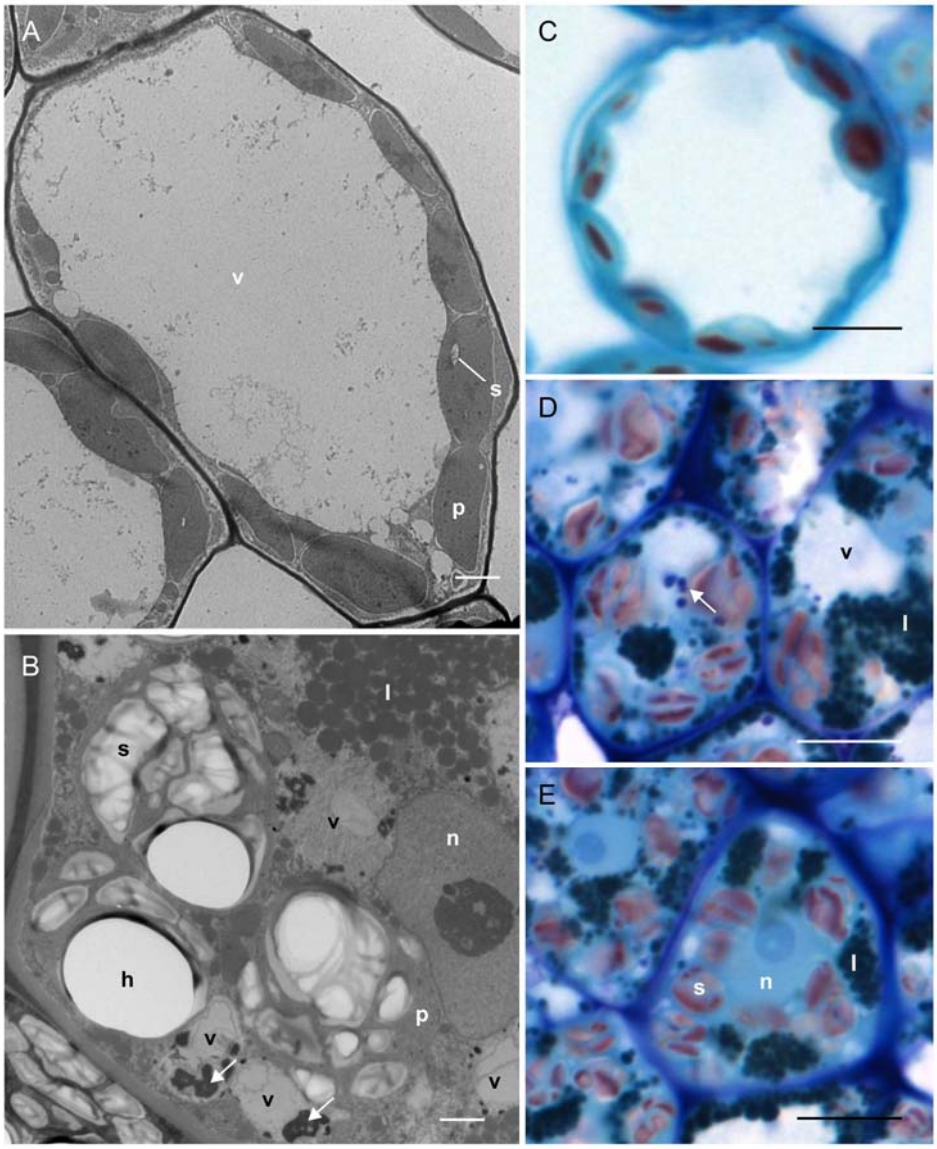

Figure 3.6 LEC2 leaf cells undergo a drastic alteration in structure and accumulate novel vesicles. Leaves were collected from LEC2 plants incubated with DMSO (A, C) or with DEX (B, D, E). Leaf sections were stained with OsO_4 and imaged by transmission electron microscopy (A, B) or were triple-stained with OsO_4, IKI and TBO and imaged by light microscopy (C-E). Arrows point to protein aggregates. h, hole; l, lipid-filled vesicles; n, nucleus; p, chloroplast; s, starch granule; v, vacuole. Bar = 2 µm (A, B), 10 µm (C, D, E). Images (C-E) are magnified from Fig. 3.5.

oleosin. Oleosin is the most abundant transmembrane protein that controls the size and shape of oil bodies in seeds (Siloto et al., 2006). No specific labeling was observed in leaf tissue derived from wild type plants (not shown) or LEC2 plants incubated with DMSO (Fig. 3.7A). In leaf sections from LEC2 plants induced by DEX, the antibody bound to the membrane of the novel vesicles (Fig. 3.7B) with the same sparse labeling pattern observed in seed sections (Fig. 3.7C), indicating that the vesicles are indeed oil bodies. The lumen of immunolabeled oil bodies appeared translucent under the TEM (Fig. 3.7B, C) in comparison to the electron dense oil bodies in OsO_4 stained tissue sections (Fig. 3.7D). OsO_4 is used to enhance contrast in TEM images by binding to lipids but is omitted during immunolabeling to avoid denaturing or masking the antigen (Bozzola and Russell, 1992). In summary, LEC2 induced leaves accumulate oil bodies which are organelles commonly observed in seed and cotyledon tissues but not in vegetative organs such as leaves (Kim et al., 2002).

3.2.2 Chloroplasts accumulate large starch granules and appear to differentiate in response to LEC2 over-expression

Microscopic observations of LEC2 induced leaves revealed a noticeable increase in the size and number of starch granules (Fig. 3.5C and Fig. 3.6B, D, E) when compared to control leaves (Fig. 3.5B and Fig. 3.6A, C). Therefore, a more detailed observation of chloroplasts in LEC2 induced leaves was undertaken. Under the TEM, wild type leaves (not shown) and leaves of LEC2 plants incubated with DMSO were observed to have oval-shaped starch granules in the chloroplast stroma (Fig. 3.8A). In LEC2 induced leaves, the starch granules appeared to increase in number and in size. The granules took on different shapes; they appeared as fat discs, were more rounded, or assumed other shapes. The granules were localized to the chloroplast stroma and appeared to push the thylakoid stacks toward the periphery of the chloroplast (Fig. 3.8B). Localized areas of the LEC2 induced leaf tissues were packed with small cells containing dense deposits of

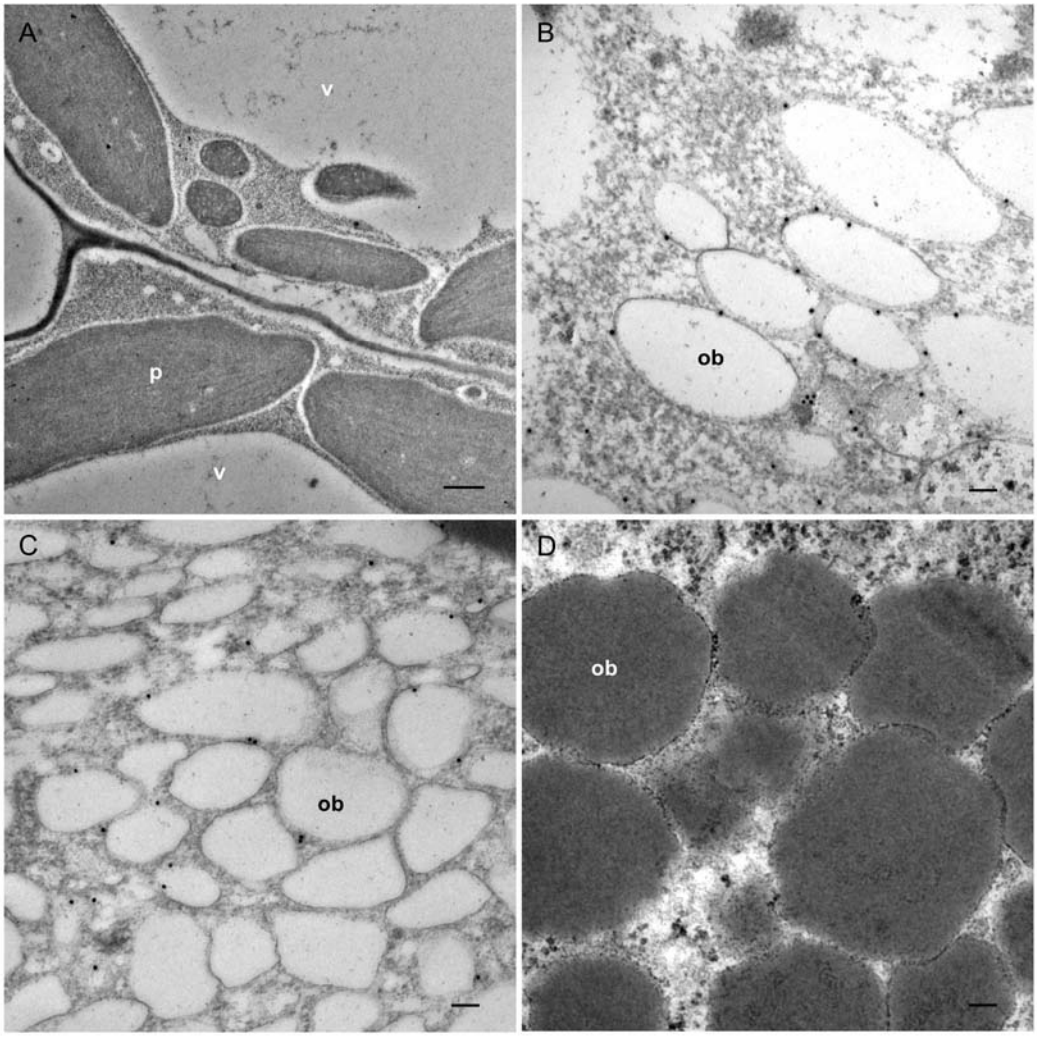

Figure 3.7 LEC2 promotes the accumulation of oil bodies in the cytoplasm of leaf cells. Leaf sections from plants incubated with DMSO (A) or DEX (B, D) and a Ws-0 seed section (C). Electron immunogold labeling of oil bodies using 15 nm gold particles (A-C). An OsO_4-stained section of a representative leaf from LEC2 plants incubated with DEX (D). ob, oil body; p, plastid; v, vacuole. Bar = 500 nm (A), 100 nm (B-D).

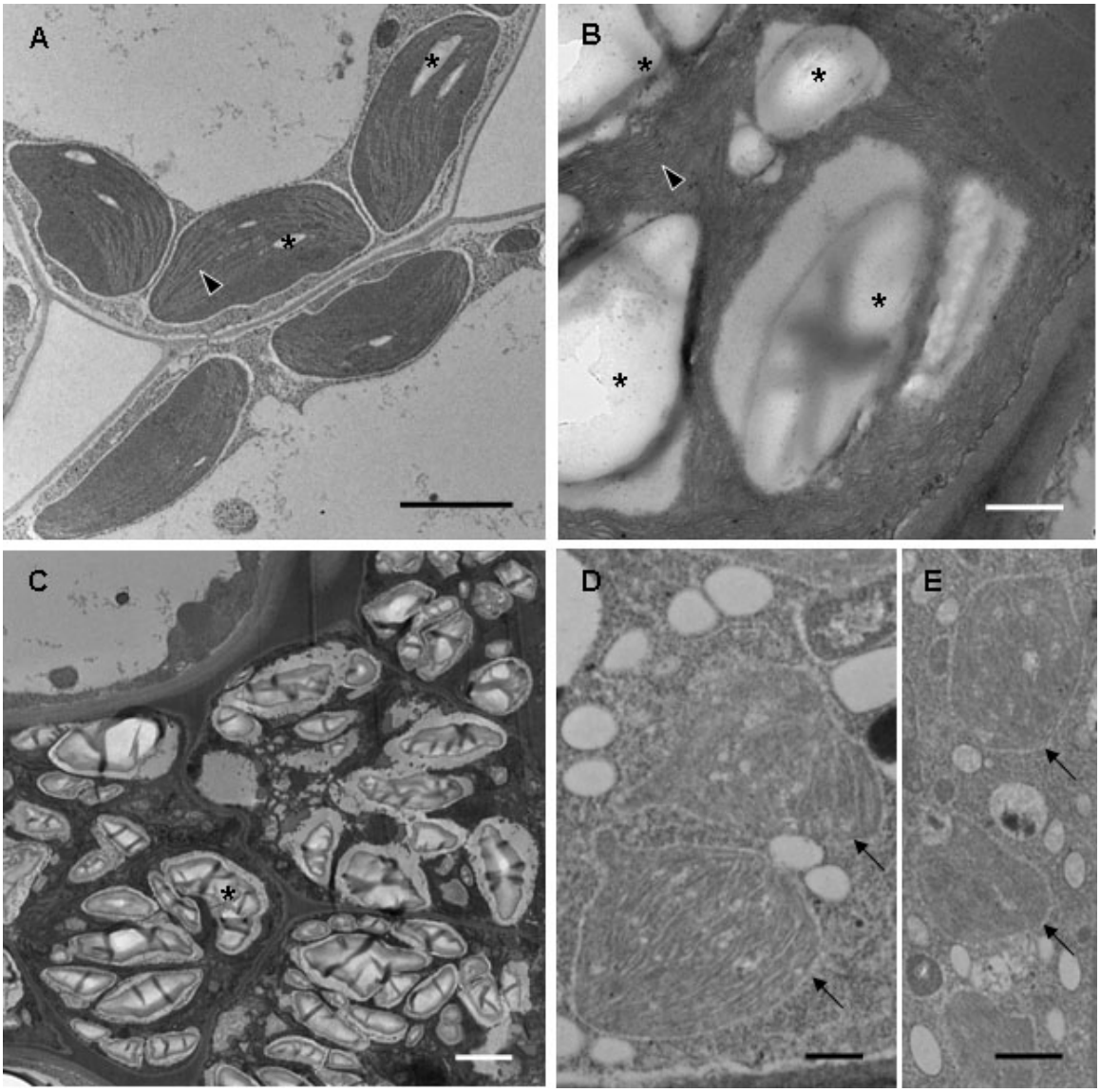

Figure 3.8 LEC2 over-expression promotes increased starch accumulation and plastid de-differentiation. Leaf sections were collected from plants incubated with DMSO (A) or 30 μM DEX (B-E). Thylakoid stacks and small starch granules were observed in large chloroplasts in leaf cells of LEC2 plants on DMSO (A). In leaves from LEC2 plants on DEX (B-E), starch granules increased in size and number and pushed the thylakoid stacks to the periphery of the stroma (B), dense deposits of starch granules were observed in small cells in discrete areas of leaf sections (C) and small, round plastids were observed that appeared to have less-developed thylakoid structures (D, E). Small electron transparent vesicles in (D, E) are oil bodies. Asterisks show starch granules. Arrowheads point to thylakoids. Arrows point to de-differentiating plastids. Bar = 2 μm (A, C), 500 nm (B, D), 1 μm (E).

starch granules (Fig. 3.8C). The chloroplasts were so tightly filled with starch granules that it was difficult to discern the chloroplasts. The dense starch deposits were also observed as red IKI-stained areas in triple-stained tissue (Fig. 3.5C) and as heavily stained blue regions in leaf sections stained with TBO and OsO_4 without IKI (Fig. 3.4C). Moreover, these localized starch deposits appeared to affect the thickness of the leaf (Fig. 3.4C). In areas of the leaf containing dense starch deposits, the cross section was typically more narrowed than the surrounding areas without starch deposits. It is well known that the synthesis of starch reserves in leaves is dependant on light (Zeeman et al., 2002). Thus, to ensure that this phenomenon was not due to variations in the length of time that plants were exposed to light, all samples were harvested at approximately the same time during the photoperiod in each experiment.

Plastids were also observed at different stages of differentiation in LEC2 induced leaves. Chloroplasts in control leaves were ~ 4 μm in diameter, lens-shaped, had well developed thylakoid stacks and the stroma contained small starch granules (Fig. 3.8A). Upon induction of LEC2 with DEX, some plastids were observed to be much smaller in size (~ 2 μm in diameter) and had a more rounded shape than chloroplasts. The presence of thylakoid stacks was not evident but internal membranes were observed in the stroma (Fig. 3.8D, E). The morphology of the de-differentiated chloroplasts in LEC2-induced leaves resembles that of pseudochloroplasts. Pseudochloroplasts belong to a differentiation stage between proplastids, the progenitors of all plastids, and chloroplasts (Rohde et al., 2000).

Starch accumulation and plastid de-differentiation were not ubiquitous events in LEC2 induced leaves. Often, chloroplasts with large starch granules were seen alongside chloroplasts with small starch deposits. Areas of the leaf containing dense starch granule deposits were commonly observed among samples but the deposits occurred in discrete areas and not along the entire leaf section (Fig. 3.4C). In addition, chloroplasts undergoing different stages of de-differentiation were observed in the same leaf sample (not shown). Generally, chloroplasts with large starch granules were observed more frequently than chloroplasts undergoing de-differentiation.

3.2.3 Seed storage proteins accumulate in LEC2 induced leaves

Given that putative protein deposits were detected in small-sized vacuoles of LEC2 induced leaves using light and electron microscopy (Fig. 3.5C, Fig. 3.6B, D) and that SSP RNA is present in *Arabidopsis* leaves over-expressing LEC2 (Braybrook et al., 2006; Santos-Mendoza et al., 2005), the accumulation of SSPs was examined in these leaves. For this, total soluble protein was extracted from leaf samples collected after 14 d incubation on MS medium alone or MS supplemented with 30 μM DEX or DMSO. Proteins were size separated by SDS-PAGE and proteins were detected with antibodies against the two major *Arabidopsis* SSPs, the 12S globulins and 2S albumins (Fig. 3.9). Both antibodies detected SSPs from seed extracts and leaf samples from LEC2 plants incubated with DEX but were unable to detect proteins in leaf extracts from LEC2 control plants or Ws-0 wild type plants. Therefore, over-expression of LEC2 caused SSPs to accumulate in leaves.

The anti-12S globulin antibody detected one band at 30 kDa, representing the alpha subunit of the 12S globulin protein (Shimada et al., 2003a) in both seed and LEC2 induced leaf samples. Detection of the predicted band size and absence of other bands suggests that the globulins were processed correctly in leaves. On the other hand, the anti-2S albumin antibody detected four bands in the seed sample and two bands in the LEC2 induced leaf sample. The antibody is specific for the 10 kDa large subunit of the 2S albumin protein (Scarafoni et al., 2001) but also detected bands at ~ 30, 17 and 12 kDa in the seed sample and one extra band at ~ 17 kDa in the LEC2 induced leaf sample. This abnormal accumulation pattern is thought to represent non-specific binding of the antibody as all of the bands were present in the seed control and seeds should not accumulate SSP precursors or abnormally processed SSPs (Gruis et al., 2004; Shimada et al., 2003b). Taken together, Western blot results show that both 12S and 2S seed proteins accumulated in their mature forms in LEC2 induced leaves.

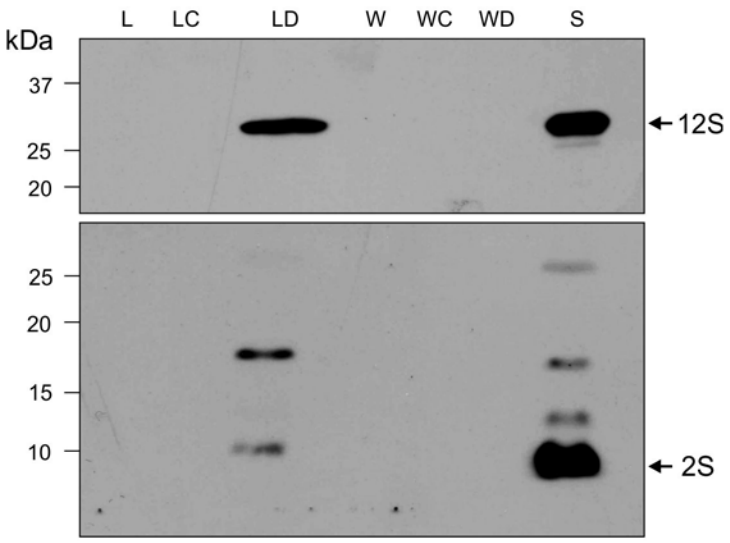

Figure 3.9 Seed storage proteins accumulate in LEC2 induced leaves. LEC2 or Ws-0 seedlings were incubated on MS medium (L and W, respectively) or in the presence of DMSO (LC, WC) or 30 µM DEX (LD, WD) for 14 d. Ws-0 seed (S) was used as a positive control. Antibodies against 12S globulins (top; 1:50,000 dilution) and 2S albumins (bottom; 1:500 dilution) were used to detect seed storage proteins in samples. For the 12 S blot, 50 µg leaf and 2.5 µg seed proteins were loaded into wells. For the 2S blot, 25 µg leaf and 10 µg seed proteins were added to wells. Arrows point to seed protein bands. Expected sizes of 12S and 2S protein subunits detected by antibodies are 30 kDa and 10 kDa, respectively.

3.2.4 Seed storage proteins are localized to vacuoles

SSPs usually accumulate in PSVs in *Arabidopsis* seeds. To determine the subcellular location of SSPs in LEC2 induced leaves, electron immunogold microscopy was used. To demonstrate that 2S and 12S antibodies could be used to immunolocalize SSPs, seed sections were used as a positive control. For both antibodies, gold particles were heavily localized to the PSV matrix (Fig. 3.10A, B). In control leaf tissues, the antibodies did not detect seed proteins (results not shown). In LEC2 induced leaf cells, both proteins were found to accumulate in electron dense aggregates within small sized vacuoles (Fig. 3.10C-G). The gold labeling was abundant within these structures. These protein deposits were often observed accumulating in clumps along the luminal side of the tonoplast and were seen suspended in clumps within the vacuole lumen (Fig. 3.10C).

The aggregates embedded many transparent vesicles and many of the vesicles contained either flocculent, electron dense material or smaller vesicles (Fig. 3.10D, F, G). The morphology of the transparent vesicles enclosing intraluminal vesicles resembled multivesicular bodies (MVBs), which are pre-vacuolar endosomes that deliver materials to vacuoles (Jiang et al., 2002). MVBs are typically ~ 250-300 nm in diameter (Reyes et al., 2011; Otegui et al., 2006) but the vesicles observed embedded in the aggregates were smaller and ranged in size from ~ 100-200 nm in diameter. The antibodies did not detect seed proteins inside the putative MVBs present within the aggregates (Fig. 3.10F, G).

3.2.5 Seed storage proteins accumulate in protein storage vacuoles in LEC2 induced leaves

In LEC2 plants induced by DEX, SSPs accumulate in electron dense aggregates within organelles that resemble small vacuoles. To confirm that these organelles were indeed vacuoles and to further characterize the type of vacuole, tonoplast intrinsic proteins (TIPs) were used as markers. TIPs are a subfamily of aquaporin proteins. These transmembrane proteins form channels to transport water and small molecules across the tonoplast. *Arabidopsis* has 10 TIP isoforms and their expression patterns are

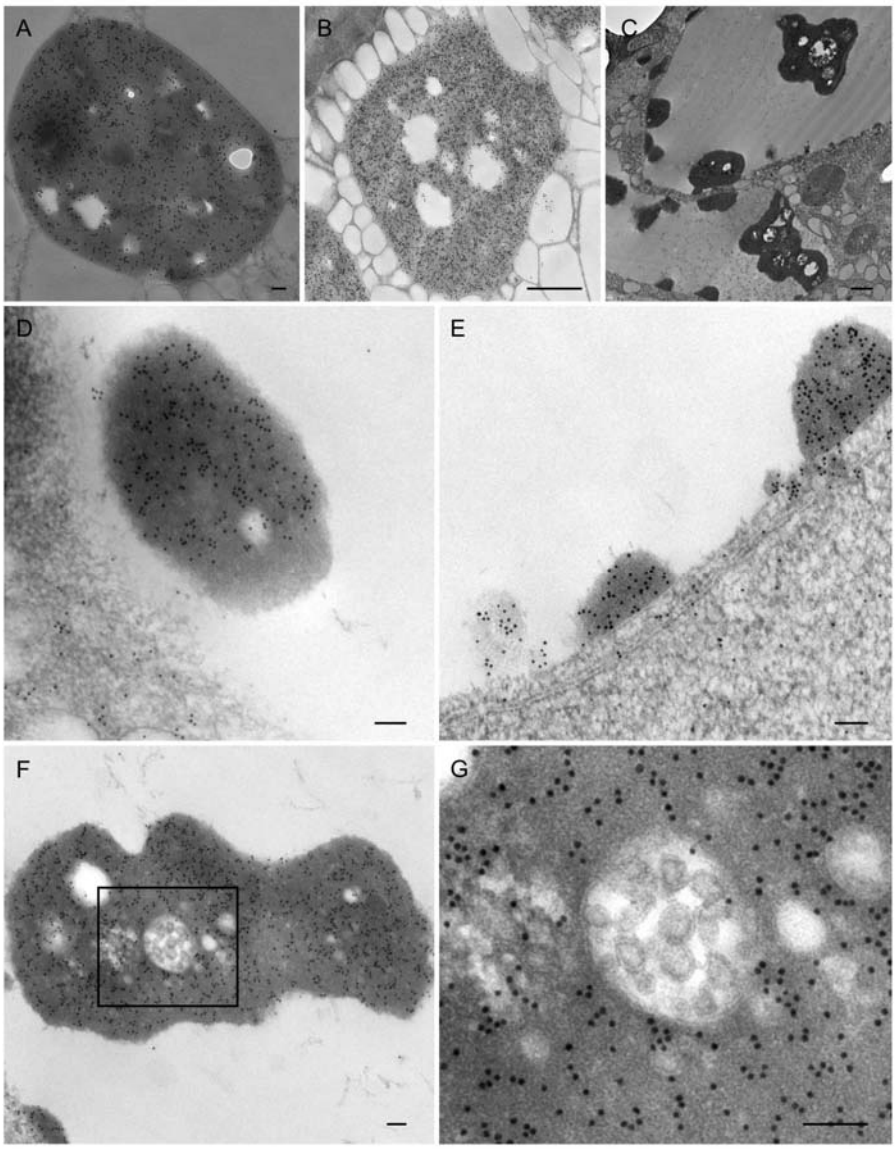

Figure 3.10 Seed storage proteins are localized to aggregates within small sized vacuoles in induced LEC2 leaves. LEC2 seedlings were exposed to DEX for 14 d before leaf collection. Immunogold labeling of Ws-0 seed tissues (A, B) and LEC2 induced leaf tissues (C-G) using antibodies against 12S globulin (A, D) and 2S albumin (B, C, E-G). 12S and 2S seed storage proteins are localized to the seed PSV matrix (A, B). Lower magnification of small vacuoles forming in LEC2 induced leaf cells (C). The vacuoles accumulate electron dense aggregates that surround transparent vesicles, many of which contain flocculent electron dense material or smaller vesicles (D, F, G). Immunogold labeling of leaf cells with antibodies against 12S globulin (D) and 2S albumin (E, F) shows that seed proteins are localized to electron dense aggregates within the vacuole lumen. A higher magnification image of the boxed area of (F) shows the exclusion of gold labeling from transparent vesicles within the aggregates (G). 10 nm gold particles were used. Bar = 100 nm (A, D-G), 500 nm (B, C).

developmentally and spatially controlled. For these reasons, TIPs have a long history of being used to distinguish between vacuole types, particularly TIP1;1 (gamma-TIP) and TIP3;1 (alpha-TIP). In *Arabidopsis*, TIP3;1 is highly expressed on the tonoplast of PSVs in embryos during seed development. During germination, TIP3;1 expression declines and is replaced by TIP1;1 expression. TIP1;1 localizes to the tonoplast of the LV and is the most widely expressed TIP in vegetative tissues (Gattolin et al., 2011; 2010; 2009; Beebo et al., 2009; Hunter et al., 2007). Therefore the specific TIP expression patterns were exploited to determine whether the small leaf vacuoles observed in plants over-expressing LEC2 were lytic or storage vacuoles.

TIP1;1 and TIP3;1 proteins were localized in induced LEC2 leaves using previously published antibodies (Jauh et al., 1998). The TIP3;1 antibody localized to the PSV tonoplast in seed tissue and did not label the LV tonoplast in leaf tissue, demonstrating its specificity for the TIP3;1 protein (Fig. 3.11A, B). Incubation of LEC2 induced leaf tissue with the TIP3;1 antibody showed specific labeling along the tonoplast of the small vacuoles where the electron dense aggregates were present (Fig. 3.11C). Labeling was not seen on small vacuoles without electron dense aggregates or around electron dense aggregates inside the vacuole lumen (results not shown). The TIP1;1 antibody localized to the LV tonoplast in leaf tissue of wild type or non-induced LEC2 plants (Fig. 3.11E). In seed tissue, the TIP1;1 antibody clearly bound to epitopes within the PSV matrix but labeling was not observed along the PSV tonoplast (Fig. 3.11D). For LEC2 induced leaf tissues, the TIP1;1 antibody did not label the tonoplast of the small vacuoles. Instead it localized to electron dense aggregates within the vacuole lumens (Fig. 3.11F, G). This labeling pattern was similar to what was observed in the seed control. For both TIP1;1 and TIP3;1 immunolabeling experiments, when the primary antibody was omitted, no specific labeling was observed (results not shown). In summary, these results suggest that in leaves over-expressing LEC2, small vacuoles containing electron dense aggregates represent a transitional stage from a LV to a PSV. Since TIP3;1 labels the tonoplast of these vacuoles and TIP1;1 does not, it suggests that the vacuoles have already transitioned from a lytic to a storage function (Jauh et al., 1998).

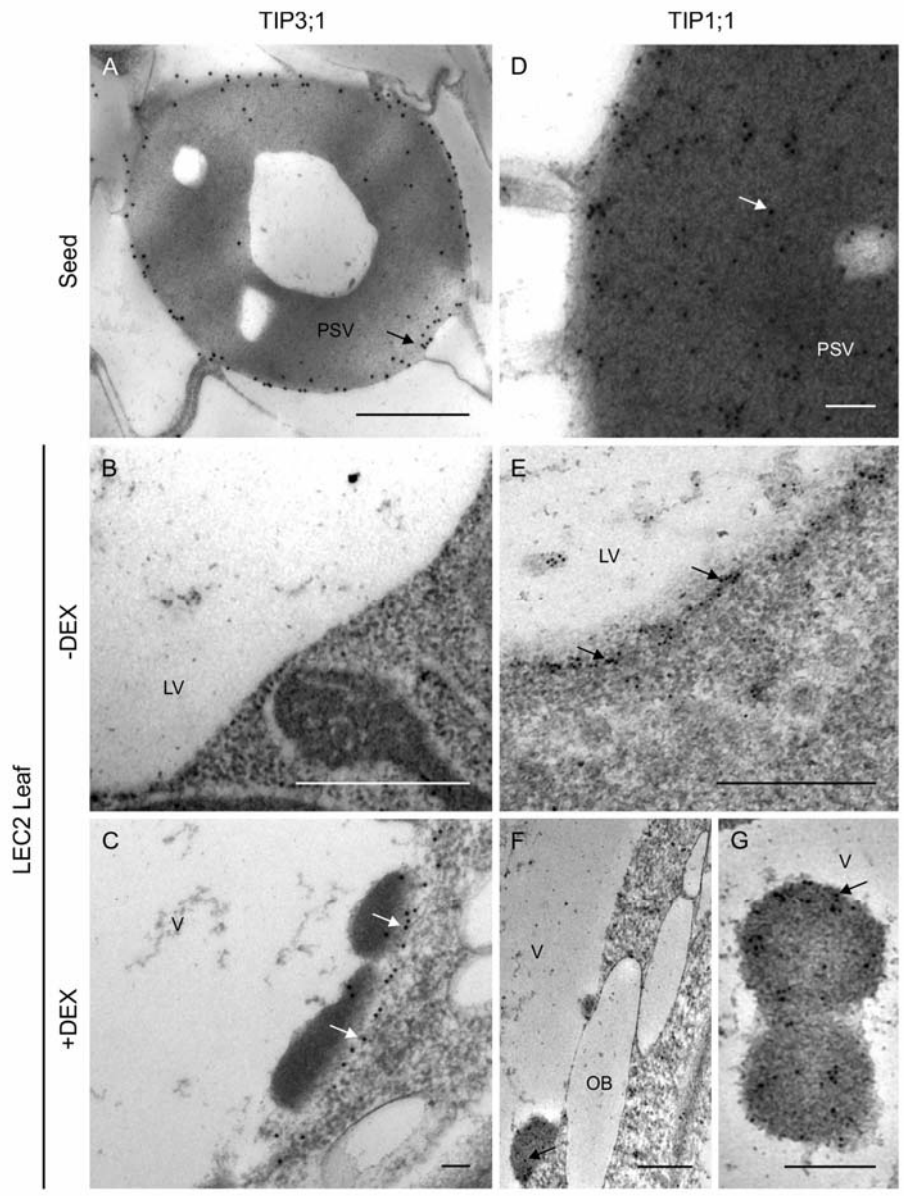

Figure 3.11 Small-sized vacuoles arising in leaf cells over-expressing LEC2 are protein storage vacuoles. The identity of the vacuoles was determined by electron immunogold labeling using antibodies against TIP1;1 and TIP3;1 isoforms. LEC2 seedlings were exposed to DEX or DMSO for 14 d before leaf collection. The antibody against TIP3;1 labeled PSV tonoplasts in seed (A) but not leaf LV tonoplasts from plants incubated on DMSO (B). TIP3;1 labeling was observed along the tonoplast of small leaf vacuoles from plants incubated on DEX (C). The antibody against TIP1;1 labeled the PSV matrix in seed tissues (D) and decorated the leaf LV tonoplast in plants exposed to DMSO (E). In plants incubated with DEX, TIP1;1 labeling was not seen along the tonoplast of small leaf vacuoles (F) but was seen on aggregates within the lumen (F, G). LV, lytic vacuole; OB, oil body; PSV, protein storage vacuole; V, vacuole. Arrows point to 15 nm (A, C) or 10 nm (D-G) gold particles. Bar = 500 nm (A, B, E), 250 nm (F, G), 100 nm (C, D).

3.3 Ectopic expression of LEC2 causes leaf cells to develop embryogenic characteristics that become more pronounced over time

3.3.1 Seed proteins are detected in leaves 11 d after induction of LEC2 with DEX while the vegetative protein marker disappears

To this point, results have illustrated a snapshot of the leaf cell biology of plants over-expressing LEC2 after 14 d on DEX. To provide a more dynamic view of the alterations to LEC2 induced leaf cells over time, samples were collected every 3-4 d over a 21 d period to examine the pattern of protein accumulation and the alteration of cell fate.

The accumulation pattern of vegetative and seed-specific protein markers was followed over time to demonstrate the progression of the transition from vegetative to embryonic characteristics caused by over-expression of LEC2 in leaves. Total protein was extracted from leaves and analyzed by immunoblots using antibodies against vegetative (TIP1;l) and seed (TIP3;1, 12S globulin and oleosin) protein markers. The experiment was repeated twice with independent samples and, overall, the pattern of protein accumulation and the number and size of bands was consistent among experiments. Representative blots are shown in Fig. 3.12. Leaves collected from LEC2 plants incubated with DMSO did not accumulate the seed-specific proteins TIP3;1, 12S globulin or oleosin over the 21 d period but did accumulate TIP1;1. In LEC2 plants incubated with DEX, seed-specific marker proteins were first detected in leaves at 11 d and the seed proteins were detected in subsequent leaf samples for the duration of the experiment. Conversely, the TIP1;1 vegetative protein marker disappeared from leaf samples after 11 d on DEX, but a low level of TIP1;1 accumulation was again detected at 21 d on DEX. The same pattern of TIP1;1 accumulation was observed in a replicate experiment (not shown).

For detection of oleosin, TIP1;1 and TIP3;1, many samples showed more than the expected band (Fig. 3.12). Both TIP1;1 and TIP3;1 are ~ 26 kDa in size (Jauh et al., 1998) but appeared to migrate close to 25 kDa (TIP3;1) or under 25 kDa (TIP1;1). The same result was reported by others (Vander Willigan et al., 2006; Jauh et al., 1999). For

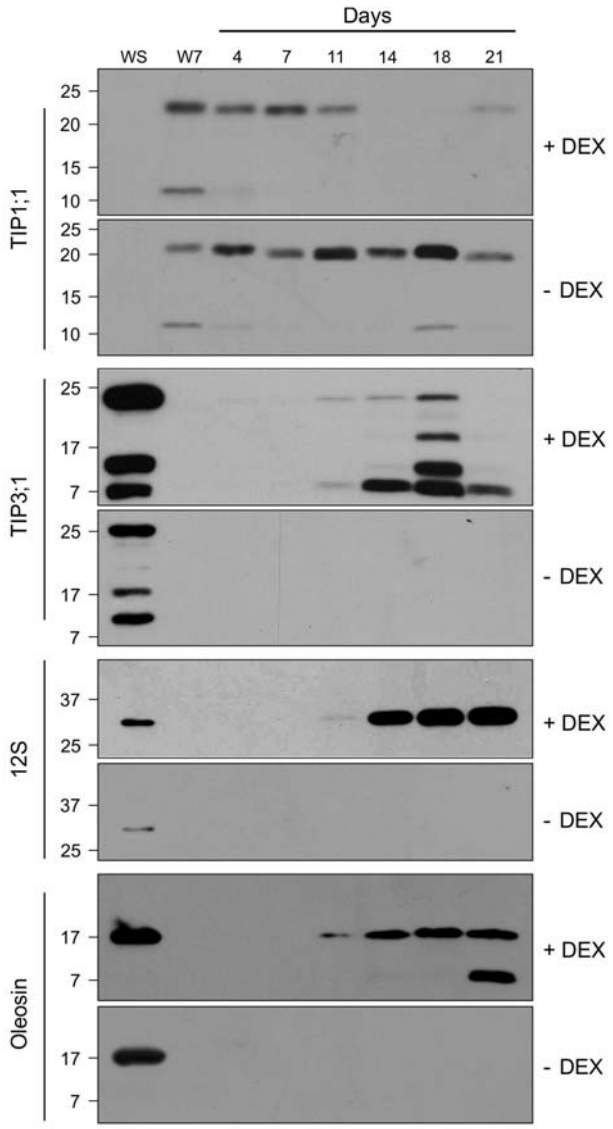

Figure 3.12 LEC2 induced leaves accumulate seed proteins while vegetative proteins disappeared over time when exposed to DEX. Seedlings germinated for 7 d on MS medium and were transferred to MS containing 30 µM DEX (+DEX) or DMSO (-DEX). Protein controls were extracted from Ws-0 seed (lane WS) and leaves from 14 d-old Ws-0 seedlings (collected at the 7 d time point) growing on MS medium (W7). Antibodies against seed-specific markers oleosin (1:5,000), 12S globulin (1:50,000) and TIP3;1 (0.24 µg/ml) and an antibody against a vegetative marker, TIP1;1 (0.2 µg/ml), were used to detect the presence of proteins in samples. For TIP1;1 and TIP3;1 blots, 50 µg leaf and 1 µg seed proteins were loaded into wells. For 12S blots, 50 µg leaf and 2.5 µg seed proteins were loaded into lanes. For the oleosin blot, 10 µg leaf and 0.5 µg seed proteins were loaded into lanes. Top heading indicates the number of days plants were exposed to DEX. Expected sizes of proteins detected by antibodies are ~ 25 kDa (TIP1;1 and TIP3;1), 30 kDa (12S globulin) and 18 kDa (oleosin).

TIP1;1 detection, a smaller and fainter band ~ 10 kDa was also observed in a number of leaf control samples but was not detected in leaf samples induced by DEX. A similar pattern was observed in a replicate blot where the ~ 10 kDa band was present in several control samples but not in leaf samples induced by DEX (not shown). The antibody against TIP3;1 detected two smaller bands (~ 7 and 14 kDa) in seed controls and up to three smaller sized bands in leaf samples on DEX (~ 7, 14 and 20 kDa). Using the same TIP antibodies, Jauh et al. (1999) also observed the presence of smaller protein bands (~ 17 and < 14 kDa) for TIP3;1 detection in pea seed extracts and < 14 kDa for TIP1;1 detection in radish root extracts. They reasoned that the extra bands were caused by proteolytic cleavage of the first transmembrane domain from the rest of the protein. The oleosin antibody recognizes an 18 kDa protein. This band was detected in extracts from seed and leaf samples from 11-21 d on DEX. At 21 d, a smaller sized band (7 kDa) of similar intensity to the 18 kDa band was present in the leaf sample that was not observed in the seed control. However, in a replicate blot, the 7 kDa band was also observed in the seed control (not shown). This band may represent a degradation product of the oleosin protein.

The protein profile of TIP3;1 accumulating in LEC2 induced leaves was different than the seed control. The antibody was generated against a short peptide sequence that was specific to the TIP3;1 isoform and this sequence is located at the C-terminal cytoplasmic tail of the transmembrane protein (Jauh et al., 1998). The TIP3;1 antibody revealed two interesting results. First, the relative intensities among bands present in LEC2 induced leaf and seed samples were different. In seeds, the antibody against TIP3;1 detected two smaller bands that were similar in intensity to the ~ 25 kDa predicted band. However, in leaf samples collected between 11-18 d on DEX, the ~ 25 kDa band was fainter than the three smaller sized bands. Second, absence of the ~ 25 kDa TIP3;1 band in leaf tissue from plants treated for 21 d with DEX is puzzling. In leaf samples, the decreased band intensity of the predicted TIP3;1 protein relative to the smaller-sized bands in the same sample and its absence from 21 d samples suggests that the TIP3;1 protein may be more susceptible to proteolytic degradation in leaves.

As LEC2 plants continue to grow on DEX induction medium, the trend is for seed protein markers to accumulate and for the TIP1;1 vegetative marker to disappear from leaves. However, it is interesting that at 21 d on DEX, the TIP1;1 vegetative marker re-appears while the predicted TIP3;1 protein band is absent. This apparent reversal of the trend toward embryogenic characteristics in leaves over-expressing LEC2 should be examined more closely.

3.3.2 Changes to leaf cell biology occur as early as 4 d after induction of LEC2 with DEX

To follow the cell fate of LEC2 over-expression over time, leaf sections were triple-stained using OsO_4, IKI and TBO. Leaf sections from control plants treated with DMSO looked similar among all collection days; cells were loosely arranged, chloroplasts contained small starch granules and the LV was large (Fig. 3.13A-F). In contrast, leaf sections from plants induced by DEX revealed phenotypic changes that became more pronounced over time. For plants over-expressing LEC2, a range of responses was observed among leaf samples collected at each time point. Therefore, representative images that best characterize the changes caused by LEC2 over-expression for each time point are shown in Figure 3.13. Already at 4 and 7 d of incubation on DEX, chloroplasts had large starch granules, small oil bodies were visible in the cytoplasm and vacuoles were slightly reduced in size (Fig. 3.13G, H). By 11-14 d of incubation on DEX, the space between cells was reduced, vacuoles were extremely reduced in size, oil bodies were more plentiful and larger in size and small protein deposits were observed in the small vacuoles (Fig. 3.13I, J). As more time was spent on DEX, the changes became more pronounced and specimens became more difficult to prepare and visualize using light microscopy (Fig. 3.13K-L). Thus, alteration of the leaf cell fate toward embryogenic characteristics was seen as early as 4 d after incubation on DEX and became more prominent over time on DEX.

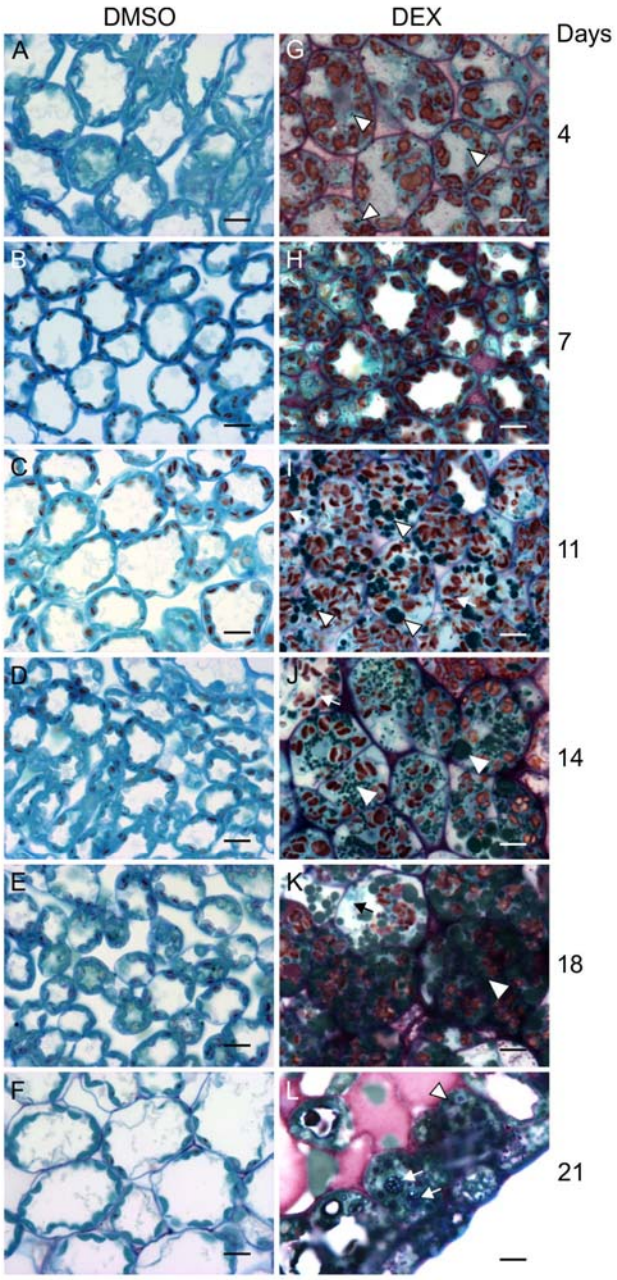

Figure 3.13 Over-expression of LEC2 promotes embryogenic characteristics in leaf cells which become more pronounced over time on DEX. LEC2 seedlings germinated for 7 d on MS medium were transferred to MS with 30 µM DEX or DMSO for 21 d and leaves were collected at 3-4 d intervals. Leaf sections were stained with TBO, OsO_4 and IKI to differentiate between the cell components. Representative images are shown for leaves collected after 4 (A, G), 7 (B, H), 11 (C, I), 14 (D, J), 18 (E, K) and 21 (F, L) d on DMSO (left panel) or DEX (right panel). Arrowheads point to green-stained oil bodies and arrows point to blue-stained protein deposits. Bar = 10 µm.

3.3.3 Transitioning leaf vacuoles are morphologically similar to seed storage vacuoles

While surveying leaf tissue from plants over-expressing LEC2, organelles resembling PSVs were observed as early as 14 d on DEX. Using histochemical staining, PSV-like structures were seen in leaf cells 21 d after DEX induction which resembled seed PSVs (Fig. 3.14A, B). Both seed PSVs and leaf PSV-like organelles appeared round in shape and were ~ 7 μm in diameter. Their lumens were stained blue to dark blue and were dotted by lightly blue-stained areas, likely representing globoid structures (Prego et al., 1998). In seed tissues, PSVs appeared to aggregate in the center of the cell. However, in leaf tissue, this localization pattern was not as evident. In triple-stained seed tissues, cells were packed with intensely stained lipids and proteins which made it difficult to discern the dark blue PSVs from the dark green coloured oil bodies. The same was true for leaves of LEC2 induced plants at 18 and 21 d on DEX (Fig. 3.13K, L). A more obvious portrayal of maturing PSVs in leaf tissues was observed by electron microscopy in leaf cells 14 d after DEX induction. Immunogold labeling with tonoplast (Fig. 3.11) and luminal (Fig. 3.10) markers established that the small vacuoles containing electron dense protein aggregates were developing PSVs. Indeed, the presence of TIP3;1 in the tonoplast (Fig. 3.11) is indicative that the leaf vacuoles have specialized to a storage function (Jauh et al., 1999; 1998). Moreover, some vacuoles undergoing transition were observed to be morphologically similar to seed PSVs. In these developing organelles, electron dense aggregates fill the vacuole lumen and begin to take on the appearance of mature PSVs in dry seeds (compare Fig. 3.14C-E and Fig. 3.14F). In these developing PSVs, the electron dense aggregates filling the vacuole lumen contained many large, round electron transparent areas, resembling globoid structures in seed PSVs. The morphological similarity of the developing leaf PSVs to seed PSVs suggests that these leaf organelles are at an advanced transitional state toward becoming a mature storage vacuole in the leaf.

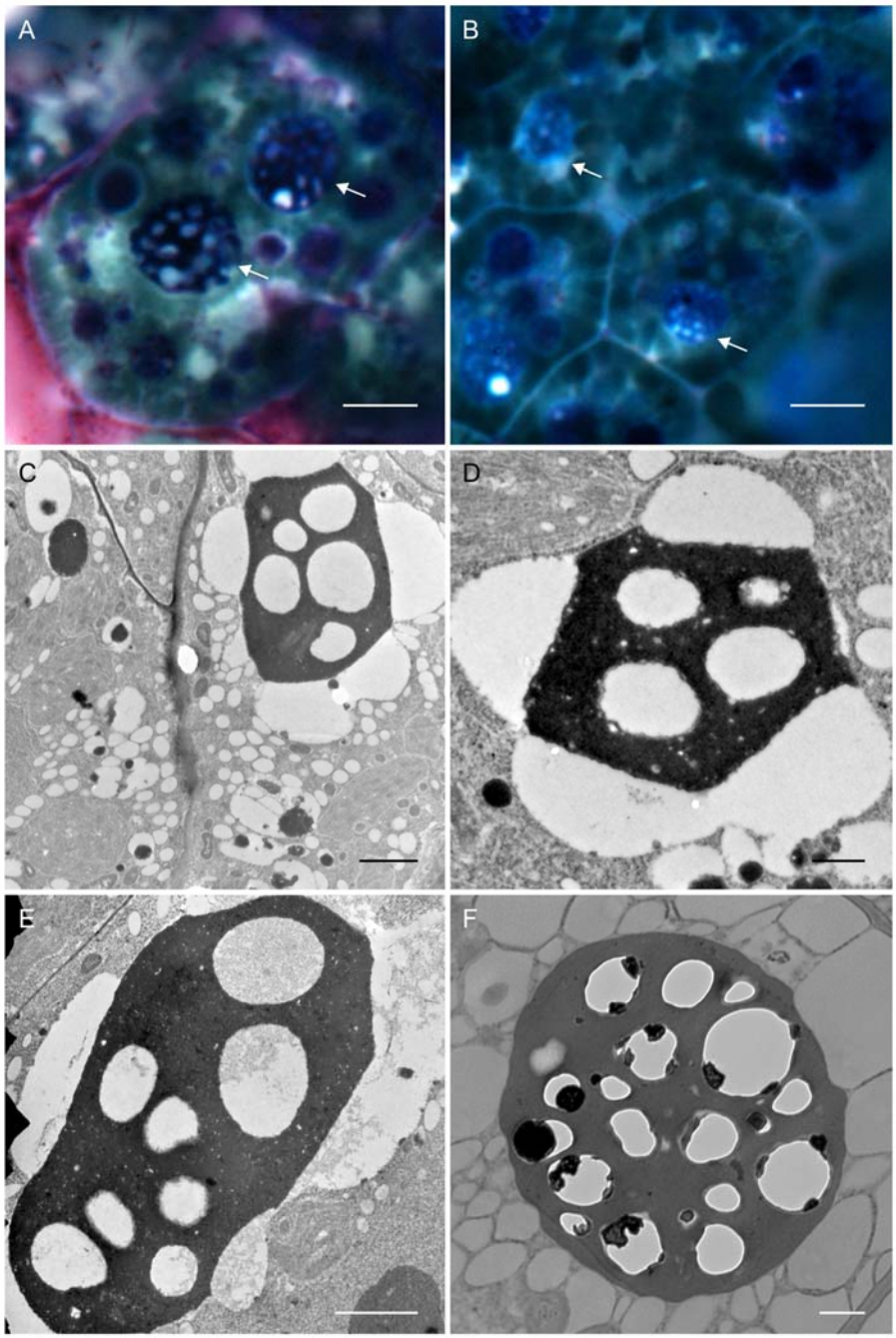

Figure 3.14 Protein storage vacuole-like organelles appeared in leaf cells over-expressing LEC2. Leaves were harvested from LEC2 plants after 14 d (C-E) or 21 d (A) on DEX. Ws-0 seed tissue was used as a control (B, F). Sections were stained with TBO, OsO_4 and IKI and observed by light microscopy (A, B) or sections were stained with OsO_4 and observed by transmission electron microscopy (C-F). PSV-like organelles in leaf cells (A, C-E) are similar in appearance to seed PSVs (B, F). Arrows point to PSV-like organelles (A) and PSVs (B). Bar = 10 µm (A, B), 2 µm (C, E), 500 nm (D, F).

3.4 Fluorescently labeled tonoplast intrinsic proteins present a dynamic view of the transition from lytic to storage vacuoles in LEC2 induced leaves

3.4.1 TIP3;1 is synthesized *de novo* in tissues following LEC2 induction

To study the LV to PSV transition in LEC2 induced plants, the expression pattern of fluorescently labeled TIP proteins was observed by confocal microscopy. The *TIP3-YFP/TIP1-RFP* construct was generated by Gattolin et al. (2011) to observe the PSV to LV transition during seed germination. Expression of both TIP isoforms is under control of their native promoters which are developmentally and spatially regulated. TIP3;1 is expressed in seed embryos and localizes to the PSV tonoplast while TIP1;1 is expressed in vegetative tissues and localizes to the LV tonoplast (Gattolin et al., 2011; 2009; Beebo et al., 2009). Thus the vacuole type present in the cell would be distinguished by the fluorescence they emit (Hunter et al., 2007). Transgenic plants harboring *TIP3-YFP/TIP1-RFP* were crossed with plants carrying *35S:LEC2-GR* (Stone et al., 2008). The resulting LEC2/TIP3-YFP/TIP1-RFP seeds were germinated on MS medium for 7 d at 20°C. Seedlings were transferred to MS containing DEX and tissues were sampled over time.

The formation of PSVs in vegetative tissues was observed by monitoring the reappearance of TIP3;1-YFP expression in the whole plant over time on DEX (Fig. 3.15A). In germinating seeds, TIP3;1-YFP fluoresced bright green but expression began to decline after radicle emergence and by ~ 4 d was largely absent from the seedling, except for some fluorescence at the root tip (not shown). These results are consistent with those of Gattolin et al. (2011) who observed a similar pattern of TIP3;1-YFP fluorescence during *Arabidopsis* seed germination using the same TIP construct. Following transfer of seedlings to DEX, PSVs first re-appeared in roots after 10 d on DEX. Under the confocal microscope, single cells or small clusters of cells in the inner root expressed the TIP3;1-YFP marker (Fig. 3.15B, C). Next, the marker was observed in cotyledons at 12 d on DEX (Fig. 3.15F, I). Following this, PSVs began to form in leaves at 14 d on DEX. TIP3;1-YFP expression appeared in older leaves of the rosette first (Fig. 3.15A, D, E, G, H). Expression tended to begin at the tip of the leaf and along the leaf margin and moved

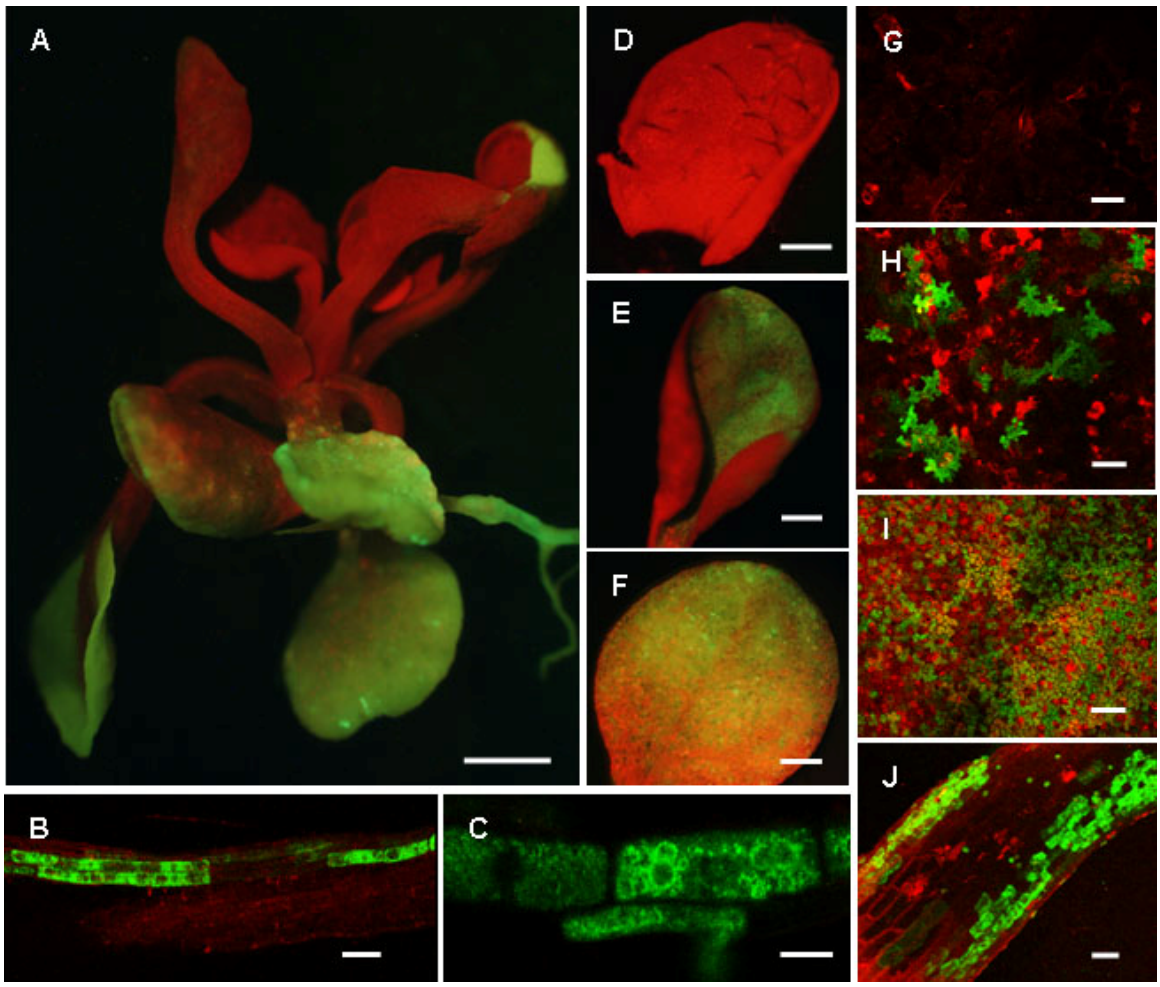

Figure 3.15 TIP3;1-YFP is synthesized *de novo* in roots, cotyledons and leaves following LEC2 induction. Transgenic LEC2/TIP3-YFP/TIP1-RFP seeds were germinated on MS medium for 7 d and then transferred to 30 µM DEX. After 15 d incubation on DEX, or unless otherwise specified, plants were imaged by fluorescence microscopy using a GFP filter: whole plant showing roots, cotyledons and young leaves expressing TIP3;1-YFP (A), young leaf (D), mature leaf (E) and cotyledon (F). Chlorophyll autofluorescence (red), YFP fluorescence (green). Tissues were also imaged by confocal microscopy: roots at 10 d incubation on DEX (B, C), young leaf (G) and mature leaf (H) at 14 d incubation on DEX, cotyledon at 12 d incubation on DEX (I) and petiole at 19 d on DEX (J). Non-specific fluorescence was enhanced to add context to images (red), YFP fluorescence (green). Bars = 1 mm (A), 10 µm (B, C), 0.5 mm (D- F), 100 µm (G-J).

inward and down the leaf toward the petiole as the leaf matured (Fig. 3.15J). For all tissues examined, TIP3;1-YFP expression initially occurred in single cells or small clusters of cells (Fig. 3.15B, H-J). Altogether, LEC2 over-expression in vegetative tissues promotes the synthesis and accumulation of TIP3;1-YFP, which is indicative of PSV formation. Typically, single cells or small clusters of cells in all organs are initially observed to respond to LEC2 by forming PSVs. Storage vacuoles first appear in roots and PSV formation ascends up the plant over time.

3.4.2 TIP1;1 expression is replaced by TIP3;1 in leaves following LEC2 induction

To study the transition of LVs to PSVs in vegetative tissues, the expression patterns of TIP1;1-RFP and TIP3;1-YFP, which are indicative of the presence of LVs and PSVs, respectively, were observed over time on DEX. In embryos germinating for 2 d, TIP3;1-YFP was highly expressed on the PSV tonoplast of cotyledon cells whereas TIP1;1-RFP expression was not detected (Fig. 3.16A-C). At 6 d, the expression of TIP markers overlapped in cotyledons; TIP3;1-YFP fluorescence was becoming less intense while TIP1;1-RFP fluorescence was strong (Fig. 3.16D-F). Co-localization of both TIPs on the same tonoplast demonstrates the transition from PSV to LV in germinating seedlings. Seedlings were then transferred to DEX after 7 d. By 14 d (7 d on DEX), TIP3;1-YFP expression was undetectable in leaves and TIP1;1-RFP expression was observed although with lower intensity, indicating a reduction in TIP1;1-RFP (Fig. 3.16G-I). The timing of the PSV to LV transition during seed germination is comparable to what was reported by Gattolin et al. (2011) using the same TIP markers. This demonstrates that TIP expression patterns during germination of LEC2 seeds are consistent with previously published results. After 21 d (14 d on DEX), TIP1;1-RFP expression was no longer detectable in leaf epidermal cells while TIP3;1-YFP expression re-appeared (Fig. 3.16J-L). However the TIP3;1-YFP expression pattern was altered in cells. The tonoplast no longer resembled that of the large LV that lined the periphery of the epidermal cell. Instead, the tonoplast appeared to occupy a large volume of the cytoplasm within each cell. This observation shows that the TIP expression patterns are altered by LEC2 over-expression

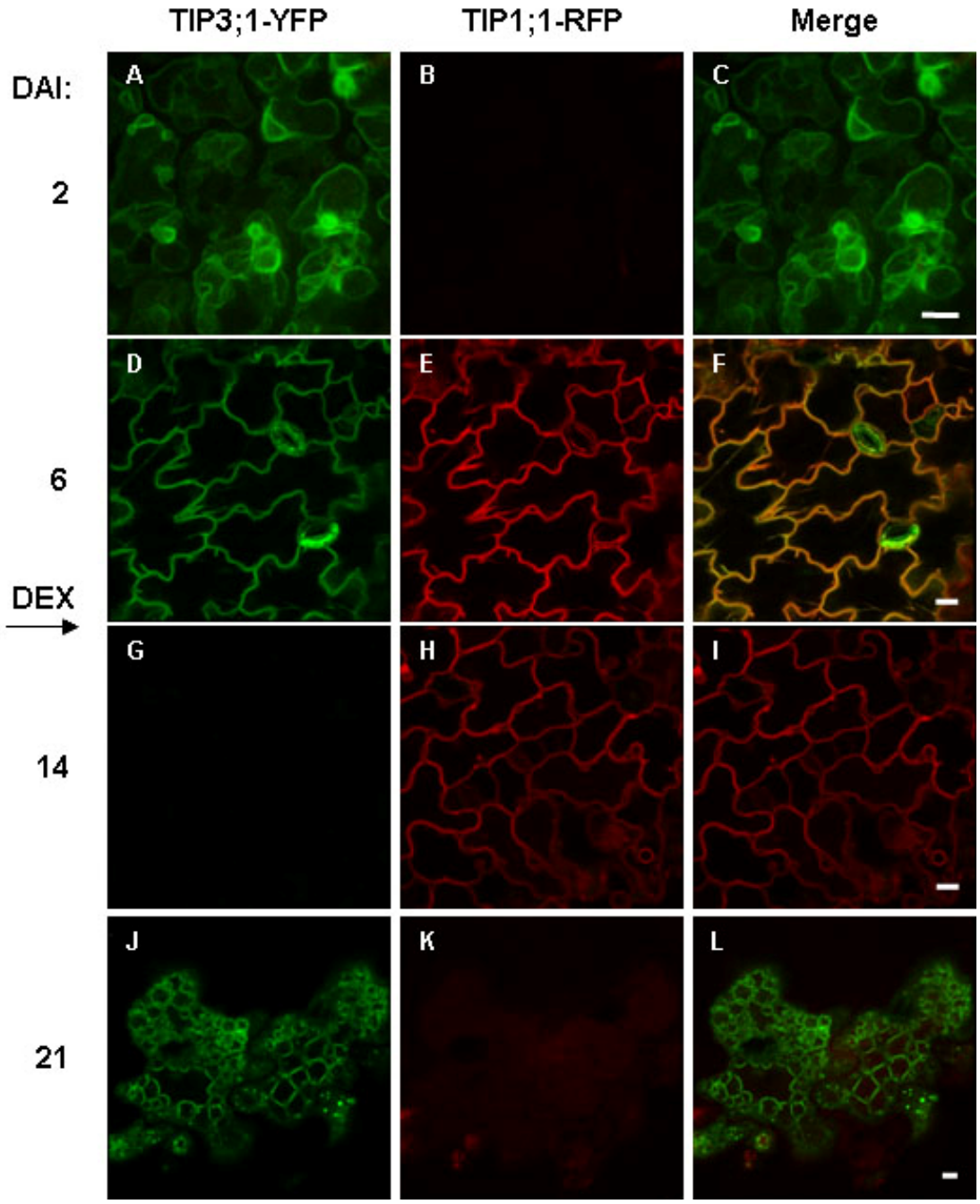

Figure 3.16 Leaf lytic vacuoles are replaced by protein storage vacuoles following LEC2 induction. *Arabidopsis* seeds co-expressing 35S:LEC2-GR, TIP3;1:TIP3;1-YFP (green) and TIP1;1:TIP1;1-RFP (red) were stratified at 4°C for 3 d and transferred to MS medium at 22°C to germinate for 7 d. Seedlings were then transferred to MS supplemented with 30 µM DEX (indicated by an arrow). At the indicated times, TIP expression was analysed in the epidermis of cotyledons (A-F) or mature leaves (G-L) by confocal microscopy. Green oval structures in (F) are stomata. DAI; days after incubation at 22°C. Bars = 5 µm (A-C), 10 µm (D-L).

and further confirms the transition of leaf LVs to PSVs in response to LEC2 over-expression.

3.4.3 TIP3;1-YFP labels the lytic vacuole, vacuolar bulbs and protein storage vacuole-like structures that are unique to LEC2 induced leaves and resemble embryonic storage vacuoles

To study the morphology of vacuoles expressing TIP3;1-YFP in their tonoplasts, leaves from LEC2/TIP3-YFP/TIP1-RFP plants incubated on DEX for 15 d were observed by confocal microscopy. Transgenic *Arabidopsis* lines harboring *TIP3;1:TIP3;1-YFP* or *35S:TIP3:1-YFP* (Hunter et al., 2007) were included as controls. To add perspective to some images, tissues were stained by neutral red (NR) which labels the vacuole lumen (Dubrovsky et al., 2006) or FM4-64 which labels the plasma membrane (Jelinkova et al., 2010; Bolte et al., 2004).

A comparison of vacuoles labeled by TlP3;1-YFP in leaves induced by LEC2 and embryonic tissues revealed a similar morphology. At the onset of PSV formation in cotyledons of transgenic TIP3;1:TIP3;l-YFP embryos, TIP3;l labeled the PSV tonoplast and the plasma membrane (Fig. 3.17A, B). Localization of TIP3;l to the plasma membrane was previously confirmed by co-localization with FM4-64 (Gattolin et al., 2011). The tonoplast displayed fluorescent folds that appeared brighter than the rest of the tonoplast. NR was used to stain the PSV lumen and highlighted the globular shape of the PSVs in embryonic cells (Fig. 3.17B). The selectivity of the NR stain for vacuole lumens relies on the low pH of the organelle. NR passes through the plasma membrane and tonoplast in an unprotonated form but once inside the acidic vacuole lumen, the stain is protonated and trapped (Dubrovsky et al., 2006).

In LEC2/TIP3-YFP/TIP1-RFP leaf epidermal cells induced by DEX, the tonoplast appeared to be labeled by TIP3;1-YFP and appeared to form many highly fluorescent folds like embryonic PSV tonoplasts (Fig. 3.17C, D). Additional highly fluorescent spherical structures called bulbs were observed (Saito et al., 2002).

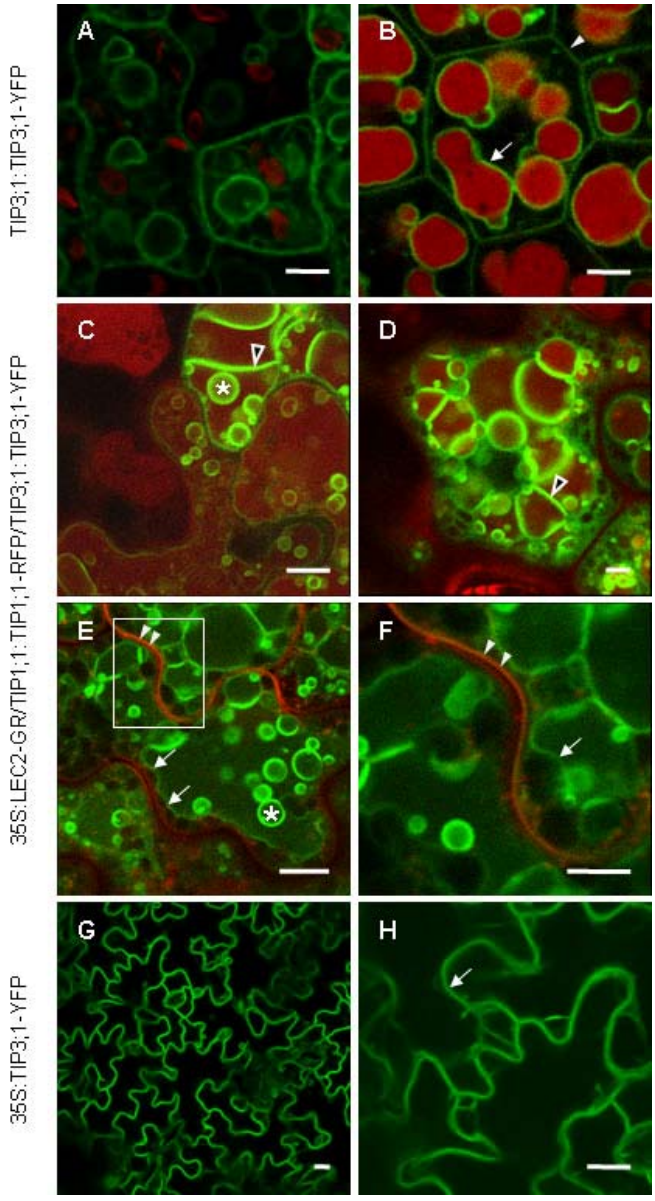

Figure 3.17 TIP3;1-YFP labels the lytic vacuole tonoplast, vacuolar bulbs and PSV-like structures that are unique to LEC2 induced leaves and resemble embryonic storage vacuoles. Cotyledons of maturing TIP3;1:TIP3;1-YFP embryos imaged at the onset of TIP3;1-YFP expression (A, B). Transgenic LEC2/TIP3-YFP/TIP1-RFP seedlings were incubated on 30 µM DEX for 15 d. Leaves were stained with neutral red (C, D) or FM4-64 (E, F). Boxed image in (E) is magnified in (F) to show co-localization of FM4-64 and TIP3;1-YFP on plasma membrane. Leaves of 21 d-old transgenic *Arabidopsis* plants expressing *35S:TIP1;1-YFP*. Leaf epidermal cells were examined in (C-H). Green color in (A-H) is YFP fluorescence. Red color in (A) is chlorophyll autofluorescence. Red color in (B-D) is neutral red. Red color in (E, F) is FM4-64. Arrows point to tonoplast, arrowheads point to plasma membrane, empty arrowheads label tonoplast folds and stars label bulbs. Bars = 10 µm in (A-E, G, H), 5 µm in (F).

These structures are often abundant in vacuoles of young plant tissues and as plants mature, the number of bulbs decreases (Hunter et al., 2007). NR produced a different staining pattern in leaf vacuoles compared to embryo vacuoles. In the embryo, only the globular PSV lumens were stained however in LEC2 leaves, the entire cell volume was typically stained, including the regions within folds and bulbs. Thus, despite the presence of TIP3;1-YFP expression which suggests that the vacuole has changed to a storage function (Jauh et al., 1999) as well as the appearance of complex tonoplast configurations which are typically observed in young tissues (Saito et al., 2002), NR staining shows that the vacuole lumen continues to fill the entire cell volume, like a LV. In addition, as observed in embryo PSVs (Fig. 3.17A, B), TIP3;1-YFP labeled the plasma membrane in LEC2 induced leaves. This was demonstrated by co-localization of FM4-64 and TIP3;1-YFP (Fig. 3.17E, F). The ability to localize to both the tonoplast and plasma membrane is unique to TIP3 seed-specific isoforms (Gattolin et al., 2011).

To exclude the possibility that the morphological changes associated with PSV formation in leaf vacuoles responding to LEC2 over-expression was not a result of TIP3;1-YFP over-expression, a control was needed. Since the TIP markers were under control of their native promoters, it was not possible to show the expression of TIP3;1-YFP in leaves without LEC2 over-expression because the promoters are developmentally regulated. Consequently, TIP3;1-YFP expression would be silenced in vegetative tissues and activated in embryonic tissues (Hunter et al., 2007). To demonstrate the morphology of leaf vacuoles labeled with TIP3;1-YFP without LEC2 over-expression, the fusion was placed under control of a 35S constitutive promoter. Under a 35S promoter, the TIP3;1 gene was no longer under developmental or spatial control by its native promoter and it should localize to the tonoplast of any vacuole that was present in the cell (Hunter et al., 2007). In plants expressing *35S:TIP3;1-YFP*, tonoplasts were fluorescently labeled by TIP3;1-YFP but had the morphology of LVs (Fig. 3.17G, H). Fluorescently labeled tonoplasts of leaf epidermal cells were characteristically puzzle-shaped, PSV-like structures were not observed and only a small number of vacuolar bulbs were seen. These results establish that the altered vacuole morphology observed in LEC2/TIP3-YFP/TIP1-RFP plants was caused by LEC2 over-expression rather than over-expression of TIP3;l-YFP.

During seed development, PSVs accumulate storage protein reserves. Similarly, SSPs were demonstrated to accumulate in developing PSVs in leaves over-expressing LEC2 (Fig. 3.10). In embryos, storage proteins autofluoresce inside PSV lumens when excited by a 405 nm UV beam (Fig. 3.18A) (Hunter et al., 2007). Therefore the contents of LEC2 induced leaf vacuoles were examined to determine whether developing PSVs are capable of autofluorescing like seed PSVs. Epidermal cells from leaves of transgenic LEC2/TIP3-YFP/TIP1-RFP plants not induced by DEX revealed LV tonoplasts fluorescently labeled with TIP1;1-RFP and lacking any autofluorescence in the lumen (Fig. 3.18B). Plants incubated on DEX for 19 d showed the characteristic PSV-like vacuole morphology as revealed by TIP3;1-YFP and FM4-64 staining, but cells were not observed to autofluoresce (Fig. 3.18C). However, in plants incubated on DEX for 23 d, autofluorescence was detected in leaf epidermal cells (Fig. 3.18D-F). These induced leaf cells expressed TIP3;1-YFP on the tonoplast of developing PSVs and were stained with FM4-64 to define the cells. The pattern of autofluorescence was similar to what was observed by NR staining. The entire cell volume was observed to autofluoresce, including the bulbs. Therefore developing PSV-like vacuoles in LEC2 induced leaves were demonstrated to accumulate seed proteins and to autofluoresce like seed PSVs but the pattern of autofluorescence suggested that SSPs were accumulating in the lumen of a large vacuole.

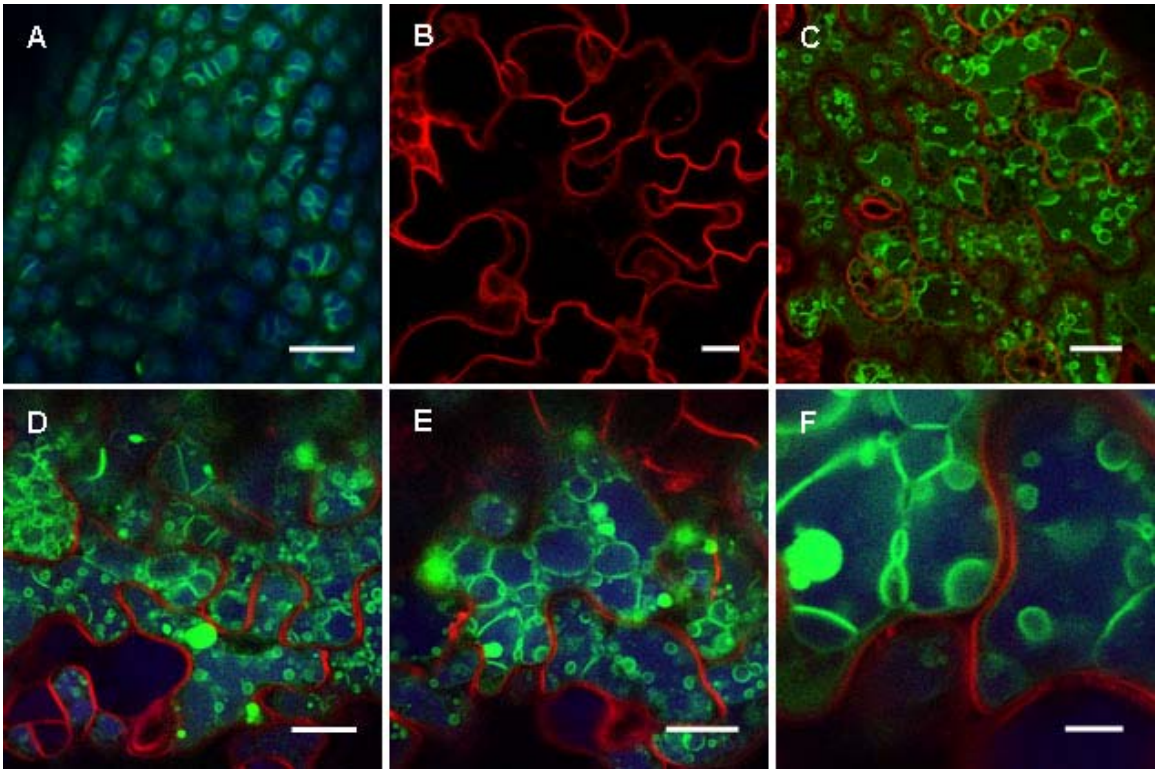

Figure 3.18 Protein storage vacuoles autofluoresce in embryos and LEC2 induced leaf cells. Transgenic embryos expressing TIP3;1-YFP had autofluorescent PSV lumens when excited at 405 nm (A). Leaf epidermal cells of transgenic LEC2/TIP3-YFP/TIP1-RFP plants were examined for vacuole autofluorescence when excited at 405 nm (B-F). Plants grown without DEX expressed TIP1;1-RFP on the LV tonoplast and vacuole lumens did not autofluoresce (B). Plants grown for 19 d on DEX expressed TIP3;1-YFP on the tonoplast but did not autofluoresce (C). At 23 d on DEX, developing PSVs were labeled by TIP3;1-YFP and vacuoles autofluoresced (D-F). PSV autofluorescence is blue and TIP3;1-YFP expression is green. Red color in (B) is TIP1;1-RFP. Red color in (C-F) is FM4-64. Bar = 16 µm (A), 20 µm (B-E), 5 µm (F).

CHAPTER 4

DISCUSSION

4.1 *Arabidopsis* plants acquire seed characteristics in response to LEC2 over-expression

4.1.1 LEC2 promotes embryogenic characteristics and callus formation

LEC2 over-expression promoted a change in leaf phenotype from vegetative to embryogenic characteristics. Leaves began to take on characteristics of cotyledons (Fig. 3.1G, H). Moreover, LEC2 over-expression was sufficient to induce the formation of callus and somatic embryo-like structures. Ledwoń and Gaj (2009) also observed intense callus formation which preceded somatic embryo formation in *Arabidopsis* tissues over-expressing LEC2. Somatic embryos closely resemble zygotic embryos except they do not originate from fertilization. Instead, they arise from somatic cells that have acquired competence to initiate embryo development (Quiroz-Figueroa et al., 2006; Fehér et al., 2003). The formation of somatic embryos on *Arabidopsis* plants over-expressing LEC2 was previously reported by Ledwoń and Gaj (2009) and Stone et al. (2001) but was not observed by Santos-Mendoza et al. (2005), who only identified abnormal structures forming on leaves. Results from this work support the results of Ledwoń and Gaj (2009) and Stone et al. (2001). Thus, LEC2 activity creates an environment that not only promotes embryogenic characteristics in vegetative tissues but is strong enough to induce vegetative cells to undergo somatic embryogenesis (SE) (Braybrook and Harada, 2008; Stone et al., 2008).

4.1.2 Induced plants do not show a homogenous response on the whole plant, tissue or cellular levels

Staining revealed a drastic change in the overall phenotype of leaf mesophyll cells in response to LEC2 over-expression; cell organization and shape were altered as well as the appearance of cell contents. These findings are consistent with those of West et al. (1994) who showed the opposite effect with a *lec1* knockout mutation. The *lec1* mutation affected the cellular organization of cotyledons and caused them to more closely resemble leaves than cotyledons of wild type plants.

Plants over-expressing LEC2 did not show a homogenous phenotypic response. A number of induced seedlings did not show a phenotype (Fig. 3.4A) and of the plants that revealed a phenotype, they did not all exhibit the same degree of induction. For this work, only those plants that were most strongly induced were studied, i.e. plants that showed the most extreme phenotypes. As well, the phenotype appeared to vary along leaf sections. In some areas, intensely stained novel cell structures accumulated whereas other areas revealed large unstained vacuoles (Fig. 3.4B, C). A similar pattern was observed in *lec1* mutant cotyledons where mesophyll cells were observed to organize into a palisade layer in parts but not throughout the cotyledon (West et al., 1994). Alternatively, the uneven phenotype could be explained by an uneven distribution along the leaf blade of the steroid used to induce LEC2 activity. It is possible that the leaves would be more evenly induced if DEX were applied directly to the leaf instead of being uptaken by the roots through contact with the DEX-containing medium (Aoyama and Chua, 1997; Schena et al., 1991).

4.2 Chloroplasts accumulate starch and de-differentiate in response to LEC2 over-expression

4.2.1 Chloroplast starch granules increase in size in response to LEC2 over-expression

A noticeable increase in the size of starch granules in LEC2 induced leaves was observed compared to control leaves. Starch granules in leaves are generally characterized as discoid in shape (Zeeman et al., 2007; 2002) but in LEC2 induced leaves the granules were larger and took on different shapes. In *Arabidopsis* leaves, sucrose and starch are synthesized as products of photosynthesis during the day. Sucrose is sent to non-photosynthetic parts of the plant while starch accumulates in the chloroplast stroma. At night, the stored starch is degraded to provide a supply of carbohydrate to support the continued growth of the plant (Smith et al., 2005). Not only does starch transiently accumulate in leaves, but it is also observed to transiently accumulate in embryos and in meristematic areas of vegetative tissues (Andriotis et al., 2010; Smith et al., 2005). Embryos and meristems represent cells that are actively dividing or have recently divided

and are in the early stages of differentiation. Although the importance and function of starch metabolism in *Arabidopsis* embryos is not well understood (Angeles-Núñez and Tiessen, 2011; da Silva et al., 1997), transient starch accumulation is thought to represent a temporary carbon reserve and is proposed to be a normal feature of cells undergoing early stages of differentiation (Andriotis et al., 2010).

The LEC2 transcription factor has been implicated in the regulation of soluble sugar and starch pathways in the developing seed (Angeles-Núñez and Tiessen, 2011). During *Arabidopsis* seed development, the early maturation phase is characterized by a rapid accumulation of starch which then disappears as oil and protein stores are synthesized (Baud et al., 2002). Over-expression of LEC2 has been shown to increase the expression of *SUCROSE SYNTHASE2* (*SUS2*), an important gene involved in starch synthesis in developing seeds (Angeles-Núñez and Tiessen, 2012; Santos-Mendoza et al., 2005). At the same time, loss-of-function *lec2* mutants accumulated significantly more starch and soluble sugars than wild type seeds (Angeles-Núñez and Tiessen, 2011). Several theories have been put forth to explain these seemingly conflicting results but two ideas may provide an explanation for the increased starch accumulation observed in leaves over-expressing LEC2. First, SUS2 acts in the cytosol and is not involved in plastidial starch metabolism. Second, LEC2 is expected to regulate many other genes of central metabolism besides *SUS2*, although many of these interactions have yet to be uncovered (Angeles-Núñez and Tiessen, 2012). Thus, the observed increase in starch accumulation in leaves over-expressing LEC2 may be explained by the involvement of LEC2 in regulating the sugar and starch metabolism in seeds as well as the ability of the transcription factor to promote differentiation of leaf cells toward an embryogenic pathway.

4.2.2 Chloroplast de-differentiation is observed in LEC2 induced leaves

In addition to increased starch granule size, some chloroplasts were observed to de-differentiate in leaves over-expressing LEC2. Generally, in strongly induced LEC2 leaves, chloroplasts with large starch granules and a developed thylakoid system were

seen (Fig. 3.8B). However, a smaller number of chloroplasts were observed to de-differentiate; they were much smaller in size, more round in shape and displayed a reduced thylakoid complexity (Fig. 3.8D, E). Since thylakoids are the photosynthetic membranes of the chloroplasts that harbor the main complexes involved in the light reaction, a reduction in thylakoid complexity suggests that chloroplasts are degenerating (Vothknecht and Westhoff, 2001). All plastids are derived from small (~ 1 μm), round, colorless and undifferentiated proplastids which are present in the embryo and meristematic cells. These organelles have a poorly defined internal membrane system that consists of some vesicles that appear to connect to the inner membrane of the plastid envelope. During cell differentiation, proplastids differentiate into other plastid types depending on the needs or specialization of the cells. In young leaf cells exposed to light, proplastids grow in size, develop a more complex internal membrane system and a more highly variable morphology during their transition to become a chloroplast (Wise, 2007; Lopez-Juez and Pyke, 2005; Pyke, 1999). Thus the level of complexity increases as the plastid differentiates into a chloroplast (Vothknecht and Westhoff, 2001).

In response to LEC2 over-expression, it appears as if the reverse was taking place. A number of leaf chloroplasts were observed to de-differentiate towards proplastids. Although it can be convenient to classify a plastid into a stage of differentiation, it is difficult to precisely classify the stage (Pyke, 1999). The morphology of the de-differentiated plastids in LEC2 induced leaves closely resembles the morphology of pseudochloroplasts as reported by Rohde et al. (2000). Pseudochloroplasts belong to a differentiation stage between proplastids and chloroplasts and are characterised by a partial differentiation of thylakoid membranes (Rohde et al., 2000). Without more in-depth morphological characterization of these plastids, it is not possible to be sufficiently certain as to what stage of differentiation the plastids observed in LEC2 over-expressing leaves belong. Regardless of whether the classification of the de-differentiated chloroplasts is accurate, the morphological observations suggest that the chloroplasts appear to de-differentiate.

The transcription factor ABI3 is a master regulator of seed development that is implicated as a regulator of plastid development (Rohde et al., 2000). During the

maturation stage, *Arabidopsis* embryos turn green for a short time as a result of chlorophyll accumulation and then they lose color and begin to desiccate. However, loss-of-function *abi3* mutant embryos are inhibited from de-greening and develop leaf primordia (Parcy et al., 1997; Nambara et al., 1995). ABI3 is expressed in apical meristems in response to conditions that favor growth-arrest, such as a short period of darkness. For example, etiolated seedlings exposed to darkness do not develop leaves (Rohde et al., 1999). By studying *abi3* mutants exposed to prolonged darkness, plastid development was found to be perturbed; wild type leaves possessed proplastids whereas *abi3* mutants possessed etioplasts, which are plastid types commonly found in plants grown in darkness for only short periods in time. Moreover, constitutive expression of *ABI3* led to the preservation of chloroplasts in dark grown leaves (Rohde et al., 2000). Even though the observations in this thesis appear to contradict those of Rohde et al. (2000), it is tempting to speculate that LEC2 affects plastid differentiation by positively regulating ABI3. Rohde et al. (2000) have clearly determined that ABI3 affects plastid differentiation however the conditions used to study the effects of ABI3 on plastid differentiation differ from this work. To support this idea, LEC2 over-expression has been shown to cause the accumulation of *ABI3* mRNA in *Arabidopsis* leaves (Santos-Mendoza et al., 2005). In addition, the expression analysis of the *ABI3* promoter fused to a *GUS* reporter in a *lec2* mutant showed that LEC2 controls *ABI3* expression during embryogenesis but is not responsible for initiating *ABI3* expression (To et al., 2006). Therefore, the appearance of de-differentiated chloroplasts could be due to the up-regulation of *ABI3* through the ectopic expression of LEC2 in leaves. It would be interesting to further investigate *ABI3* expression in LEC2 induced leaves.

4.3 Oil bodies accumulate in the cytoplasm of leaf cells over-expressing LEC2

The over-expression of LEC2 promotes accumulation of oil bodies in leaves. Oil bodies are organelles that specialize in the storage of lipids in seeds and anthers but are not observed in other tissues. They are composed of a matrix of TAGs surrounded by a phospholipid monolayer embedded with oleosin proteins (Hsieh and Huang, 2007; Kim

et al., 2002; Huang et al., 1992). The identity of these organelles was confirmed by staining with the lipophilic OsO_4 stain which specifically bound the oil body lumen (Fig. 3.5, Fig. 3.6) and by identifying the subcellular localization of oleosin by electron immunogold labeling (Fig. 3.7). Without LEC2 over-expression, a small number of tiny lipid droplets were observed in leaf cells (not shown), but oleosin protein accumulation was not detected (Fig. 3.12).

The results presented here further support previous work demonstrating that LEC2 over-expression promotes oil body formation in vegetative tissues. In an inducible LEC2 over-expression line, LEC2 was shown to activate oleosin mRNA expression in vegetative tissues (Braybrook et al., 2006; Santos-Mendoza et al., 2005). Unfertilized ovules constitutively expressing LEC2 were shown to accumulate oil reserves by staining with Sudan black B, which allows visualization of lipids (Stone et al., 2008; Bronner, 1975). In addition, the biochemical composition of lipids accumulating in LEC2 induced leaves was shown to be similar to seed tissues as measured by fatty acid methyl ester analysis (Stone et al., 2008) and gas chromatography (Santos-Mendoza et al., 2005).

In this work, oil body formation was observed as small lipid droplets in the cytoplasm of leaf cells as early as 4 d after exposure to DEX (Fig. 3.13G). The oil bodies grew in size and number over time and were quite obvious by 11 d on DEX (Fig. 3.13I, G-L). However, oleosin protein accumulation was first detected at 11 d after DEX treatment (Fig. 3.12). The apparent discrepancy between visualization of oil bodies and oleosin accumulation could be due to a low level of accumulation of oleosin proteins earlier than 11 d that went undetected by Western blot analysis. In seeds, oleosin and TAG synthesis is co-ordinated but oleosin synthesis has been demonstrated to occur independently of TAG synthesis (Beaudoin and Napier, 2000). It is possible that the activation of either of these pathways is differentially influenced by the activity of LEC2 and its downstream regulatory targets. Indeed, oleosin is a direct gene target of LEC2 (Braybrook et al., 2006; Santos-Mendoza et al., 2005) while TAG synthesis is indirectly regulated by LEC2 through its activation of *WRINKLED1*, a transcription factor necessary for the regulation of lipid biosynthesis in seeds (Santos-Mendoza et al., 2008; Baud et al., 2007). However, this theory would require further study.

4.4 Seed storage proteins accumulate in LEC2 induced leaves

LEC2 activates expression of a large number of genes involved in the maturation stage of seed development. In *Arabidopsis* seeds, a prominent group of RNAs induced by LEC2 comprise the 2S albumin and 12S globulin SSP families, which represent the major SSPs in *Arabidopsis* (Baud et al., 2008; Kroj et al., 2003). Ectopic expression of LEC2 in *Arabidopsis* was shown to up-regulate the RNA expression of both SSP families in seedlings (Braybrook et al., 2006) and leaves (Santos-Mendoza et al., 2005). In addition, the constitutive expression of LEC2 was demonstrated to promote the accumulation of 2S and 12S seed proteins in unfertilized ovules (Stone et al., 2008). This work supports the previous results by showing that ectopic expression of LEC2 causes the accumulation of 12S globulin and 2S albumin seed proteins in leaves.

During seed maturation, SSPs undergo complex post-translational modifications which alter their solubility properties to allow them to aggregate and endure long-term storage (Gruis et al., 2004). SSPs are synthesized on the rough ER as pro-proteins and are converted into their mature forms once they reach the vacuole. Pro-globulins are cleaved to produce alpha (~ 30 kDa) and beta (~ 20 kDa) subunits that are linked by a disulfide bond (Shimada et al., 2003b; Sjödahl et al., 1991). Pro-albumins undergo a more complicated maturation process; they are cleaved at four sites resulting in the removal of three pro-peptides. The remaining small (3-5 kDa) and large (8-12 kDa) subunits are linked by disulfide bonds (De Clercq et al., 1990; Krebbers et al., 1988). Two proteinase families are primarily involved in proteolytic processing of storage proteins; vacuolar processing enzymes (VPEs) which are members of a family of cysteine proteinases and, to a smaller extent, an aspartic proteinase (Gruis et al., 2004; Shimada et al., 2003b).

Both 12S globulin and 2S albumin proteins accumulate in their mature forms in LEC2 induced leaves. However, the antibody against 2S albumins specifically detected the 10 kDa large subunit but it also detected three additional non-specific bands in the seed sample (~ 30, 17, 12 kDa) and one extra band at ~ 17 kDa in the LEC2 induced leaf sample (Fig. 3.9). The 17 kDa band appeared slightly larger than the predicted 15 and 16 kDa 2S albumin precursor forms observed by Shimada et al. (2003b) in *vpe* knockout

seeds and the ~ 15 kDa 2S precursor observed in developing *Brassica napus* embryos using the same anti-2S antibody (Otegui et al., 2006). The proximity in band size could suggest that it represents a 2S precursor form. However it is doubtful because the band is also present in the seed control. Fully mature, wild type seeds should not accumulate SSP precursors (Gruis et al., 2004; Shimada et al., 2003b). Furthermore, a non-specific band of similar size appeared in Coomassie stained SDS gels of wild type seeds (Shimada et al., 2003b). Thus, the ~ 17 kDa band, along with the other two bands, likely represent non-specific binding of the antibody.

4.5 LEC2 over-expression promotes the formation of protein storage vacuole-like organelles in *Arabidopsis* leaves

4.5.1 Characterization of protein storage vacuoles in LEC2 induced leaves

Over-expression of two *LEAFY COTYLEDON* transcription factors was shown to promote the formation of novel leaf organelles that accumulated protein. The structures were labeled as protein bodies but were not further characterized. Stone et al. (2008) discovered protein bodies in roots and unfertilized ovules in transgenic plants constitutively expressing *LEC2* but did not detect the organelles in the same non-transgenic tissues. Similarly, Gazzarrini et al. (2004) revealed protein body formation in leaves of their FUS3 over-expression line. In the work presented here, LEC2 over-expression was demonstrated to promote the formation of protein-accumulating vacuoles in leaves. These vacuoles were further characterized to show that they resemble PSVs and accumulate SSPs. A detailed characterization of the vacuoles relied on histochemical stains with different binding affinities to distinguish between the cell components and to facilitate the detection of protein aggregates (Fig. 3.5, Fig. 3.6), morphological observations (Fig. 3.14) as well as vacuole luminal (Fig. 3.10, Fig. 3.17 and Fig. 3.18) and tonoplast (Fig. 3.11, Fig. 3.16 and Fig. 3.17) markers. Results showed that the large LV, typically present in leaf cells, was transitioning toward a PSV in response to LEC2 over-expression.

4.5.2 Storage proteins accumulate as densely stained aggregates in the vacuole lumen

In response to the ectopic expression of LEC2, the large LV, typically observed in leaf cells, was replaced by numerous small sized vacuoles that contained protein aggregates as demonstrated by TBO staining (Fig. 3.5C, Fig. 3.6D). These protein deposits appeared electron dense under the TEM (Fig. 3.6B, Fig. 3.10C) and accumulated 2S albumin and 12S globulin seed proteins (Fig. 3.10D-G).

Within larger protein aggregates, small transparent vesicles were often present. Many of these vesicles appeared to have a surrounding membrane and contained electron dense aggregates or smaller internal vesicles (Fig. 3.10F, G). The distinct morphology of the vesicles containing intraluminal vesicles bears a resemblance to multivesicular bodies (MVBs), although they are smaller in size. MVBs are PVCs and, as their name suggests, they contain internal vesicles which allow trafficking of membrane proteins and provide distinct domains for separation of cargo proteins (Jiang et al., 2002). In seeds, MVBs receive SSPs and their processing enzymes from the Golgi and transport them to the PSV (Reyes et al., 2011). In this work, immunogold labeling of SSPs was generally not observed in the internal vesicles and densely stained aggregates of the putative MVBs (Fig. 3.10G). Proteolytic processing of SSPs begins in the MVB and Otegui et al. (2006) elegantly demonstrated that both unprocessed protein precursors and processed SSPs are present in the MVB lumen. Therefore, the 12S and 2S antibodies used in this study should recognize processed SSPs in the MVB lumen. The absence of seed proteins in the putative MVBs could indicate that they transport a different cargo. Another puzzling observation is that the putative MVBs appear to remain intact in the vacuole. As MVBs are internalized into seed PSVs, their membranes fuse and release their contents into the PSV lumen (Jiang et al., 2002). Taken together, SSP aggregates accumulating in small sized vacuoles contain vesicles that are morphologically similar to MVBs, although they are smaller, do not appear to contain SSPs or fuse with the vacuole tonoplast. In future work, MVB-specific membrane sorting proteins could be used as markers to characterize these vesicles (Scheuring et al., 2011).

4.5.3 Vacuoles containing protein deposits in LEC2 induced leaf cells are protein storage vacuoles

The distribution of TIP markers along the tonoplast of small vacuoles that appeared in LEC2 induced leaves indicates that they are PSVs. However, TIP1;1, a vacuolar transmembrane protein, was localized to the seed PSV matrix (Fig. 3.11D) and the protein aggregates accumulating in leaf PSVs (Fig. 3.11F, G). This peculiar TIP1;1 localization pattern was observed in other studies using the same antibodies. Gillespie et al. (2005) observed TIP1;1 labeling in the PSV matrix of *Brassica napus* and *Arabidopsis* seeds. In addition, punctate structures within the PSV were labeled by the TIP1;1 antibody in *Arabidopsis* seeds (Bolte et al., 2011). On the contrary, over-expression of fluorescently labeled TIP1;1 was not detected in *Arabidopsis* embryonic tissues by confocal microscopy (Gattolin et al., 2011). As well, immunogold localization of TIP1;1 in tobacco root tip PSVs using an antibody obtained from a different source showed no TIP1;1 labeling of the PSV matrix (Zheng and Staehelin, 2011). Moreover, using the same antibodies to identify vacuoles during the PSV to LV transition in pea root tips, Olbrich et al. (2007) detected both TIP3;1 and TIP1;1 along the tonoplast of transitioning vacuoles but did not report TIP1;1 localization within protein aggregates. Therefore, it is possible that TIP1;1 labeling of the *Arabidopsis* PSV matrix could be a consequence of non-specific binding of the antibody used in this and other studies to an unknown *Arabidopsis* PSV matrix protein.

4.5.4 The morphology of vacuoles during lytic to storage vacuole transition in LEC2 induced leaves is similar to the morphology of vacuoles transitioning in developing seeds and root tips

The features of vacuoles in transition in LEC2 induced leaves closely resemble those reported in previous studies. In pea and barley root tip PSVs that were transitioning to LVs, Olbrich et al. (2007) observed vacuoles that accumulated densely stained aggregates along the luminal side of the tonoplast and immunogold labeling showed that the aggregates were composed of SSPs. Morphologically similar structures were observed

during the LV to PSV transition in developing *Arabidopsis* embryo cotyledons (Mansfield and Briarty, 1991) and in developing pea cotyledons (Hoh et al., 1995). However in pea cotyledon cells the protein deposits were contained within a tube-like membrane system that surrounded the vacuole (Hoh et al., 1995). This tubular membrane system was not observed surrounding vacuoles in leaves over-expressing LEC2.

A recently published report by Zheng and Staehelin (2011) studying the PSV to LV transition in tobacco root tips emphasized the importance of tissue fixation on the quality of specimens by comparing PSV preservation using high-pressure freezing and freeze substitution (HPF-FS) or chemical fixation. They demonstrated that the clumped, densely-stained, aggregated storage proteins in PSVs, as observed in LEC2 induced leaf samples in this work, were an artifact of chemical fixation. This differs from the uniform distribution of densely stained PSV luminal contents produced by the HPF-FS method. Their results make a strong case that HPF-FS should be used to preserve vacuoles. The authors reason that chemical fixation is an inadequate means of preserving cell structures; due to the slow rate of fixation, membrane structures are not effectively preserved and the distribution of proteins in PSVs is affected. Earlier work by Lonsdale et al. (1999), studying PSV ultrastructure in barley protoplasts, arrived at the same conclusions. In this study, samples were prepared by chemical fixation because the specialized equipment required for HPF-FS was not accessible. Ultimately, it is reasonable to consider that results by Zheng and Staehelin and Lonsdale et al. (drawing attention to the differences in PSV ultrastructure between both fixation methods) and the resemblance of vacuole ultrastructure images of this study to previously published works using chemical fixation (Olbrich et al., 2007; Hoh et al., 1995; Mansfield and Briarty, 1991) provide a better understanding of what the chemically fixed images of LEC2 induced leaf LV to PSV transition convey.

In advanced stages of PSV formation in LEC2 induced leaves, the densely stained protein aggregates were observed to fill the vacuole lumen and appeared to associate with the tonoplast at several sites (Fig. 3.14C-E). The morphology of the late-stage leaf PSVs closely resembles that of PSVs in *Arabidopsis* seed tissues. Electron transparent structures inside the aggregates resemble large globoids. Globoid inclusions in seed PSVs

are typically composed of phytic acid deposits which appear electron dense. However, they are brittle and often shatter from the resin during tissue sectioning, usually leaving behind small deposits along the edge of the globoid (Fig. 3.14F) (Zheng and Staehelin, 2011; Otegui et al., 2002). The globoid-like structures seen in LEC2 induced leaf PSVs did not appear to contain electron dense phytic acid crystals. Therefore, the resemblance of electron transparent structures to globoid inclusions is likely coincidental and may have resulted as an artifact of chemical fixation during tissue preparation (Zheng and Staehelin, 2011; Lonsdale et al., 1999).

4.6 Seed protein accumulation correlates with alterations in cell biology in response to LEC2 over-expression in leaves

Light microscopy of leaf samples collected after LEC2 induction show a progressive alteration of the leaf cell biology over time on DEX (Fig. 3.13). Histochemical staining revealed a noticeable change in the cell and tissue morphology and a drastic increase in starch and oil accumulation whereas the accumulation of protein deposits in leaf cells was less obvious using this microscopy approach. The intensity of the overall staining did not allow good visualization of the blue TBO-stained protein deposits. The change in leaf phenotype toward embryogenic characteristics as illustrated in Fig. 3.13, showed a relationship to the pattern of seed protein accumulation in leaf samples (Fig. 3.12). In Western blots, the accumulation of 12S globulin, oleosin and TIP3;1 proteins was detectable at 11 d on DEX and persisted up until 21 d on DEX. At the same time, TIP1;1 protein was absent in leaves after 11 d on DEX (Fig. 3.12). Stained leaf tissues observed by light microscopy followed a similar pattern. At 11 d on DEX, alterations to the leaf cell biology were obvious. Large LVs were replaced with small vacuoles which correlated with the disappearance of TIP1;1 and the appearance of TIP3;1, respectively. Oil bodies were prominent in the cytoplasm and protein deposits were observed in vacuoles (Fig. 3.13I), which showed a relationship with the appearance of oleosin and 12S globulin proteins in immunoblots, respectively (Fig. 3.12). Taken together, the alteration of cell biology toward seed characteristics illustrated by light microscopy

correlates well with the seed protein accumulation patterns in response to LEC2 over-expression.

4.7 Lytic vacuoles are observed to transition toward protein storage vacuoles in leaves over-expressing LEC2

4.7.1 TIP3;1-YFP appears *de novo* in vegetative tissues

The formation of PSVs in seedlings was observed by following the expression of TIP3;1-YFP in response to activation of LEC2 by DEX. This TIP isoform is exclusively expressed on the tonoplast of PSVs in embryonic tissues (Gattolin et al., 2011). The formation of PSVs was first observed in roots at 10 d on DEX and was visible in cotyledons by 12 d on DEX and then in leaves at 14 d on DEX. It is possible that the timing and pattern of TIP3;1-YFP expression would be different if DEX were applied directly to the plant. In this study, DEX was administered to the plant through the growing medium. As a result, the roots were primarily in contact with the steroid and the aerial organs would have gained exposure to the chemical as it was taken up through the roots by transpiration (Aoyama and Chua, 1997; Schena et al., 1991).

It is interesting that TIP3;1-YFP expression first occurred in single cells or small clusters of cells within the tissues studied but was not observed to be expressed in all cells. This pattern is reminiscent of the uneven LEC2 phenotype observed in leaf tissues (Fig. 3.4B, C). In these tissues, many cells showed an altered phenotype while others appeared unaffected. The TIP3;1-YFP expression pattern might be explained by an uneven distribution DEX within the tissues which could affect LEC2 activity at the cellular level. Alternatively, this induction pattern may be clarified by considering the process of somatic embryogenesis (SE). The induction of SE requires somatic cells to acquire embryogenic competence to undergo SE. Only these competent cells are capable of changing their fate and initiating embryo development (Braybrook and Harada, 2008; Namasivayam, 2007). This involves modification of their existing gene expression patterns with an embryogenic gene expression pattern. Somatic embryos are usually

induced to form by treatment of tissues with auxin or other chemical and physical treatments. An exogenously applied auxin source such as 2,4-dichlorophenoxyacetic acid (2,4-D) may stimulate endogenous indoleacetic acid (IAA) auxin accumulation and lead to embryogenic competence (Fehér, 2006; Fehér et al., 2003; Zimmerman, 1993). 2,4-D has also been demonstrated to stimulate LEC2 activity (Ledwoń and Gaj, 2009) which is involved in activating the expression of endogenous auxin biosynthetic genes (Stone et al., 2008). As demonstrated in this work (Fig. 3.2) and in previous reports (Ledwoń and Gaj, 2009; Stone et al., 2001), ectopic expression of LEC2 promotes somatic embryo formation without the requirement for exogenously applied auxin. Thus, LEC2 creates an environment in somatic cells that make them competent to respond to stimuli and undergo SE (Braybrook and Harada, 2008). However, the means by which LEC2 achieves somatic embryo formation is not well understood. It likely involves the regulation of auxin levels but may also include the regulation of ABA and GA levels (Ledwoń and Gaj, 2009; Braybrook and Harada, 2008; Stone et al., 2008). During SE, a limited number of the total cell population becomes competent in response to an inductive signal such as an exogenously supplied auxin. Only these competent cells proliferate as embryogenic cells and differentiate to form somatic embryos (Namasivayam, 2007). Perhaps the patchy appearance of TIP3;1-YFP fluorescence in single cells or small cell clusters may reflect what is observed during SE where the ability of LEC2 to promote cell competence is initially limited to a small number of responsive cells.

4.7.2 Expression of TIP1;1-RFP is replaced by TIP3;1-YFP in LEC2 induced leaves

The transition between LV to PSVs in leaves was observed by monitoring the expression and co-localization of TIP1;1-RFP and TIP3;1-YFP on leaf cell tonoplasts in response to LEC2 over-expression. Markers clearly demonstrated the PSV to LV transition in germinating seeds. As TIP1;1-RFP replaced TIP3;1-YFP, both markers were observed to co-localize to the same tonoplast for a short time period around 6 d after germination. This is supported by Gattolin et al. (2011) who observed the vacuolar transition of

germinating seeds over a 7 d period using the same TIP constructs. Similarly, Hunter et al. (2007) followed the transition from the PSV to LV during seed germination by over-expressing individual constructs (*TIP1;1:TIP1;1-RFP* and *TIP3;1:TIP3;1-YFP*). However, the timing was slightly skewed; Hunter et al. (2007) observed both the disappearance of TIP3;1-YFP expression and the appearance of TIP1;1-RFP expression ~ 3 d earlier than what was observed for LEC2 leaf LV to PSV transition in this study. This discrepancy could be due to the position of the tissues that were examined. Gattolin et al. (2011) demonstrated that although TIP1;1 is expressed throughout the whole seedling during late germination, as its expression is initiated, it follows a specific accumulation pattern beginning in roots and extending to the cotyledons. Alternatively, the difference in timing of the vacuolar transition may reflect the different systems that were studied. Hunter et al. (2007) studied vacuole biogenesis in germinating seeds while this work examined an experimental system whereby leaves are induced to develop embryogenic characteristics by over-expression of LEC2.

The timing of the LV to PSV transition in leaves over-expressing LEC2 was comparable between fluorescently labeled TIP isoforms observed by confocal microscopy and endogenous TIP isoform protein accumulation detected by Western blots. At 14 d after germination (7 d on DEX), immunoblots and confocal microscopy analysis demonstrated that TIP1;1 accumulated in leaves but TIP3;1 was not detected. By 21 d after germination (14 d on DEX), leaf LVs were replaced by PSVs; TIP1;1-YFP fluorescence was undetected and was replaced by TIP3;1-YFP fluorescence (Fig. 3.16J-L). The same pattern was observed for endogenous TIP protein accumulation (Fig. 3.12). These results demonstrate that over-expressed TIP isoforms follow the same temporal expression patterns as endogenous TIPs. However, to provide a more complete interpretation of the timing of the LV to PSV transition in leaves, observations should be made at additional time points between 14-21 d after germination (7-14 d on DEX). Taken together, the pattern of endogenous and over-expressed TIP1;1 and TIP3;1 protein markers correlates, and demonstrates that leaf LVs transition to PSVs in response to LEC2 over-expression.

4.7.3 The lytic to storage vacuole transition was observed by over-expression of TIP markers but the origin of protein storage vacuoles remains a mystery

Over-expression of both TIP3;1-YFP and TIP1;1-RFP under their native promoters demonstrated that the LV was replaced by a PSV when LEC2 was over-expressed in leaves. However, the fine details of the vacuolar transition were not captured, specifically, the origin of the developing PSV. It is thought that PSVs could form *de novo* or could derive from a reprogramming of the LV (Zouhar and Rojo, 2009; Robinson and Hinz, 1997; Hoh et al., 1995). In tobacco root tip cells undergoing PSV to LV transition, LVs were shown to be derived from PSVs by careful electron microscopical analysis (Zheng and Staehelin, 2011). The origin of PSVs is still under consideration but may involve *de novo* formation of a tubular membrane system that enlarges and engulfs the LV (Hoh et al., 1995).

Western blot results showed that accumulation of both TIP isoforms overlapped at 18 d after germination (11 d on DEX) (Fig. 3.12). Therefore, this point in time represents the vacuole transition point or the precise time at which it would be possible to observe the two vacuole types present in the cell using the TIP markers. Visualization of the transition point in leaf cells was attempted by observing TIP3;1-YFP and TIP1;1-RFP expression while over-expressing LEC2. However, at time points around the transition point, both markers were not detected concurrently by confocal microscopy (Fig. 3.16G-L). Without simultaneous expression of both LV and PSV markers during this transitory period, it is not possible to speculate on the PSV origin. Thus TIP1;1 may not be the most suitable LV marker with which to observe the transition from LV to PSV in leaves over-expressing LEC2. However, as acknowledged above, it is possible that the transition point at which both TIP3;1-YFP and TIP1;1-RFP were expressed was overlooked. Further observations around this transition point are necessary.

4.7.4 Lytic to storage vacuole transition is accompanied by a change in vacuole morphology in LEC2 induced leaves

Not only were the TIP fluorescent markers capable of distinguishing between vacuole types, they also revealed a change in the vacuole morphology in response to LEC2 over-expression. In control LEC2 leaves without DEX, TIP1;1-RFP labeling of the LV tonoplast appeared puzzle-shaped in epidermal cells (Fig. 3.18B). A similar tonoplast morphology was observed in epidermal cells in transgenic 35S:TIP3;1-YFP plants (Fig. 3.17G, H). However, when TIP1;1-RFP fluorescence was replaced by TIP3;1-YFP in LEC2 induced leaf epidermal cells, the tonoplast revealed a novel morphology that more closely resembled seed PSVs. The tonoplast was folded over itself and numerous, bright vacuolar bulbs were present (Fig. 3.17C-F). Bulbs have been characterized as projections of cytoplasm in the vacuole surrounded by tonoplast (Saito et al., 2002). They are observed in a diversity of cell types but their occurrence is generally associated with tonoplast rearrangements during cell expansion in maturing tissues such as cotyledons and young leaves (Beebo et al., 2009; Hunter et al., 2007; Saito et al., 2002). When labeled by fluorescent TIP fusions, bulbs are generally brighter than adjacent tonoplast and it is thought that TIPs are enriched in these tonoplast regions. Bulbs have a complex structure and are classified into five types based on their structural diversity, however their origins are unknown (Beebo et al., 2009; Saito et al., 2002).

Developing leaf PSVs expressed TIP3;1-YFP on the tonoplast as well as the plasma membrane (Fig. 3.17E, F). These results support the findings of Gattolin et al. (2011) who observed the localization of the two *Arabidopsis* TIP3 isoforms (TIP3;1 and TIP3;2) to the PSV tonoplast and plasma membrane during seed maturation and germination. However the TIP3 isoforms were not detectable on the plasma membrane in fully mature, dry seed or when expression levels decline after germination. This dual localization was discovered to be unique to TIP3 isoforms. If TIP1;1-YFP is expressed under control of the TIP3;2 native promoter, it localizes to the tonoplast only (Gattolin et al., 2011).

As leaf LVs transitioned to PSVs in response to LEC2 over-expression, they bore a morphological resemblance to both embryo and leaf vacuoles. Highly fluorescent TIP3;1-YFP-labeled tonoplast folds and bulbs appeared that are characteristic vacuolar morphologies of young tissues (Saito et al., 2002). At the same time, TIP3;1-YFP also revealed a tonoplast configuration characteristic of the LV while NR staining showed that the vacuole lumen fills the entire cell volume. In addition, accumulating SSPs exhibited autofluorescence within the large vacuole lumen. These results suggest that as the LV transitions toward a PSV, the tonoplast remodels before the large vacuole is replaced by smaller sized PSVs.

CONCLUSIONS AND PERSPECTIVES

Over the past decade, a great deal of progress has been made to understand the effects of the LEC2 transcription factor on gene expression (Stone et al., 2008; 2001; Santos-Mendoza et al., 2008; 2005; Baud et al., 2007; Braybrook et al., 2006). This project takes a different approach and focuses on examining the effects of LEC2 at the cellular level. An attempt was made to associate what has been learned about the transcriptional regulation of gene expression by LEC2 to the cellular changes observed in vegetative cells over-expressing the transcription factor. Though many of the observed cellular phenotypes can be explained, some results appear contradictory to what was anticipated from the literature. Perhaps the cellular effects of LEC2 are obscured by its over-expression in vegetative tissues, i.e. LEC2 is introduced into a vegetative regulatory network and factors within this network may interfere or interact with LEC2 action. Alternatively, these conflicting results may not yet have an explanation as the regulatory networks controlling plant development are complicated and there is much unknown.

LEC2 promotes an alteration of the leaf cellular phenotype to resemble embryonic tissues as demonstrated by the morphological changes of cells and tissues, the differentiation of leaf organelles, the appearance of seed organelles and the detection of seed proteins in leaves. The main question of this work was whether PSVs can exist in leaves by over-expression of the LEC2 transcription factor. Leaf PSVs were observed

that appear similar to seed PSVs using light microscopy (Fig. 3.14A, B). However, most of the observations were LVs transitioning toward PSVs in leaves. Under the TEM, late-staged leaf PSVs did not appear to accumulate phytic acid or look as if they possessed the rounded shape characteristic of seed PSVs (Fig. 3.14C-F), but that may be due to the chemical fixation method used in this study. Confocal microscopy revealed the leaf vacuole tonoplast to be extensively remodeled but the LV tonoplast configuration persisted and the large vacuole lumen filled the cell volume. The scarcity of typical PSV formation in leaves over-expressing LEC2 suggests that leaf LVs may not be capable of completely converting to seed PSVs. Perhaps the transition is inhibited because the leaf vacuoles cannot be released from one of their primary roles in vegetative tissues; to maintain cell size and control turgor pressure. This determines the rigidity of the cell to support plant growth and survival (Staehelin and Newcomb, 2000). Therefore, the question arises as to how well LEC2 can promote fully functional or fully formed PSVs in vegetative cells.

Based on the results from this research, a model of vacuole development in seed plants is proposed (Fig. 4.1). The model posits that vacuole development is controlled by a balance between a complex network of regulatory factors that determine cell identity. During embryonic development, an unknown maturation signal acts on regulatory factors to determine the epigenetic state of the cell. The regulatory factors are examples of known seed gene repressors such as BRM (Tang et al., 2008), FIE (Bouyer et al., 2011), VAL1 and VAL2 (Suzuki and McCarty, 2008; Suzuki et al., 2007; Tsukagoshi et al., 2007), HDAC6 and HDAC19 (Tanaka et al., 2008) and PKL (Aichinger et al., 2009; Zhang et al., 2008) (refer to section 1.4.1). The seed gene repressors are thought to act by modifying chromatin structure to cause a repressive chromatin conformation at seed gene promoters (Zhang and Ogas, 2009). In developing seeds, the maturation factor suppresses the action of repressors causing an active chromatin state around the promoters of seed genes thus causing expression of the central regulators of embryonic development (LEC1, LEC2, FUS3 and ABI3). These transcription factors interact with metabolic signaling pathways and plant growth regulator ratios and they upregulate the expression of seed genes and secondary seed transcription factors to determine the seed environment

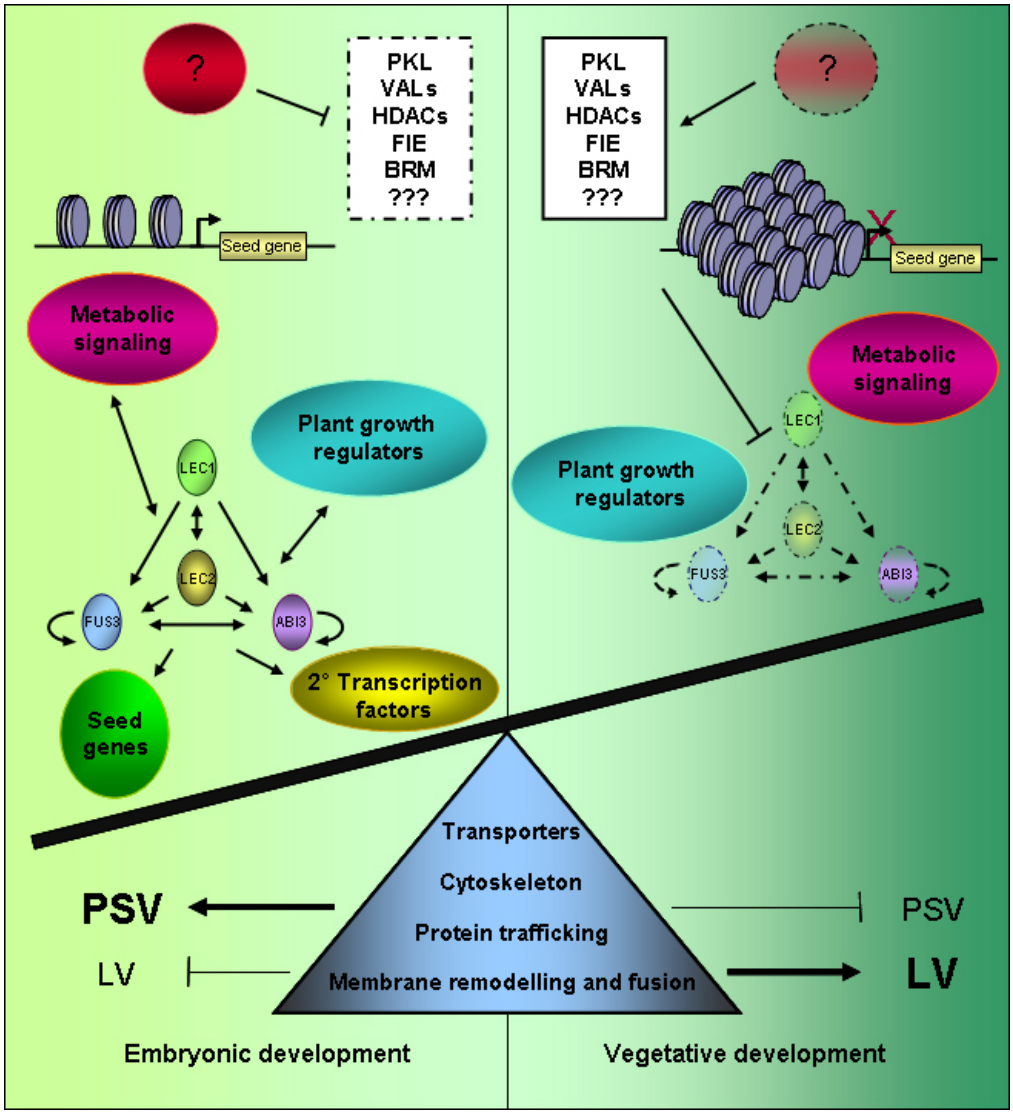

Figure 4.1 Model of vacuole development in seed-bearing plants. Vacuole development is controlled by a balance between a complex network of regulatory factors that determine cell identity. A discussion of the model is presented in the text. Left panel; embryonic development, right panel; vegetative development, red circle; unknown maturation signal, white box; examples of known seed gene repressors, LEC1, LEC2, FUS3 and ABI3; central regulators of embryonic development, PSV; protein storage vacuole, LV; lytic vacuole. Arrows and T bars indicate positive and negative interactions, respectively. Solid lines indicate that interactions are occurring while hatched lines indicate that interactions are not occurring. Solid colored shapes represent active regulatory factors and hatched faded shapes indicate silenced regulatory factors. LEC1; LEAFY COTYLEDON1, FUS3; FUSCA3, ABI3; ABSCISIC ACID INSENSITIVE3, PKL; PICKLE, VALs; VP1/ABI3-LIKE1 and 2, HDACs; HISTONE DEACETYLASE6 and 19, FIE; FERTILIZATION INDEPENDENT ENDOSPERM, BRM; BRAHMA.

(refer to section 1.2.4). In vegetative tissues, the maturation signal is not present thus triggering seed gene repressors to promote a repressive chromatin state which silences the central regulators. At the same time, metabolic signaling pathways and plant growth regulator ratios are modified to promote vegetative development (Finkelstein, 2010; Rolland et al., 2006; Gibson, 2005). Vacuole identity is proposed to be determined by the balance between the regulatory signals. Control by the complex network of regulatory factors is mediated by factors that directly affect vacuole development (Carter et al., 2004). These mediators may include cytoskeleton elements (Oda et al., 2009), transporters (Krebs et al., 2010), protein trafficking elements and factors involved in membrane modelling and fusion (Zouhar and Rojo, 2009; Sanmartin et al., 2007; Sohn et al., 2007). If the balance is tipped toward embryogenic development, the mediators will respond by favoring the formation of PSVs. On the other hand, if the balance is tipped toward vegetative development, the mediators will respond by favoring the formation of LVs. In this research, LEC2 is over-expressed in vegetative tissues, thus tipping the balance toward embryonic development. However, the vegetative network does not allow a complete commitment toward embryonic development therefore vacuoles that have both PSV and LV characteristics are observed.

The LEC2 over-expression system represents a new tool that may be used to study vegetative to embryonic developmental transitions. *Arabidopsis* has been widely adopted as a model plant to study genetics, physiology and molecular genetics (Meyerowitz, 2001). However, *Arabidopsis* seed tissues are inherently difficult to handle and consequently less effort is focused on the analysis of *Arabidopsis* seeds than other species (Ibl and Stoger, 2011; Girke et al., 2000). Despite the convenience of the LEC2 system, it cannot completely eliminate the need to confirm results in seeds, the original biological system.

This work can be further extended to study events that occur during vegetative to embryonic transitions such as organelle biogenesis and differentiation. While vacuoles were the focus of this study, it was not possible to ignore the appearance of numerous oil bodies accumulating in the cell cytoplasm or the alterations experienced by chloroplasts,

the second largest organelle in plant cells. However, several other organelles were overlooked and it would be interesting to investigate their fate. As alluded to in earlier results, the LEC2 over-expression system may be a good approach to study protein trafficking and vesicular transport. During seed development, SSPs are abundantly expressed and transported in bulk through the secretory system to the vacuole for storage. For these reasons, the study of seed proteins is favored (Fujiwara et al., 2002). This work has established that SSPs are synthesized and accumulated in leaf PSVs. Thus, it would be interesting to investigate the trafficking pathways and whether they are modified during the transformation of LVs to PSVs.

Another interesting possibility that could be investigated using the LEC2 over-expression system would be to compare the transcriptome, epigenome and proteome of plants before and after LEC2 induction. This analysis may uncover exciting results that may further our understanding of the regulatory mechanisms governing developmental phase changes and may perhaps lead others to uncover the identity of the unknown maturation factor that is proposed to silence seed gene repressors to allow seed development to occur (Fig. 4.1).

An important application for this work is to exploit the LEC2 system to accumulate recombinant proteins in plants. It is often desirable to accumulate proteins in leaves (Conley et al., 2011). This research indicates that vegetative cells could be converted to protein storage cells upon LEC2 over-expression. To my knowledge, this strategy has not yet been tested for protein accumulation. If the leaf vacuole responds to LEC2 by switching to a storage function but maintaining its large volume, it could have considerable implications for recombinant protein accumulation. Thus, a study comparing protein yield and vacuole changes in leaves in response to LEC2 over-expression would be worthwhile.

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APPENDIX I

Statement of contributions

The following thesis contains material which is contributed by colleagues and some work was accomplished through collaboration:

Transgenic seeds harboring *35S:LEC2-GR* were generously donated by Dr. John Harada (University of California, Davis, CA, USA).

Dr. Lorenzo Frigerio (University of Warwick, Coventry, UK) kindly donated transgenic seeds containing *TIP3;1:TIP3;1-YFP* and *TIP1;1:TIP1;1-RFP*.

Confocal and fluorescence microscopy results (Fig. 3.15-3.18) were obtained through collaboration with Dr. Lorenzo Frigerio. Confocal images of TIP3;1:TIP3;1-YFP seeds (Fig. 3.17A, B and Fig. 3.18A) and 35S:TIP3;1-YFP leaves (Fig. 3.17G, H) were taken by Dr. Lorenzo Frigerio.

Anti-12S globulin and anti-2S albumin antibodies were contributed by Dr. Ikuko Hara-Nishimura (Kyoto University, Kyoto, Japan).

Dr. Alessio Scarafoni (University of Milan, Milan, Italy) generously donated anti-napin antibody.

Dr. John Rogers (Washington State University, Pullman, Washington) kindly shared his anti-alpha-TIP and anti-gamma-TIP antibodies.

The anti-*Arabidopsis* oleosin D9 antibody was kindly provided by Dr. Cory Nykiforuk (SemBioSys Genetics Inc., Calgary, Alberta, Canada).

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