Post-Translational Regulations of FUSCA3
in *Arabidopsis thaliana*

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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2013

Abstract

Seed formation consists of two major stages: embryo pattern formation and maturation. During seed maturation, the embryo accumulates storage material, acquires desiccation tolerance, and enters a stage of dormancy. Genetic analyses have identified several master regulators that orchestrate late embryogenesis, including the B3-domain transcription factor FUSCA3 (FUS3). In Arabidopsis, FUS3 has been shown to be a central regulator of hormonal pathways; it positively regulates late embryogenesis by increasing abscisic acid (ABA) level while repressing gibberellin (GA) synthesis. In turn, FUS3 protein level is positively and negatively regulated by ABA and GA, respectively. However, the mechanism of how this regulation occurs has not been well characterized. In this study, FUS3 has been shown to be an unstable protein rapidly degraded by the proteasome through a PEST instability motif. To further characterize the mechanisms involved in FUS3 homeostasis, FUS3-interacting proteins were identified. The SnRK1 kinase AKIN10 was shown to interact with and phosphorylate FUS3 at its N-terminus. Furthermore, overexpression of AKIN10 delays FUS3 degradation, suggesting AKIN10 positively regulates FUS3 protein accumulation. Overexpression of AKIN10 delays developmental phase transitions, and causes defects in lateral organ development. These defects were partially rescued by the loss-of-function fus3-3 mutation, suggesting FUS3 and AKIN10 genetically interact to regulate these developmental processes. SnRK1/AMPK/Snf1 kinases are
regulators of energetic stress responses. Overexpression studies suggest both FUS3 and AKIN10 positively regulate ABA signaling, but differ in sugar responses during germination; AKIN10 mediates glucose sensitivity, while FUS3 regulates osmotic stress responses. Overexpression of AKIN10 and FUS3 results in glucose and osmotic stress hypersensitivities, respectively, both of which are partially dependent on de novo ABA synthesis. Thus, FUS3 and AKIN10 act in overlapping pathways and combine different environmental signals to generate a common ABA-dependent response. In summary, novel mechanisms that regulate FUS3 homeostasis and function were identified. A model explaining the interaction between FUS3 and AKIN10 during embryonic and vegetative development, and the function of these two central developmental regulators in hormonal and stress signaling pathways is discussed.
Acknowledgments

It’s been almost 20 years since Jurassic Park was released in theatres – that’s how long I’ve known the acronym DNA, long before I learned what it stands for. Funny how a movie buzzword back then is now a routine part of my day job. Looking back, I consider myself lucky that I get to work on something I’m passionate about since childhood. It’s been a long way from a boy’s fascination with dinosaurs to a Ph.D. in molecular biology, and I have the following people to thank for helping me getting this far.

I thank my supervisor Dr. Sonia Gazzarrini, for having faith in me to take on this project, for her scientific inputs and writing critiques throughout the project, and for putting up with the tantrums I throw from time to time. More importantly, I thank Sonia for showing me that being a scientist is more than just about pipetting things and following protocols, and for inspiring me to continue the path of academia.

I thank my supervisory committee members Dr. Dario Bonetta and Dr. Darrell Desveaux, Ph.D. transfer committee member and Ph. D. internal examiner Dr. C. Daniel Riggs, Ph. D. internal examiner Dr. Peter McCourt, and Ph. D. external examiner Dr. Shelley Hepworth, for taking time out of their teaching/research/life in general to provide me with their advices throughout the project.

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I thank the members of Tunes. Beats. Awesome. acapella 2007~2012, for being the best kind of friends a scientist can ask for outside of the lab – the kind that sings well and has fun, and for the incredible musical adventures we shared that helped me keeping life in perspective.
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<tr>
<td>5PTase</td>
<td>myoinositol polyphosphate 5-phosphatase</td>
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<td>ABA</td>
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<tr>
<td>ABF</td>
<td>ABRE-binding factor</td>
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<td>ABI</td>
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<td>YABBY</td>
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<td>YUC</td>
<td>YUCCA</td>
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Contributions

Allen Yi-Lun Tsai’s contribution to Lu et al. (2010) includes the determination and quantification of the FUS3-GST degradation kinetics in a cell-free system (Figure 5a, b), and writing of the manuscript relative to these experiments.

Contribution to Tsai and Gazzarrini (2012a) includes: determination of the physical interaction between FUS3 and AKIN10 (Figure 1); confirmation of FUS3 phosphorylation by AKIN10 and determination of AKIN10 phosphorylation sites on FUS3 (Figure 2); in vitro degradation kinetic of FUS3 (Figure 3); characterization of genetic interactions between FUS3 and AKIN10 in developmental phase transitions (Figures 4, 5) and lateral organ development (Figure 6); co-writing of the manuscript.

Contribution to Tsai and Gazzarrini (2012b) includes: seed germination kinetics of plants overexpressing FUS3 or AKIN10 in MS media, exogenous ABA, fluridone or glucose (Figure 1); seed germination and seedling growth of plants overexpressing FUS3 or AKIN10 in exogenous sugar (Figure 2); characterizing the role of ABA in the germination delay imposed by exogenous glucose or sorbitol (Figure 3); co-writing of the manuscript.
Chapter 1

Introduction
1.1 Plant life cycle and developmental phase transitions

Plant development can be divided into several stages with distinct morphological and physiological profiles, generally known as developmental phases. The timing and regulation of the transition between these phases dictate the overall progression of the plant life cycle. A plant’s life cycle begins with embryonic development, where the zygote from parental gamete fusion forms the embryo within the parental plant; in the case of spermatophytes this results in the formation of seeds. Seed formation is a critical adaptation for the terrestrial colonization of plants. As sessile organisms, plants face several challenges on land compared to animals. Plants must adapt to environmental stresses that cannot be avoided. Furthermore, plants must disperse their offspring to explore new habitats and to minimize competition. Spermatophytes have evolved seed formation to overcome these challenges. The formation of seeds allows plants to temporarily cease growth between generations in adverse conditions (dormancy), thus protecting the developing embryo, and resume growth when the environment becomes favourable. In addition, the dormant nature of the seeds allows them to be spread over great distances by wind, water current, animals or even ballistic forces. In general, the formation of seeds allows plants to overcome spatial and temporal barriers to the propagation of their progeny.

Upon seed germination, the plant enters the vegetative phase of development, and begins to make new organs from the apical meristems. This phase can be further divided into juvenile and adult stages, as plants display different lateral organ morphology with age (reviewed by Poethig, 2003). Under the appropriate conditions, the plant switches to the reproductive phase of development and becomes competent of reproduction; in the case of angiosperms this denotes the initiation of flowering. In addition, reproductive development is often associated with the elongation of stem known as bolting. After one (in annual plants) or multiple (in perennial plants) flowering events, the plant concludes its life cycle by entering senescence and propagates the next generation.

The control of developmental phase transitions is profoundly linked to the plant’s ability to respond to the environment. For example, flowering must be timed to ensure proper development and dispersal of seeds, while in non-self-fertilizing plants flowering must be
synchronized among individuals (reviewed by Srikanth and Schmid, 2011). Different developmental phase transitions respond to different internal and external cues; however, certain factors such as plant hormones, carbohydrate levels and microRNA-mediated gene silencing exert control over multiple developmental phase transitions. For example, the phytohormone gibberellin promotes both germination and flowering, while ethylene promotes flowering and senescence (reviewed by Thomas and Sun, 2004; Lin et al., 2009). Glucose regulates germination, flowering and senescence (reviewed by Gibson, 2005). Lastly, microRNAs down-regulate several negative regulators of vegetative phase change and flowering, thereby promoting these transitions (reviewed by Poethig, 2009). Nonetheless, the precise mechanism of how plants integrate multiple signals to regulate developmental phase transitions, and how different phase transitions are related to one another currently remain elusive.

1.2 Embryo pattern formation

Embryogenesis refers to the process where a single-celled zygote develops into a multi-cellular embryo bearing the basic organismal body plan. Several traits render embryogenesis unique in plants. Firstly, plant embryogenesis is initiated by a process known as double fertilization. Two sperm cells are released from the pollen tube. One sperm cell fuses with the female central cell to form the endosperm, while the other fuses with the egg to form the zygote (Lerstern, 2004). Further, plant cells are considered to be totipotent. Many somatic plant cells have the potential to form an embryo under the appropriate condition, making plant embryogenesis much more plastic as compared to animals (Toonen and de Vries, 1996).

Plant embryogenesis is an intricate, though still poorly understood process. Similar to other plant cellular processes, plant embryogenesis is most extensively characterized in the Brassicaceae model organism Arabidopsis thaliana (Arabidopsis). Although embryogenesis has been described in other plant species, the general framework described in Arabidopsis is well conserved in other angiosperms. In Arabidopsis, embryonic development can be roughly divided into two stages: pattern formation and maturation.

Pattern formation refers to the differentiation of cell fates in initially equivalent cells, which results in an embryo with discernible major organs and tissues from a zygote (reviewed by
Capron et al., 2009). This is primarily achieved by establishing polar axes, such that the embryo becomes an organized structure instead of a nondescript mass of cells. Since plant cells are immotile, pattern formation is primarily achieved by tightly regulated and reproducible cell divisions (reviewed by Capron et al., 2009). This is already evident in the first zygotic division, where a smaller, cytoplasm-dense apical cell and a larger, vacuole-rich basal cell are formed (Figure 1a). The progeny of the apical cell will form the bulk of the embryo proper, while that of the basal cell will contribute to a small portion of the root tip and the suspensor, which connects the embryo to the maternal tissues. After the apical cell undergoes three rounds of divisions, the apical-basal polarity is further elaborated. The 8-cell or the octant-stage embryo has distinct upper- and lower-cell tiers, each will follow different division patterns to adopt different fates (Figure 1b). The upper tier will form the shoot apical meristem (SAM) and the cotyledons, while the lower tier will form the provasculature and the root. After one round of periclinal division, the radial axis is formed in the 16-cell or the dermatogen stage embryo (Figure 1c). The embryo now has distinct outer and inner cell layers, which will be further elaborated into the protoderm, the ground tissue and the provasculature from outside to inside (Figure 1d). Bilateral symmetry is established by the formation of two cotyledon initials, rendering the embryo heart-shaped. Upon the completion of pattern formation, the embryo gains recognizable apical-basal and radial polarity, bilateral symmetry, and the major organs and tissue layers found in the adult plant are formed (Figure 1e, g).
Figure 1. Arabidopsis embryogenesis and seedling structure.
Schematic diagram depicting the progression the Arabidopsis embryo from the zygote to the heart-stage embryo and the seedling. A: apical and basal cells, B: octant stage, u.t.: upper tier (descendents marked with green), l.t. lower tier (descendents marked with brown), C: dermatogen stage, D: globular stage, E: transition stage, F: heart stage, G: seedling. Lightly shaded regions refer to the epidermis (except in G), dark regions refer to the ground tissues. Blue and red mark the shoot and root apical meristems, respectively. Image reprinted from Capron et al. (2009) with permission, copyrighted by the American Society of Plant Biologists.
1.2.1 Auxin

One of the main regulators of pattern formation is the phytohormone auxin. Phytohormones, or plant hormones, are chemicals that specifically affect plant growth at low concentrations. Auxin, named after the Greek word αὐξεῖν meaning ‘to grow’, is the first of these chemicals described. The activity of auxin was first reported by Francis and Charles Darwin in grass coleoptile phototropism, where they concluded a signal must have been sent to cause the bending of the coleoptile in response to light (Darwin and Darwin, 1887). By 1926, Frits Went documented the first isolation of auxin. In general, auxin controls cell division and elongation, and therefore affects tropic growth response; it also regulates vascular development, lateral organ initiation and root growth. Genetic analysis has highlighted the role of auxin in establishing embryo apical-basal polarity since mutations that affect auxin signaling, synthesis or transport resulted in embryos lacking cotyledons, the hypocotyl or the root (Berleth and Jürgen, 1993; Friml et al., 2003; Cheng et al., 2007; Stepanova et al., 2008).

1.2.2 Auxin synthesis, transport and signal transduction

As the first phytohormone described, the synthesis, transport and perception of auxin have been extensively characterized. Auxin is an indole-derived compound, with indole acetic acid (IAA) being the most common natural form. Auxin can be synthesized either from tryptophan by tryptophan aminotransferase (Stepanova et al., 2008), or directly from indole by the YUCCA (YUC) monooxygenases, which consists of 11 predicted proteins in Arabidopsis (Zhao et al. 2001). Genetically reducing auxin synthesis during embryogenesis disrupts the formation of hypocotyl and the root meristem, which often results in only one cotyledon forming with no venation (Cheng et al., 2007). Similarly, genetically reducing auxin synthesis during vegetative development reduces plant stature, causes curly rosette leaves and defective floral organs, both lacking venations (Cheng et al., 2006). These phenotypes indicate auxin synthesis is required for pattern formation, organogenesis and vasculature formation.

The sites of auxin synthesis are not necessarily the same sites where auxin action takes place (Woodward and Bartel, 2005). Therefore, the transport of auxin becomes crucial in the regulation of auxin activity. Auxin transport is polar: auxin is transported into cells via the LIKE-AUX1 (LAX) family of influx transporters (Petrasek and Friml, 2009), and out of cells via the PINFORMED (PIN) family of efflux transporters (Galweiler et al., 1998; Friml et al.,
Hence, the direction of auxin flow is dictated by the localization of these transporters. The polarity of auxin transport at the single-cell level in turn defines the polarity of tissues and organs by a mechanism known as the canalization hypothesis (Sachs, 1981; Sachs, 1991). Genetic or chemical disruption of polar auxin transport, such as mutations in the PIN genes or treatment with 1-N-naphthylphthalamic acid (NPA) leads to defects in root establishment and cotyledon development (reviewed by Chandler, 2008).

Auxin molecules are perceived by a family of cognate receptors, including TRANSPORT INHIBITOR RESPONSE1 (TIR1), and mediate TIR1 binding to the auxin/indole-acetic acid (AUX/IAA) family of transcriptional repressors (Figure 2) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Calderón Villalobos et al., 2012). The auxin molecule physically occupies the hydrophobic regions between TIR1 and AUX/IAA, acting as a “molecular glue” to mediate the binding of TIR1 to AUX/IAA (Figure 2) (Tan et al., 2007). TIR1 and other auxin receptors are S-phase Kinase-associated Protein1 (SKP1)-Cullin (CUL)-F-box (SCF)-type E3 ubiquitin ligases, and they facilitate the ubiquitination and degradation of AUX/IAA (Figure 2). The degradation of AUX/IAA de-represses several AUXIN RESPONSE FACTORS (ARFs) which triggers transcriptional responses (Figure 2) (Chapman and Estelle, 2009). Mutations that cause auxin insensitivities, such as monopteros (mp), bodenlos (bdl) and auxin resistant6 (axr6), were originally identified as embryo pattern formation mutants. Indeed, embryos of these mutants often lack a cotyledon, the hypocotyl or the root (Berleth and Jurgen, 1993). These mutants certainly map to genes that mediate auxin signaling (Berleth and Jurgen, 1993); MP encodes an ARF (Hardtke and Berleth, 1998), BDL encodes an AUX/IAA protein (Hamann et al., 1999), and AXR6 encodes the cullin subunit of the SCF E3 ligase complex (Hobbie et al., 2000). Altogether, these findings point to a role of auxin in pattern formation, embryo polarity regulation, and the establishment of lateral organs and vasculature.
**Figure 2. Molecular model of auxin perception**

Schematic diagram depicting the perception and signal transduction of auxin. At basal auxin concentrations, the AUX/IAA transcriptional repressor binds the transcription factor ARF and prevents the transcription of auxin-responsive gene. At elevated concentrations of auxin, the auxin molecule mediates the binding of the F-box protein TIR1 with AUX/IAA. TIR1 poly-ubiquinates AUX/IAA, which leads to its degradation by the proteasome. The degradation of AUX/IAA de-represses the ARF, leading to the expression of auxin-responsive gene.
Basal auxin level

Elevated auxin level
1.2.3 Cotyledon development

Cotyledons are considered to be embryonic leaves, as they are the first aerial organs to appear in plants and are formed during embryogenesis. The number of cotyledons distinguishes monocotyledonae and eudicotyledonae, the two major clades of angiosperms, though exceptions do occur in each clade. Functionally, monocotyledons are storage organs that remain below ground and are incapable of photosynthesis. Cotyledons of eudicots, in contrast, are usually above ground and are capable of photosynthesis. The emergence of the cotyledons in dicotyledonous plants marks a significant milestone, as the embryo radial symmetry is replaced by bilateral symmetry. The two cotyledons are typically 180° apart, and their emergence is sequential. The initiation of the first cotyledon imposes lateral inhibition to the second cotyledon, limiting its position at the opposite end (Woodrick et al., 2000).

Several pieces of evidence indicate auxin promotes cotyledon initiation. Indeed, the application of auxin on the embryo can artificially stimulate cotyledon growth (Reinhardt et al., 2000). In the heart stage embryo, auxin has been detected in the cotyledon initials by immunolocalization (Benkova et al., 2003). In agreement with this, the activity of the D1-4 auxin response element 5' of Gretchen Hagen 3 (GH3) (DR5, an auxin maxima reporter) and expression of YUC genes were similarly detected in cotyledon initials (Ulmasov et al., 1997; Cheng et al., 2006; Chandler, 2008). Together, this indicates auxin synthesis and perception indeed play critical roles in cotyledon initiation. The PIN1 transporters in the protoderm move auxin upward to the sites of cotyledon emergence, thereby marking the cotyledon initials, and subsequently move auxin away through the vasculature (Benkova et al., 2003). Auxin de-represses the ARF proteins, MP and NON-PHOTOTROPIC HYPOCOTYL 4 (NPH4), which promote the expression of DORNRÖSCHE (DRN) and DRN-LIKE (DRNL) transcription factors to initiate the cotyledon development program (Chandler et al., 2007; Cole et al., 2009). Consequently, mutations in auxin synthesis, perception and transport all cause abnormal numbers of cotyledons or fused cotyledons (reviewed by Chandler et al., 2008).

In contrast to auxin, negative regulatory mechanisms are exerted to ensure cotyledons are not initiated in inappropriate locations. In Arabidopsis, the main regulators of this inhibition are the CUP-SHAPE COTYLEDON (CUC) transcription factors from the NO APICAL MERISTEM (NAM), ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF), CUC2 (NAC)
domain protein family. Arabidopsis has three CUC gene paralogues (CUC1, CUC2 and CUC3). The cuc1 cuc2 cuc3 triple mutant displays a single cup-shaped cotyledon, presumably by fusing the two existing cotyledons (Aida et al. 1997). CUC genes are expressed in regions between the cotyledon initials and suppress cell division and differentiation, thereby inhibiting cotyledon initiation (Aida et al., 1999). In the mp and pin1 pinoid (pid) mutants, the expression patterns of the CUC genes are altered, resulting in abnormal cotyledon initiation (Aida et al., 2002; Furutani et al., 2004). This suggests auxin regulates the expression of the CUC genes, and supports the model where the CUC genes antagonize the role of auxin in the initiation of cotyledon development, thereby regulating the number and boundary of the cotyledons.

The origin of the cotyledons in dicots remains controversial, as it is unclear whether these organs are derived from the SAM. A major phenotype of the cuc mutants is the lack of SAM, suggesting the definition of cotyledon boundary is required for SAM establishment (Aida et al., 1999). Indeed, the CUC genes promote the expression of SHOOT MERISTEMLESS (STM), a positive regulator of SAM identity. STM in turn negatively regulates the CUC genes, thus limiting the size of SAM itself (Aida et al., 1999). Specifically, the establishment of the SAM is linked to the adaxial-abaxial polarity of the cotyledon. Class III homeodomain-leucine-zipper (HD-ZIP) family transcription factors PHAVOLUTA (PHV), PHABULOSA (PHB) and REVOLUTA (REV) are expressed in the adaxial side of the cotyledon primordia (McConnell et al., 2001; Prigge et al., 2005). Aside from the loss of leaf adaxial identity, the phv phb rev triple mutant is also missing the SAM, suggesting adaxial identity is required for SAM establishment (Emery et al., 2003). Overexpression of abaxial cell fate regulators FILAMENTOUS FLOWER (FIL), the YABBY (YAB) and KANADI (KAN) families similarly abolishes the SAM by eliminating adaxial cell fate programs (Seigfried et al., 1999; Eshed et al., 2001). Remarkably, the phv phb rev triple mutant also demonstrates the lack of one or both cotyledons, suggesting the formation of the SAM positively regulates cotyledon initiation (Prigge et al., 2005). Thus, genetic analysis strongly suggests that cotyledon and SAM development are tightly linked.

Cotyledons are structurally homologous to leaves, and they also share part of their developmental program (reviewed by Chandler, 2008). Loss-of-function mutations in the FUSCA3 (FUS3), LEAFY COTYLEDON 1 (LEC1), LEC2, HYDRA1 (HYD1) or HYD2 genes can partially convert cotyledons into vegetative leaves (Keith et al., 1994; Meinke et al., 1994;
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Topping et al., 1997; Jang et al., 2000). Conversely, loss-of-function mutations in the EXTRA COTYLEDON 1 (XCT1), XCT2, or ALTERED MERISTEM PROGRAM1 (AMP1) genes partially convert vegetative leaves into cotyledons (Conway and Poethig, 1997; Chaudhury et al., 1993). In addition, FIL, YAB and KAN are regulators of the adaxial-abaxial polarity in leaves and cotyledons (Sawa et al., 1999; Siegfried et al., 1999; McConnell et al., 2001; Eshed et al., 2004; Prigge et al., 2005). These examples suggest that the lateral organ development program is conserved between cotyledons and vegetative leaves.

1.3 Seed maturation and dormancy

During the second half of plant embryogenesis, seed maturation begins in preparation for seed dormancy. The hormone abscisic acid (ABA) plays a major role in the regulation of late embryonic functions. Upon entering the maturation stage, the embryo ceases mitotic activity and continues growing by cell expansion filling up the entire seed. The cells enlarge by accumulating carbohydrates, lipids, and seed storage proteins (SSP), which will serve as nutrients for the seedling upon germination. The embryo also accumulates late embryogenesis abundant (LEA) proteins, dehydrins, and osmocompatible solutes, to protect the embryo from desiccation (Ingram and Bartel, 2003; Verslues et al., 2006). The embryo subsequently loses up to 90% of its water content, acquires desiccation tolerance and enters a period of dormancy, during which it remains metabolically inactive. Seed germination resumes upon external and internal cues. External germination cues include light, water and a pre-chilling period. Internal cues for germination are largely orchestrated by a number of hormones, including gibberellin (GA) and ABA; the former promotes, while the latter inhibits germination. It is the ratio between these two hormones that triggers the break of dormancy and the onset of germination. Failure to establish dormancy can have dire consequences, as the seed may germinate at inappropriate times, exposing the seedling to hostile environments. The extent of seed dormancy is truly remarkable, as some seeds remain viable even after being dormant for decades.

1.3.1 ABA

The hormone ABA was discovered in several instances independently: as abscisin II from cotton, promoting abscission; as dormin from sycamore, promoting dormancy and as β-inhibitor
from *Aegopodium* tuber, inhibiting growth (Bennet-Clark et al., 1953; Ohkuma et al., 1963; Cornforth et al., 1965). All three chemicals were later confirmed to be identical and were renamed ABA after their abscission-promoting activity. Currently, the knowledge regarding ABA function is no longer limited to abscission. Rather, ABA is best known for its role in stomata closure in response to abiotic stresses, particularly against drought, salt and osmotic stresses (reviewed by Qin et al., 2011). ABA also plays a prominent role in seed maturation and dormancy establishment (reviewed by Finkelstein et al., 2008). Recently, ABA has also been shown to be involved in mediating biotic stress responses (Ton et al., 2009).

### 1.3.2 ABA signal transduction

Genetic, molecular and chemical genomic studies have led to the identification of various components of the ABA signaling pathway. The mechanism of ABA perception remained elusive until recently, due to the lack of identification of an ABA receptor. Although several ABA-binding proteins had been identified in the past, their merits as physiologically-relevant ABA receptors remain debatable (reviewed by Cutler et al., 2010). The current model of ABA signaling places the PYRABACTIN RESISTANT (PYR)/PYR-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTOR (RCAR) family as the ABA receptors (Figure 3) (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). Chemical genomic screens were conducted with pyrabactin, an ABA agonist that mimics ABA specifically during germination, and *pyr1* was identified to be insensitive to pyrabacine (Park et al., 2009). PYR1 was shown to bind and inhibit group A protein phosphatases 2C (PP2Cs) in the presence of both pyrabactin and ABA (Figure 3) (Park et al., 2009). Ma et al. (2009) took a different approach and identified PYR/PYL, designated RCAR, as interactors of group A PP2Cs. The PYR/PYL/RCAR proteins contain the steroidogenic acute regulator-related lipid transfer (START) domain (Iyer et al., 2001; Radauer et al., 2008). As shown by various crystallography data, the helix-grip motif of the START domain binds ABA, and PP2C binding prevents dissociation of the ABA molecule from PYR/PYL/RCAR (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al. 2009).

The ABA signal transduction cascade downstream of PYR/PYL/RCAR receptors consists of a series of phosphorelay by the Sucrose non-fermenting 1 (Snf1)-related kinases 2 (SnRK2) and PP2C phosphatases (Figure 3). Class A PP2Cs are Mg$^{2+}$/Mn$^{2+}$-dependent monomeric
phosphatases and at least six of them negatively regulate ABA signaling: ABSCISIC ACID INSENSITIVE 1 (ABI1), ABI2, HOMOLOGY TO ABI 1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION 1 (AHG1), and AHG2/AtPP2CA (Leung et al., 1997; Saez et al., 2004; Yoshida, T. et al., 2006; Nishimura et al., 2007). PYR/PYL/RCARs bind to and inhibit PP2Cs, and this interaction is enhanced by ABA. However ABA may not be required for all permutations of PYR/PYL/RCAR-PP2C interactions (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). Several substrates of PP2Cs have been characterized, but the ones most intimately involved in ABA signaling are the SnRK2s (Figure 3). Three members of the SnRK2 subfamily play critical roles in ABA signaling: SnRK2.2, SnRK2.3 and SnRK2.6 (Yoshida R. et al., 2006; Park et al., 2009). These three kinases belong to the subclass III SnRK2 and are all upregulated by ABA (Kobayashi et al., 2004). Knocking out all three kinases nearly abolishes ABA perception, suggesting together these three kinases account for the bulk of ABA signal transduction (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009).

The ABA signaling cascade results in gene regulation mediated by transcription factors. A wide range of transcription factors have been documented to respond to ABA, though the best characterized ones include the basic leucine zipper (bZIP) transcription factors ABI5 and ABA responsive element (ABRE) binding protein (AREBP). SnRK2 kinases phosphorylate and activate these transcription factors, thereby relaying the ABA signal (Figure 3) (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007). The bZIP transcription factors target the ABRE featuring the conserved ACCTGGC motif (Kaplan et al., 2006). Aside from transcription factors, SnRK2 kinases mediate ABA signaling in guard cells by phosphorylating other substrates, such as the anion channel SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1), as a mean to regulate stomata closure by altering cell osmolarity (Geiger et al., 2009).

A simple ABA core signaling network can be constructed from the findings to date (Figure 3). In the absence of ABA, PP2C dephosphorylates SnRK2 kinases and AREBP/ABI5 proteins remain inactive. In the presence of ABA, PYR/PYL/RCAR proteins bind and inactivate PP2Cs. This allows the SnRK2 kinases to be activated, thereby phosphorylating and activating AREBP/ABI5 proteins, which in turn initiate transcriptional responses. Despite the understandings of the ABA core signaling network, the functions of many other ABA signaling components remain to be delineated (reviewed by Cutler et al., 2010).
Figure 3. Molecular model of ABA perception

Schematic diagram depicting the perception and signal transduction of ABA. At basal ABA concentrations, PP2C de-phosphorylates and inactivates SnRK2. Without SnRK2, AREBP remains inactivated thus ABA-responsive genes are repressed. At elevated ABA concentrations, PYR/PYL/RCAR binds the ABA molecule. ABA-binding increases the affinity of PYR/PYL/RCAR to bind and inactivate PP2C. In the absence of PP2C, SnRK2 can be phosphorylated and activated, which in turn phosphorylates and activates AREBP. Active AREBP then mediates the transcription of ABA-responsive gene.
Basal ABA level

Elevated ABA level
1.3.3 Sugar and ABA crosstalk

Sugars molecules are fundamental for all organisms, as they provide a source of energy for cells. Beside their role as nutrient sources, sugars can also act as signaling molecules. In general, low sugar promotes source activities such as photosynthesis, mobilization and export of stored nutrients, whereas sink activities such as growth and nutrient storage are repressed. In contrast, high sugar promotes sink activities while inhibiting source activities (reviewed by Rolland et al., 2006; Rook et al., 2006; Hanson and Smeekens, 2009). However, plants show different sugar responses throughout development, as they have different nutritional requirements at different stages of development (reviewed by Gibson, 2005).

Sucrose is the main product of photosynthesis, and is the most common form of sugar transported through plants. The mechanism of sucrose sensing currently remains obscure, and glucose metabolic intermediates such as trehalose and trehalose-6-phosphate (T6P) may be the true signaling molecules (Schluepmann et al., 2003; Hanson and Smeekens, 2009). Sucrose can be hydrolyzed into fructose and glucose by sucrose invertases, therefore sucrose feeding inevitably leads to glucose signaling to some extent. Nonetheless, some of the sucrose responses such as bZIP11 translation inhibition and transcriptional regulation of various sucrose-inducible genes cannot be replicated with glucose, suggesting the existence of sucrose-specific signaling pathways that are distinct from glucose (Wiese et al., 2004; Rolland et al., 2006). The main glucose receptor is HEXOKINASE1 (HXK1) (Rolland et al., 2006). HXK1 catalyzes the first step of glycolysis by phosphorylating glucose, but plants expressing a catalytically inactive hxx1 mutant protein can still rescue the glucose signaling defect in the gin2/hxx1 mutant, suggesting the enzymatic and signaling activities of HXK1 are independent (Moore et al., 2003).

Exogenous sugar inhibits seed germination, cotyledon expansion, development of roots and true leaves (Jang et al., 1997; Nemeth et al., 1998; Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000), chloroplast development (To et al., 2003), and mobilization of seed storage lipids (Martin et al., 2002; To et al., 2002). In order to identify sugar signaling components, mutants insensitive to exogenous sugar during germination were identified. These mutants include the glucose-insensitive (gin), which tolerate 6% glucose, and the sugar-insensitive (sis) mutants, which tolerate 300 mM (~10%) sucrose (Zhou et al., 1998; Laby et al., 2000). Other sugar signaling mutants were screened based on the transcriptional responses of sugar-inducible
genes. These include the *sucrose-uncoupled* (*sun*) mutants, where the expression of sugar-repressible genes failed to be repressed by sugars, and the *impaired sucrose induction* (*isi*) mutants, where the expression of sugar-inducible genes fails to be induced by sugars (Dijkwel et al., 1997; Rook et al., 2001). Surprisingly, many mutations identified in these screens were mapped to ABA synthesis or signaling genes. The *gin6*, *sun6*, *sis5* and *isi3* mutations are allelic to *abi4*, defective in an APETALA2 (AP2)-type transcription factor required for ABA signaling but independent of the ABA core signaling network (Finkelstein et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al. 2001). *ABI4* also regulates trehalose response, chloroplast retrograde signaling, and its own expression in a glucose-dependent manner (Koussevitzky et al., 2007; Ramon et al., 2007; Bossi et al., 2009). The *sis4*, *gin1* and *isi4* mutations are allelic to *aba2*, while the *gin5* mutation is allelic to *aba3* (Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002). *ABA2* and *ABA3* encode a short chain dehydrogenase/reductase and a molybdenum cofactor sulfurase, respectively; both are enzymes involved in ABA synthesis (Schwartz et al., 1997).

Other genetic evidence also strengthen the link between ABA and sugar signaling. Several ABA synthesis or signaling mutants not identified in the sugar signaling screens also demonstrate sugar signaling defects. For example, loss-of-function mutations in *ABI8*, *ABI5* and *ABA1* cause glucose insensitivity (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Arroyo et al., 2003; Brocard-Gifford et al., 2004). *ABA1* encodes a zeaxanthin epoxidase required for ABA synthesis; *ABI5* encodes a bZIP factor required for ABA signaling (Finkelstein and Lynch, 2000; Xiong et al., 2002), while the molecular function of *ABI8* is still uncharacterized (Brocard-Gifford et al., 2004; Kong et al., 2012). In addition, ABRE-binding factor 2 (*ABF2*), a bZIP factor mediating ABA responses, was also shown to mediate glucose responses, as the loss-of-function mutant is glucose-insensitive while overexpressing *ABF2* leads to glucose hypersensitivity (Kim et al., 2004). Similarly, overexpressing *ABF3* and *ABF4* also leads to sugar hypersensitivity, suggesting multiple transcription factors mediating ABA responses regulate glucose responses (Kang et al., 2002).

All these studies demonstrate that reduced ABA level or signaling results in insensitivity to high sugar during germination. Interestingly, sugars exert control over ABA level and response. Indeed, exogenous sugar supply increases ABA level while triggering the expression of *ABI3*,

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**Additional Information:**

- *sucrose-uncoupled* (*sun*) mutants
- *impaired sucrose induction* (*isi*) mutants
- *abi4*, APETALA2 (AP2)-type transcription factor
- *ABA2* and *ABA3* enzymes
- *ABI8*, *ABI5*, *ABA1* genes
- *ABF2*, *ABF3*, *ABF4* transcription factors
ABI5 and LEA genes, reminiscent of an ABA response (Cheng et al., 2002; Dekker et al., 2008; Bossi et al., 2009). Furthermore, expression of the ABA biosynthesis regulator FUS3 is also upregulated by sugar, although in a sugar-sensitized background where repressors of sugar-inducible genes HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE (HSI2) [VIVIPAROUS1/ABI3-LIKE (VAL1)] and HSI-LIKE1 (HSL1) (VAL2) are mutated (Tsukagoshi et al., 2005; Tsukagoshi et al., 2007). In summary, these studies indicate ABA and sugar likely act in overlapping pathways by mutually promoting the signaling of each other.

Despite the prominent link between the ABA and sugar signaling pathways, ABA synthesis/signaling defects don’t entirely correlate to sugar signaling defects. Unlike ABI4 and ABI5, the roles of ABI1, ABI2 and ABI3 in sugar signaling are not as clearly defined, despite all five genes being involved in ABA signaling. This can be attributed in part to the age/dormancy of the seeds, the ecotype and the allele used in these studies (Laby et al., 2000; Arroyo et al., 2003; Arenas-Huertero et al., 2000; Huijser et al., 2000; Dekker et al., 2008; Nambara et al., 2002). Nevertheless, these findings show that different abi3 alleles show different degree of sugar insensitivity and imply ABI3 may indeed interact with sugar signaling, at least to establish the glucose-mediated ABA resistance (Nambara et al., 2002; Dekker et al., 2008). The fact that not all ABA synthesis and signaling mutants show altered sugar sensitivity suggests the ABA and sugar signaling pathways only partially overlap, and each pathway contains distinct signaling modules.

Not all sugar signaling mutant alleles map to ABA synthesis or signaling genes. The gin4 and sisl mutant alleles mapped to constitutive triple response1 (ctr1), a negative regulator of ethylene signaling (Kieber et al., 1993; Gibson et al., 2001; Cheng et al., 2002). In agreement with this, loss-of-function mutants of ethylene synthesis and signaling genes are hypersensitive to sugar (reviewed by Gibson, 2005; Rolland et al., 2006). This suggests ethylene negatively regulates sugar signaling during germination. However, since ethylene also counteracts ABA signaling, the antagonism between ethylene and sugar may be mediated by ABA (reviewed by McCourt and Gazzarrini, 2001; McCourt and Gazzarrini, 2003; Rolland et al., 2006; Rook et al., 2006).
Even with the wealth of knowledge about the ABA-sugar crosstalk, many aspects about this interaction remain obscure. In contrast to the hypothesis that ABA and sugar act in overlapping pathways, sugar antagonizes the germination and growth repression induced by ABA (Garcia-Rubio et al., 1997; Finkelstein and Lynch, 2000; Price et al., 2003; Dekkers et al., 2004). Thus, ABA may facilitate sugar signaling only under certain conditions or developmental stages. ABA-sugar crosstalk also shows variation between different ecotypes. ABI4 and sugar was shown to induce the expression of *DELAY OF GERMINATION 1 (DOG1)*, a major locus in dormancy control, though this induction only occurs in the Cape Verde Islands (Cvi) ecotype (Teng et al., 2008). On the other hand, the Cvi *DOG1* allele promotes the sugar induction of *ABI4* (Teng et al., 2008). These findings suggest ABA-sugar crosstalk remains a very complicated and context-dependent interaction.

### 1.4 The *ABI3*, *FUS3* and *LEC* genes

Several lines of evidence suggest the requirement but not sufficiency of ABA to regulate seed maturation and dormancy. Tobacco (*Nicotiana tabacum*) seeds with ABA depleted by antibody accumulate fewer seed storage proteins and lipids, germinate precociously and are desiccation intolerant (Phillips et al., 1997). In maize (*Zea mays*), mutations that lead to vivipary, or premature germination of kernels while still attached to the mother plant, have been mapped to loci named *VIVIPAROUS (VP)* (Neill et al., 1986; McCarty, 1995). *VP* genes can be divided into three classes: carotenoid synthesis enzymes, which synthesize the ABA precursor, ABA synthesis enzymes, and *VP1*, which encodes a B3-domain transcription factor (McCarty et al., 1989; Singh et al., 2003; Porch et al., 2006; Suzuki et al., 2006). Mutations in genes of the first two classes lead to decreased ABA level in seeds, whereas the *vp1* mutant does not show ABA level defect, but is insensitive to ABA. The *vp* mutants highlight the necessity of ABA synthesis and perception in the establishment of seed dormancy. In Arabidopsis, seeds of the ABA biosynthetic mutants, *aba2* and *aba3*, are also less dormant, while those overexpressing *ABA2* increase dormancy (Léon-Kloosterman et al. 1996; Lin et al., 2007). However, no defects in desiccation tolerance or accumulation of seed storage compounds have been reported in these mutants. The *snrk2.2 snrk2.3 snrk2.6* triple mutant seeds are also less dormant (Fujii et al., 2007; Nakashima et al., 2009), however, they do show some desiccation intolerance (Nakashima et al., 2009). This appears to be a consequence of water loss due to ABA insensitivity rather
than defects in seed storage compound accumulation. Thus, analyses of ABA auxotrophic and signaling mutants conducted in various species suggest ABA is required, but may not be sufficient to trigger seed maturation and dormancy.

In order to genetically dissect seed maturation and dormancy, new mutants associated with maturation defects were identified in Arabidopsis and included *abi3*, *lec1*, *lec2*, and *fus3* (Giraudat et al., 1992; Meinke, 1992; Baumlein et al., 1994; Keith et al., 1994; West et al., 1994). Recessive mutations in *abi3* were originally identified in screens aiming to identify mutants insensitive to ABA during germination (Koornneef et al., 1984). The *LEC* genes and *FUS3* were originally identified as regulators of cotyledon identity, as recessive mutations in these genes partially convert the cotyledons into leaves, hence, the name *LEAFY COTYLEDONS* (Meinke, 1992). The name FUSCA is derived from Greek meaning ‘to darken’, and was designated to mutant loci with dark seeds (Muller, 1963; Muller and Heidecker, 1968). The dark color of the seed is due to the accumulation of anthocyanins in the embryo. Recessive mutations in *ABI3*, *FUS3* and *LEC* genes (AFL hereafter) cause the most dramatic defects in seed maturation, therefore these genes are collectively known as the master regulators of late embryogenesis and seed maturation (Parcy et al., 1997; Holdsworth et al., 1999; Vicent et al., 2000; Harada, 2001).

The AFL genes share overlapping but not identical mutant phenotypes and expression patterns. Recessive mutants in these genes show reduced dormancy and precocious germination of immature (green) seeds. These mutants also fail to accumulate seed storage and osmoprotective compounds and, as a consequence, the seeds are desiccation intolerant with the exception of *lec2* (Giradaut et al., 1992; Keith et al., 1994; Meinke et al., 1994; Stone et al., 2001). This suggests all four genes act to initiate and maintain seed maturation, and some are required for the establishment of desiccation tolerance. There are also subtle differences between the mutant phenotypes that set these genes apart. *abi3* is the only mutant showing ABA insensitivity and defects in chlorophyll degradation (Giraudat et al., 1992). The *fus3* and *lec* mutations cause partial conversion of cotyledons into vegetative leaves, due to the appearance of trichomes and the partial leaf ultrastructure (Meinke, 1992; Keith et al., 1994). The *fus3* and *lec* embryos also accumulate anthocyanins, therefore the seeds appear dark red (Meinke, 1992; Keith et al., 1994). The phenotypes of *fus3* and *abi3* are considered to be due to heterochronic shifts, as the SAM and
the root meristem have been initiated prematurely in the embryo, with leaf primordia and root already developed before germination (Keith et al., 1994; Nambara et al., 1995). In contrast, the \textit{lec} mutant phenotypes are considered to be homeotic, as no premature SAM development phenotypes were found (Meinke, 1992).

Analyses of AFL gain-of-function mutants also suggest these genes have overlapping but non-identical roles in the seed maturation. Ectopic expression of any AFL gene during vegetative development causes plants to ectopically express seed storage compounds (Parcy et al., 1994; Lotan et al., 1998; Stone et al., 2001; Gazzarrini et al. 2004; Kagaya et al., 2005a/b). Ectopic \textit{FUS3} or \textit{LEC1} expression partially converts leaves into cotyledons, and both \textit{LEC} genes cause somatic embryo to develop from vegetative tissues (Lotan et al., 1998; Gazzarrini et al., 2004; Stone et al., 2001). Furthermore, plants ectopically expressing \textit{FUS3} show delayed germination, vegetative and reproductive phase transitions through regulation of cell cycling; they also increase ABA sensitivity during germination (Gazzarrini et al., 2004). In contrast, ectopically expressing \textit{ABI3} did not significantly alter post-embryonic development, but showed increased ABA sensitivity (Parcy et al., 1994; Tamminen et al., 2001; Zhang et al., 2005). In general, the analyses of AFL mutants indicate the AFL genes are positive regulators of embryogenesis, cotyledon identity and seed storage protein expression, although each gene still retain unique functions.

1.4.1 Structure, DNA-binding activity and targets of the AFL genes

All four AFL genes have been shown to act as transcriptional factors, and their DNA-binding mechanisms have been well characterized. \textit{LEC2}, \textit{ABI3} and \textit{FUS3} belong to the B3-domain transcription factor family, which are only found in plants (Figure 4) (McCarty et al., 1991; Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). The conservation of B3 DNA-binding domain in part explains why these regulators have overlapping functions, as they are likely to share downstream targets. There are currently 87 known B3 domain transcription factors in Arabidopsis, divided into five families: ARF, ABI3/VP1 (which include the AFL genes), RELATED TO ABI3/VP1 (RAV), HSI/VAL and REPRODUCTIVE MERISTEM (REM) (Figure 4) (Romanel et al., 2009). The B3 DNA-binding domain of the ABI3, FUS3 and LEC2 was shown to bind to the alternating pyrimadines and purines (RY) cis-regulatory motif
CATGCA (Ezcurra et al., 2000; Mönke et al., 2004; Yamasaki et al., 2004), found in the promoters of various seed storage proteins (Stalberg et al., 1993; Conceição Ada and Krebbers, 1994; Ellerstrom et al., 1996). LEC2, ABI3 and FUS3 have been shown to bind specifically to the RY motif \textit{in vitro}, while ABI3 has also been shown to bind the RY motif \textit{in vivo} (Reidt et al., 2000; Kroj et al. 2003; Mönke et al., 2004; Braybrook et al., 2006, Mönke et al., 2012). In contrast, LEC1 belongs to a different protein family, homologous to the CCAAT-binding transcription factor HAP3 subunit (Lotan et al., 1998; Lee et al., 2003). LEC1 was shown to trigger the expression of cruciferin C likely by acting as a co-factor of ABRE-binding bZIP transcription factors (Yamamoto et al., 2009).

The AFL genes act synergistically to regulate common downstream targets. Indeed, they work co-operatively to promote the expressions of the SSP, 12S cruciferins and 2S napin, and LEA proteins (Parcy et al., 1997; Vicient et al., 2000; Kroj et al., 2003; Kagaya et al., 2005a/b). Further, \textit{LEC1}, \textit{ABI3} and \textit{FUS3} work together to regulate fatty acid synthesis, and \textit{FUS3} expression also correlates with seed oil accumulation (Wang et al., 2007; Mu et al., 2008).
Figure 4. Structures of the B3-domain transcription factor family.

Schematic diagrams depicting the structures of the B3 domain transcription factors. The ABI3/VP1, HSI/VAL, ARF, RAV and REM families are shown, ABI3/VP1 family includes ABI3, FUS3 and LEC2. Labelled domains include basic domain 1 (B1), B2 and B3, the PEST motif, the PHD-like zinc finger domain (CW), transcriptional repressor domain (EAR), the AUX/IAA domain, and APETALA2-like domain (AP2).
1.4.2 Expression patterns of the AFL genes

In agreement with their embryonic mutant phenotypes, all four AFL genes are primarily expressed in the embryo. *LEC1* expression is initially limited to the protoderm, but *LEC1* transcript can be detected throughout the embryo by the bent-cotyledon stage (Lotan et al., 1998). *LEC2* transcripts are present throughout the embryo in early stages but become limited to the hypocotyl and the embryo axis at later stages (Kroj et al., 2003; To et al., 2006). *ABI3* expression is uniform throughout the embryo except the root meristem, and this pattern persists throughout embryogenesis (To et al., 2006). *FUS3* transcripts can be detected in all cells of the embryo proper and the suspensor at the globular stage, and become limited to the protoderm from the heart stage (Tsuchiya et al., 2004). In the mature embryo, *FUS3* transcripts are localized in the vasculature, root tip and aleurone (Tsuchiya et al., 2004). *LEC2* and *ABI3* are also expressed in vegetative tissues, where *ABI3* is involved in lateral meristem formation (Rohde et al., 2000, Stone et al., 2001; Kroj et al., 2003; To et al., 2006). Expression of the AFL genes is also temporally regulated, with *LEC1* being expressed at early stages of embryogenesis, followed by *LEC2* (Stone et al., 2001; Kroj et al., 2003). *FUS3* and *ABI3* expression levels peak after *LEC2*, with *FUS3* levels decreasing before maturation and *ABI3* levels remaining high throughout embryogenesis (Luerssen et al., 1998; Parcy et al., 2004).

Currently, *FUS3* and *ABI3* are the only AFL genes with characterized protein expression patterns. *ABI3* antibody detects the ABI3 protein in seeds throughout development (Parcy et al., 1997; Kroj et al., 2003). However, the tissue-specific localization of the ABI3 protein has yet to be characterized. The cellular and subcellular localization of FUS3 is determined using a FUS3:FUS3-green fluorescent protein (GFP) translational reporter, which rescues the *fus3* mutant (Gazzarrini et al., 2004). At the early heart stage, the FUS3-GFP protein is detected in the entire embryo, mainly in the apical region, and also in the suspensor. By the heart to walking-stick stages, the FUS3-GFP protein localizes primarily to the protoderm, cotyledon and root tip. Despite the presence of *FUS3* transcripts in the mature embryo, the FUS3 protein has not been detected at this stage (Gazzarrini et al., 2004, Tsuchiya et al. 2004). This suggests post-transcriptional regulation mechanisms exist to downregulate FUS3 in mature embryo.
1.4.3 Hormonal regulation of the AFL genes

Several hormones regulate the expression and protein levels of the AFL genes, which in turn modulate the synthesis or perception of these hormones in a feedback mechanism (Figure 3). FUS3 and ABI3 expression is induced by auxin and FUS3 expression pattern during embryogenesis resembles the expression pattern of the DR5 promoter (Gazzarrini et al., 2004; Brady et al., 2003). Gain-of-function and genomic studies indicate LEC2 and FUS3 are positive regulators of auxin biosynthesis via the YUC genes, and LEC2, FUS3 and ABI3 also induce the expression of auxin-responsive genes (Brady et al., 2003; Nag et al., 2005; Braybrook et al., 2006; Stone et al., 2008; Yamamoto et al., 2010; Junker et al., 2012). LEC1, LEC2 and FUS3 genetically interact to regulate somatic embryogenesis by acting downstream of auxin (Gaj et al., 2005). This suggests a positive regulatory loop by which auxin promotes the expression of the AFL genes, which then interact to activate the synthesis of and/or response to auxin.

ABI3 positively regulates ABA signaling, while ABA antagonizes ABI3 protein stability. Indeed, ABA promotes the expression of ABI3-INTERACTING PROTEIN2 (AIP2), the ubiquitin E3 ligase that mediates ABI3 degradation (Zhang et al., 2005). Although the fus3 and lec2 mutations don’t cause ABA insensitivity, they enhance the ABA insensitivity of abi3 suggesting FUS3 and LEC2 genetically interact with ABI3 to regulate ABA responses (Parcy et al., 1997). FUS3 promotes ABA biosynthesis; ABA levels increase in seedlings ectopically expressing FUS3, while fus3-3 embryos contain less ABA (Nambara et al., 2000; Gazzarrini et al., 2004). As a feedback regulation, ABA increases FUS3 protein level by an unknown mechanism (Gazzarrini et al., 2004). In general, ABA positively regulates the activity of the AFL genes to promote the seed maturation program.

ABA and GA play opposite roles during seed dormancy and germination. Interestingly, FUS3 represses GA biosynthesis by down-regulating the GA synthesis genes, gibberellin-3-beta-dioxygenase 1 (AtGA3ox1), AtGA3ox2 and gibberellin-20-oxydase1 (AtGA20ox1), and GA in turn de-stabilizes the FUS3 protein (Curaba et al., 2004; Gazzarrini et al., 2004). Consequently, the precocious germination and ectopic trichome phenotypes of fus3-3 are due to excessive GA production (Curaba et al., 2004; Gazzarrini et al., 2004; Tsuchiya et al., 2004). Precocious germination of fus3-3 has also been linked to prolonged cell division activity in the fus3-3 embryo (Raz et al., 2001). In agreement with this, overexpression of FUS3 delays vegetarian
and reproductive phase transitions through repression of cell cycling in a GA-dependent manner, as they can all be rescued by exogenous GA (Gazzarrini et al., 2004).

Lastly, FUS3 also acts as a negative regulator of ethylene action. Several ethylene response genes are downregulated by ectopic FUS3 expression, and RY motifs are indeed found in the promoters of these genes (Lumba et al., 2012). Further, the advanced vegetative phase transition phenotypes of fus3-3 can be attributed to excessive ethylene activity, as it can be rescued by genetically or chemically inhibiting ethylene signaling (Lumba et al., 2012). Based on this evidence, FUS3 is considered to be an important integrator of hormone signaling during embryonic development and early vegetative growth.

### 1.4.4 Transcriptional regulation of the AFL genes

The AFL genes also control the expression of each other and some self-regulate their own expression. For example, LEC1 and LEC2 act upstream of ABI3 and FUS3, and promote their expressions (Figure 5) (Meinke et al., 1994; Parcy et al., 1997; Kagaya et al., 2005b; Santos-Mendoza et al., 2005; To et al., 2006; Wang et al., 2007). LEC1 and LEC2 also regulate each other’s expression (Figure 5) (Meinke et al., 1994; Santos-Mendoza et al., 2005). Meanwhile, ABI3 and FUS3 regulate the expression of each other, as well as their own expression (Figure 5) (Parcy et al., 1997; To et al., 2006). To further complicate this matter, cross-regulation of the AFL gene expression is spatially controlled. For example, in the root FUS3 is regulated by LEC2 and FUS3 itself, while in the embryo axis FUS3 is regulated by LEC2 and ABI3 (reviewed by Santos-Mendoza et al., 2008). The cross-regulation between these embryonic regulators partially explains the complex and overlapping phenotypes displayed by each mutant.

Genetic analysis of seed dormancy has revealed the importance of epigenetic mechanisms in the control of seed dormancy and repression of germination (reviewed by Suzuki and McCarty, 2008). Recessive mutations in the VAL/HSI transcriptional repressors show upregulation of AFL genes and development of embryo-like phenotypes in seedlings, suggesting VAL genes negatively regulate their expressions post-embryonically to promote vegetative development (Figure 5) (Suzuki et al., 2007). The VAL proteins contain a B3 domain as well as a plant homeodomain-like and CW domains (Perry and Zhao, 2003; Bienz, 2006). In addition, VAL proteins also contain the ethylene responsive transcription factor-associated amphiphilic
repression (EAR) domain (Tsukagoshi et al., 2005). The VAL genes have been independently identified as HSI genes, transcriptional repressors of sugar-inducible genes (Tsukagoshi et al., 2005). Interestingly, in the hsi2 hsl1 sensitized background the expression of AFL genes can be induced by sugar, suggesting the AFL genes may be involved in sugar signaling (Tsukagoshi et al., 2007). Another repressor of the AFL genes postembryonically is PICKLE (PKL), a chromatin organization modifier factor (Figure 5) (Ogas et al., 1999). pkl plants display embryo-like phenotypes and up-regulation of the AFL genes (Dean Rider et al., 2003). The embryo-like phenotypes of pkl and val mutants are dependent on GA, suggesting PKL and VAL not only suppress the AFL genes, but regulate the GA pathway as well (Ogas et al., 1997; Henderson et al., 2004). PKL also represses auxin signaling by down-regulating ARF7 and ARF19 during lateral root formation (Fukaki et al., 2006). Together, this highlights chromatin remodeling as a mechanism to repress AFL expression during vegetative development, and emphasizes the importance of AFL gene repression after germination, to acquire the appropriate hormone balance for vegetative growth.

Histone modification is another mechanism that down regulates AFL genes during dormancy and vegetative development. Histone methylation leads to repression of gene expression, while acetylation activates gene expression (reviewed by Berr et al., 2011). FUS3 is repressed by the Arabidopsis Polycomb group protein homologues MEDEA and CURLY LEAF/SWINGER, which mediate the tri-methylation of K27 of histone H3 (Makarevich et al., 2006). On the other hand, the histone deacetylase 6 (hda6) hda19 double loss-of-function mutant shows growth arrest and somatic embryo formation, phenotypes similar to overexpressing the LEC genes (Tanaka et al., 2008). Further, these phenotypes can be suppressed by the lec1, abi3 and fus3 mutations (Tanaka et al., 2008). These findings suggest LEC1, ABI3 and FUS3 expressions are repressed by HDA6 and HDA19 (Tanaka et al., 2008). LEC2 and FUS3 expressions are also regulated by microRNA, as mutation of DICER-LIKE1 cause premature expression of the maturation program during early embryogenesis with up-regulation of LEC2 and FUS3, suggesting the microRNA processing machinery negatively regulates the expression of LEC2 and FUS3 during early embryogenesis (Willmann et al., 2011). Together, the epigenetic regulation of the AFL genes highlights the importance of repressing the AFL genes postembryonically to allow the transition to vegetative development.
Figure 5. The interactions between the AFL genes and hormones during embryogenesis.

Schematic diagram depicting the AFL-hormones regulatory interaction network during embryogenesis. *LEC1* and *LEC2* act upstream of *ABI3* and *FUS3*. *LEC1* and *LEC2* regulate the expression of each other. *ABI3* and *FUS3* regulate the expression of each other and themselves. *LEC2* and *FUS3* promote auxin synthesis, while auxin positively regulates the expression of *FUS3*, *LEC* and *ABI3*. *FUS3* positively regulates ABA synthesis while negatively regulating GA and ethylene biosyntheses and/or signaling; ABA and GA stabilize and de-stabilize the FUS3 protein, respectively. Auxin plays critical roles to specify organ initiation during pattern formation. ABA level rises during maturation and promotes dormancy while inhibiting germination, while GA and ethylene antagonize ABA by promoting germination.
1.4.5 Function of AFL orthologues

Orthologues of AFL genes have been characterized in both monocots and eudicots, and most have similar properties in the establishment of seed maturation, dormancy and the regulation of phase transitions. In beans (*Phaseolus vulgaris*), ectopic expression of the ABI3-like factor induces expression of the seed-specific phaseolin (Ng and Hall, 2008). In pea (*Pisum sativum*), *PsFUS3, PsLEC1* and *PsABI3* are highly expressed in the embryo, and their down-regulation leads to defects in storage compound accumulation (Radchuk et al., 2006). In addition, *PsFUS3* is downregulated in ABA-deficient pea seeds, suggesting ABA accumulation triggers *FUS3* expression in pea as well (Radchuk et al., 2010b). The function of *FUS3* has been conserved in monocotyledonous plants as well. Ectopic expression of rice (*Oryza sativa*) *LEC2* and *FUS3-like1* (*OsLFL1*) delays flowering, similar to Arabidopsis (Gazzarrini et al., 2004; Peng et al., 2008). *FUS3* from barley (*Hordeum vulgare*) (*HvFUS3*) binds the RY motif and can rescue *fus3-3* defects in anthocyanin and seed storage protein accumulation (Moreno-Resueno et al., 2008). In trees, the axillary meristems can enter dormancy in response to adverse conditions, a process analogous to seed dormancy and similarly regulated by the ABI3/VP1 transcription factors. The expression level of the poplar (*Populus* spp.) orthologue of *ABI3, PtABI3* in the axillary buds indeed correlates with the ABA level (Rohde et al., 2002).

The AFL genes are widespread among plant species and are evolutionarily ancient. While *FUS3* is present only in spermatophytes and *LEC2*-like genes only in dicots, the *ABI3/VP1* lineage can be traced back to the bryophyte *Physcomitrella patens* and green algae (Swaminathan et al., 2008; Romanel et al., 2009; Li et al., 2010). The *P. patens ABI3* orthologue *PpABI3* is able to partially rescue the Arabidopsis *abi3-6* mutant in chlorophyll degradation and ABA-responsive gene regulation, but is unable to rescue the seed desiccation intolerance (Marella et al., 2006). However, loss-of-function mutant analyses shows the *P. patens ABI3* orthologues are required for ABA-induced desiccation tolerance by maintaining the expression of ABA-responsive genes (Khandelwal et al., 2010). This evidence suggests the *ABI3/VP1* transcription factors predate the evolution of seeds, and the original functions of the *ABI3/VP1* transcription factors likely involve ABA-mediated abiotic stress response.
1.5 Snf1/AMPK/SnRK1 kinases

Protein phosphorylation is among the best-characterized post-translational modifications. Protein phosphorylation was discovered about 60 years ago, referring to the transfer of a phosphate group from nucleoside triphosphate to a protein molecule (Harris, 1946; Burnett and Kennedy, 1954). This process is catalyzed by kinases, while de-phosphorylation involves phosphatases. Since phosphate groups are negatively charged, it is conceivable that phosphorylation can dramatically alter protein’s folding structure, physical properties and thus enzyme activity (Sutherland and Wosilait, 1955). More importantly, kinases themselves can be phosphorylated and regulated by other kinases (Fischer and Krebs, 1955); this forms the basis of a phosphorylation cascade that relays signals within cells.

Phosphorylation is a widely conserved mechanism among organisms across all kingdoms. Prokaryotes phosphorylate histidine residues in the two component regulatory system to relay environmental signals. Eukaryotes have two classes of kinases with different substrates: tyrosine kinases and serine/threonine kinases. Tyrosine kinases are prominently featured in animals, while serine/threonine kinases are more common in plants; nonetheless both classes are present in both kingdoms. Plants have exploited regulation by phosphorylation more prominently than other eukaryotes; the Arabidopsis genome features over a thousand kinases, whereas human has 518 (reviewed by Halford and Hey, 2009).

The yeast Sucrose non-fermenting 1 (Snf1), mammalian AMPK (5’ adenosine mono-phosphate activated protein kinase) and plant Snf1-related kinase 1 (SnRK1) kinases are evolutionarily conserved regulators of metabolism. These kinases act as global regulators of carbon metabolism in response to low energy level, by promoting catabolism while inhibiting anabolism (reviewed by Baena-Gonzáles and Sheen, 2008; Halford and Hey, 2009; Ghillebert et al. 2011). The yeast Snf1 was identified as a growth regulator in response to low glucose level (Celenza and Carlson, 1984; Celenza and Carlson, 1986). Later work showed Snf1 is involved in other processes including development, longevity and stress response (Honigberg and Lee, 1998; Kuchin et al., 2002; Hong and Carlson, 2007). The mammalian AMPK controls food intake and body weight (Minokoshi et al., 2008), and regulates insulin secretion in response to blood sugar level (da Silva Xavier et al., 2009). The plant SnRK1 was named after its ability to
complement the yeast \(\Delta snf1\) mutation (Aldersen et al., 1991). Similar to Snf1 and AMPK, SnRK1 regulates carbon metabolism in response to energy deficits by promoting catabolism and inhibiting anabolism (Radchuk et al., 2006; Baena-Gonzáles et al., 2007; Radchuk et al., 2010a). These findings show that the role of Snf1/AMPK/SnRK1 kinases in metabolic regulation is conserved in eukaryotes.

1.5.1 Structure and function of the Snf1/AMPK/SnRK1 complex subunits

The Snf1/AMPK/SnRK1 kinases function as heterotrimeric complexes comprised of one \(\alpha\)-type subunit, one \(\beta\)-type subunit and one \(\gamma\)-type subunit (Table 1) (reviewed by Halford et al., 2003; Polge et al., 2008).

The \(\alpha\) subunit is the catalytic subunit with the kinase domain at the N-terminus; this is also the most conserved domain among different species (Halford et al., 2003). The C-terminus of the \(\alpha\) subunit contains an auto-inhibitory domain, the binding sites for the \(\beta\) and \(\gamma\) subunits, and a nuclear localization signal (Figure 6) (Kazgan et al., 2010). The yeast and animal \(\alpha\) subunits dimerize, though its significance remains unknown (Nayak et al., 2006; Scholz et al., 2009).

The \(\beta\) regulatory subunit regulates the sub-cellular localization of the entire heterotrimer complex by N-terminal myristoylation (Hedbacker and Carlson, 2006; Vincent et al., 2001). In both yeast and plant, the \(\beta\) subunit N-myristoylation targets the complex to the cell membrane resulting in its inactivation (Lin et al., 2003; Pierre et al., 2007). The \(\beta\) subunit N-terminus also features the kinase interaction sequence / glycogen binding domain (KIS/GBD) that interacts with the \(\alpha\) subunit, while the C-terminus contains the association with Snf1 complex (ASC) domain that interacts with the \(\gamma\) subunit (Figure 6) (Jiang and Carlson, 1997).

The \(\gamma\) subunit is the activating subunit. In yeast, interaction with the \(\gamma\) subunit relieves the \(\alpha\) subunit auto-inhibition (Jiang and Carlson, 1996; Jiang and Carlson, 1997). In addition, the \(\gamma\) subunit has been proposed to expose the \(\alpha\) subunit active site under activating conditions (Scott et al., 2007). The \(\gamma\) subunit also contains two pairs of cystathionine-beta-synthase (CBS) repeats, which in animals binds AMP to activate the AMPK complex (Figure 6) (Hedbacker and Carlson, 2008). Neither yeast nor plant \(\gamma\) subunit orthologues have been shown to bind AMP,
but in plants AMP nonetheless promotes SnRK1 kinase activity by indirect mechanisms (Sugden et al., 1999a; Adams et al., 2004; Ghillebert et al., 2011).

Multiple paralogues of α, β and γ subunits exist, therefore the formation of different permutations of heterotrimers is possible (Table 1). For example, yeast has one α subunit (Snf1), three β subunits [Snf1-Interacting Protein1 (Sip1), Sip2 and Galactose metabolism83 (Gal83)] and one γ subunit (Snf4). Plants have three α subunits, three β subunits and at least three γ subunits. In addition, transcripts of the Snf1/AMPK/SnRK1 subunits may undergo alternative splicing in higher eukaryotes, thus adding more possible variations (Steinberg and Kemp, 2009).

1.5.2 Arabidopsis SnRK1 complex subunits

In Arabidopsis, Arabidopsis Snf1-related protein kinase 10 (AKIN10)/SnRK1.1, AKIN11/SnRK1.2 and AKIN12/SnRK1.3 encode the α subunits of the SnRK1 complex (Table 1). AKIN12 is considered to be a pseudo-gene due to the lack of expression and detectable kinase activity, leaving two active members in Arabidopsis (Fragoso et al., 2009; Jossier et al., 2009). AKIN10 is the dominant phosphorylating subunit in leaves, accounting for 90% of the total SnRK1 kinase activity (Jossier et al., 2009). In addition, AKIN10 is the main subunit mediating phosphate starvation response, as AKIN11 is degraded specifically during phosphate starvation (Fragoso et al., 2009). As critical regulators of metabolism, AKIN10 and AKIN11 have been shown to directly phosphohorylate various metabolic enzymes. Some known substrates include 3-hydroxy-3-methylglutaryl-coenzyme A reductase for sterol synthesis (Ball et al., 1995; Sugden et al., 1999b), sucrose phosphate synthase for sucrose synthesis (Sugden et al., 1999b), and nitrate reductase for nitrogen assimilation (Douglas et al., 1997; Sugden et al., 1999b). The consequences of phosphorylation (activation/inactivation) may be different for each substrate, but in general AKIN10/AKIN11-mediated phosphorylation lead to energy conservation during nutrient deprivation. Some, but not all of the AKIN10/AKIN11 substrates are also conserved in yeast and animals.

Arabidopsis has three β subunits (Table 1). The overall structures and functions of AKINβ1 and AKINβ2 are similar to those of their yeast counterparts (Bouly et al., 1999; Polge et al., 2008). AKINβ1 expression is repressed by sugars and overexpressing AKINβ1 renders the cotyledon expansion and true leaf initiation hypersensitive to sucrose, consolidating the role of the SnRK1
complex in carbohydrate metabolism (Li et al., 2009). On the other hand, AKINβ1 expression inversely correlates to nitrate reductase activity and is induced by amine nitrate, supporting the role of AKIN10/AKIN11 as inhibitors of nitrate reductase (Sugden et al., 1999b; Li et al., 2009). However, the activity of sucrose phosphate synthase, another known AKIN10/AKIN11 substrate, is not affected by AKINβ1 level, suggesting other β subunits may be involved in its regulation (Li et al., 2009). Plants also possess the atypical AKINβ3 subunit, which lacks the KIS/GBD domain (Figure 6), though it can still interact with the γ subunit and can functionally complement the yeast β subunit sip1Asip2Aga183A mutant, indicating it is a functional β subunit (Gissot et al., 2004).

The Arabidopsis γ subunits are even more diverse with three sub-families: the AKINβγ, AKINγ of the Phaseolus vulgaris 42 (PV42) family and the SnRK1 interacting protein1 (SnIP1) family (Table 1). AKINβγ is comprised of a β subunit KIS/GBD domain fused to the N-terminus of a standard γ subunit; it is also able to complement the yeast γ subunit snf4A mutant, indicating it is a functional γ subunit (Figure 6) (Lumbreras et al., 2001; Gissot et al., 2006). AKINβγ is also shown to interact with two putative pathogen resistant proteins, suggesting it may be involved in pathogen defense (Gissot et al., 2006). On the contrary, the PV42 and SnIPs families fail to complement the snf4A mutation in yeast, and they share very little homology with the standard γ subunits (Slocombe et al., 2002; Halford and Hey, 2009). Silencing AtPV42a and AtPV42b reduces silique size and seed set, suggesting these genes may play a role in reproduction as well (Fang et al., 2011). In summary, the complex structural organization of SnRK1 heterotrimer subunits suggests that plant SnRK1 function may be more intricately regulated compared to other eukaryotes.
Table 1. Subunits and upstream activating kinases of the Snf1/AMPK/SnRK1 kinases complexes

The yeast Snf1, mammalian AMPK and Arabidopsis SnRK1 complexes are each composed of one \( \alpha \) (catalytic), one \( \beta \) (localization) and one \( \gamma \) (activation) subunit. In all organisms shown here, the catalytic subunits can be activated by upstream kinases phosphorylation (reviewed by Ghillebert et al., 2011).
<table>
<thead>
<tr>
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<th>Yeast (Snf1)</th>
<th>Mammal (AMPK)</th>
<th>Arabidopsis (SnRK1)</th>
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<tbody>
<tr>
<td>α subunit (catalytic)</td>
<td>Snf1</td>
<td>AMPKα1</td>
<td>SnRK1.1/AKIN10</td>
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<td></td>
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<td>AMPKα2</td>
<td>SnRK1.2/AKIN11</td>
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<td>SnRK1.3/AKIN12</td>
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<tr>
<td>β subunit (localization)</td>
<td>Sip1</td>
<td>AMPKβ1</td>
<td>AKINβ1</td>
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<td>Gal83</td>
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<td>AKINβ3</td>
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<tr>
<td>γ subunit (activation)</td>
<td>Snf4</td>
<td>AMPKγ1</td>
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<td>AMPKγ3</td>
<td>SnIP</td>
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<tr>
<td>Upstream activating kinase</td>
<td>Sak1, Elm1</td>
<td>LKB1</td>
<td>GRIK1/SnAK2</td>
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<td></td>
<td>Tos3</td>
<td>CaMKKβ</td>
<td>GRIK2/SnAK1</td>
</tr>
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</table>
**Figure 6.** Structures of the Snf1/AMPK/SnRK1 complex subunits

Schematic diagrams of the Snf1/AMPK/SnRK1 α, β and γ subunits and their domains. The protein kinase domain (PK), auto-inhibitory domain (AIS) and β-subunit binding domain (β) are shown in the α subunit. The kinase interaction sequence / glycogen binding domain (KIS/GBD) and the association with Snf1 complex (ASC) domain are shown in the β subunit. Two pairs of cystathionine-β-synthase (CBS) repeats, are shown in the γ subunit. Plants also possess an atypical β3 subunit, which lacks the KIS/GBD, and the βγ subunit, which consists of an KIS/GBD N-terminally fused to a standard γ subunit. Plant-specific subunits are denoted with asterisks (*).
1.5.3 Activation and regulation of Snf1/AMPK/SnRK1 kinases

Two mechanisms control the activation of the Snf1/AMPK/SnRK1 α subunit; these include phosphorylation of the T-loop and regulation by metabolites. Activation of the α subunit by T-loop phosphorylation is mediated by a conserved group of kinases (Table 1) (Hawley et al., 1996; Wilson et al., 1996; Sugden et al., 1999a). The yeast Snf1 is activated by Snf1-activating kinase1 (Sak1), Elongated morphology1 (Elm1), and Target of Sbf3 (Tos3) (Table 1) (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003), whereas mammalian AMPK is activated by liver kinase B1 (LKB1) and calcium-dependent protein kinase kinase 2 (CaMKKβ) (Table 1) (Hardie, 2007; Steinberg and Kemp, 2009). Arabidopsis SnRK1s are activated by SnRK1 activating kinase 1 (SnAK1) and SnAK2 (Hey et al., 2007). SnAK kinases were originally shown to mediate immune response against Geminivirus infection, therefore they are also termed Geminivirus rep-interacting kinase1 (GRIK1) and GRIK2 (Table 1) (Shen and Hanley-Bowdoin, 2006; Shen et al., 2009). Both plant SnAK2/GRIK1 and SnAK1/GRIK2 and animal LKB1 show homology with yeast Sak1, Elm1 and Tos3 (Hey et al., 2007).

As global regulators of carbon metabolism, it is conceivable that Snf1/AMPK/SnRK1 kinases are directly regulated by metabolites. Yeast Snf1 responds to deficits in glucose, the preferred carbohydrate source, and is responsible for activating pathways that metabolize alternative carbohydrates (Celenza and Carlson, 1984; Celenza and Carlson, 1986). In the presence of glucose, Snf1 is de-phosphorylated and inactivated (Rubenstein et al., 2008). Mammalian AMPK is activated by high AMP/ATP ratio, which may result from glucose deprivation, hypoxia, or oxidative stress (Hardie, 2007; Steinberg and Kemp, 2009). AMP binds the γ subunit, which then binds and activates the AMPK α subunit (Hedbacker and Carlson, 2008).

The exact mechanism of how AKIN10 and AKIN11 are regulated by carbon metabolites is not entirely clear to date, but several lines of evidence suggest trehalose-6-phosphate (T6P) plays a major role in AKIN10/AKIN11 inhibition. Trehalose is the disaccharide formed from two glucose molecules and is a common carbohydrate made in all organisms except vertebrates (Avonce et al., 2006). T6P is an intermediate in trehalose synthesis, where T6P is de-phosphorylated by T6P phosphatase to form trehalose. T6P itself is formed by joining glucose-6-phosphate and uridine-diphosphoglucose, catalyzed by T6P synthase. T6P appears to be more than a trehalose synthesis intermediate, and may be actually a signaling molecule. T6P level
increases in response to sucrose, and T6P may act as a proxy of energy level (Schluempmann et al., 2004). Arabidopsis T6P auxotroph is embryonic lethal (Eastmond et al., 2002; Gomez et al., 2005; Gomez et al., 2006). When T6P synthase expression is silenced during vegetative development, the plants show growth retardation and fail to flower. This suggests T6P is also required for vegetative growth and reproductive transition (van Dijken et al., 2004). SnRK1 kinase activity is inhibited by T6P, and AKIN10/AKIN11 downstream targets are indeed regulated by T6P level (Zhang et al., 2009). Interestingly, T6P does not inhibit SnRK1 by direct binding, as T6P is unable to inhibit purified SnRK1 (Zhang et al., 2009). Rather, an unknown factor is required for T6P-mediated SnRK1 inhibition. This factor is likely to be proteins as it is heat-labile (Zhang et al., 2009). The T6P inhibition on AKIN10/AKIN11 is also observed in potato, as alterations of T6P level in potato (Solanum tuberosum) tubers affects the expression of AKIN10/AKIN11 target genes (Debast et al., 2011). Interestingly, AKIN10 phosphorylates T6P synthase, possibly as a feedback mechanism to control its own activity (Glinski and Weckwerth, 2005; Harthill et al., 2006).

Several novel regulatory mechanisms of AKIN10/AKIN11 have been identified as well. AKIN10 was shown to interact with myoinositol polyphosphate 5-phosphatase 13 (5PTase13), an enzyme that de-phosphorylates inositol 1,4,5-trisphosphate (IP3) to terminate the IP3 signaling (Ananieva et al., 2008). However, 5PTase13 was shown to be a positive regulator of AKIN10, as AKIN10 degrades more rapidly in the 5TPase13 loss-of-function mutant, and consequently the 5TPase13 mutant showed lower AKIN10 kinase activity (Ananieva et al., 2008). In agreement with the previous findings suggesting AKIN10 act as a positive regulator of ABA and sugar signaling pathways, 5TPase13 mutant seed germination is insensitive to ABA, glucose and sucrose (Ananieva et al., 2008). The precise biological contexts of this interaction are not yet understood, though IP3 signaling pathway is generally associated with abiotic stress response and sugar metabolism (reviewed by Valluru and Van den Ende, 2011).

The tomato (Solanum lycopersicum, formerly Lycopersicon esculentum) SnRK1 α subunit (SISnRK1) is shown to interact with AvrPto-dependent Pto-interacting protein3 (Adi3) (Avila et al., 2012). Adi3 was also shown to interact and phosphorylate the tomato Gal83 orthologue (SIGal83), the cognate β subunit for the tomato SnRK1 complex (Avila et al., 2012). The Adi3 interaction with SISnRK1 and phosphorylation of SIGal83 both negatively regulates SnRK1
kinase activity, putting Adi3 as a negative regulator of SnRK1 functions (Avila et al., 2012). Adi3 is a serine/threonine kinase and a negative regulator of programmed cell death, suggesting SnRK1 may be a mediator of programmed cell death regulation as well (Devarenne et al., 2006). SlGal83 is most closely related to Arabidopsis AKINβ1, and the phosphorylated serine residue is conserved, therefore it is possible for this regulation to occur in Arabidopsis.

1.5.4 SnRK1 downstream targets

Changes in transcriptional responses mediated by Snf1/AMPK/SnRK1 kinases have been shown in human and yeast (Young et al., 2003; Oliveras-Ferraros et al., 2009). In yeast, Snf1 phosphorylates and inactivates Multicopy inhibitor of GAL gene expression1 (Mig1), a transcription factor that mediates glucose repression (Treitel et al., 1998; Smith et al., 1999). Arabidopsis AKIN10 elicits transcriptional responses against stresses that limit photosynthesis and respiration, such as darkness, hypoxia and herbicide (Baena-González et al., 2007; Baena-Gonzáles and Sheen, 2008). Some transcriptional responses induced by AKIN10 appear to be mediated by bZIP transcription factors (Baena-González et al., 2007). Indeed, overexpression of bZIP proteins and AKIN10 together synergistically enhance the expression of AKIN10 marker genes (Baena-González et al., 2007). In agreement with this, gain-of-function mutant analyses have also identified bZIP transcription factors as transcriptional mediators of energy deficit stress response (Dietrich et al., 2011). Furthermore, purified SnRK1s phosphorylate AREBP-derived peptides containing SnRK1 target sites in vitro, suggesting SnRK1s likely phosphorylates AREBP (Zhang et al., 2008). Finally, AKIN10 was recently shown to phosphorylate the bZIP transcription factors, ABI5 and ENHANCED EM LEVEL (Bitrián et al., 2011). Together, this evidence suggests SnRK1 regulates a subset of target genes by phosphorylating and regulating bZIP transcription factors.

Recently, a different mechanism for Snf1/AMPK/SnRK1 regulation of transcription has been proposed. Snf1 has been shown to recruit various acetyltransferases to modulate histone subunits and regulate downstream target gene expressions in yeast (Lo et al., 2001; van Oevelen et al., 2006). AKIN10 and OsSnRK1 were shown to directly bind the chromatin region of two marker genes upregulated during hypoxia (Cho et al., 2012). Mammalian AMPK also has been demonstrated to transcriptionally regulate target genes by chromatin binding and
phosphorylation of histone H2B (Bungard et al., 2010). The evidence suggests SnRK1 can regulate target gene expression by directly modifying the chromatin.

1.5.5 Expression pattern and turnover of SnRK1

Microarray data suggest AKIN10 and AKIN11 are ubiquitously expressed, with higher expression in siliques (Fragoso et al., 2009). In agreement with this, AKIN10 protein is detected in almost all organs and tissues, although expression appears to be higher in lateral root and leaf primordia (Bitrián et al., 2011). Interestingly, AKIN10 transcript level doesn’t appear to be influenced by stress or hormones (Bradford et al., 2003; Fragoso et al., 2009; Bitrián et al., 2011). On the contrary, expression of the β and γ subunits is affected by stress and other stimuli (Bradford et al., 2003; Buitink et al., 2004).

The sub-cellular localization of AKIN10/AKIN11 in Arabidopsis has been somewhat controversial. When expressed under the 35S promoter, GFP-tagged AKIN10 was shown to localize to chloroplasts (Fragoso et al., 2009). However, OsSnRK1 is nuclear when transiently expressed in Arabidopsis protoplasts under the 35S promoter (Cho et al., 2012). Since the nuclear localization of OsSnRK1 is required for the induction of target genes by hypoxia, the nuclear localization is likely physiologically relevant (Cho et al., 2012). Furthermore, since both AKIN10 and OsSnRK1 bind chromatin, it would be logical to predict the nuclear localization of AKIN10 is conserved among rice and Arabidopsis (Cho et al., 2012). On the other hand, AKIN10-GFP translational fusion expressed under the endogenous promoter showed that in most cell types AKIN10 localizes to the plasma membrane and cytoplasm, while in guard cells, pollen, root and ovules AKIN10 is also nuclear-localized (Bitrián et al., 2011).

AKIN10 is degraded by the proteasome, the protein complex that degrades target proteins marked by ubiquitin. AKIN10 was found to interact with the WD40 repeat protein, PLEIOTROPIC REGULATORY LOCUS1 (PRL1) (Bhalerao et al., 1999). AKIN10 kinase activity increases in the absence of PRL1, suggesting PRL1 is a negative regulator of AKIN10 (Bhalerao et al., 1999). PRL1 interacts with the DNA damage binding protein1-cullin4-Regulator of cullin1 (DDB1-CUL4-ROC1) complex, one of the ubiquitin E3 ligase complex involved in ubiquitination of proteasome substrates. In addition, AKIN10 protein degradation is delayed in the prl1 or cul4 mutant backgrounds (Lee et al., 2008). These findings suggest PRL1
acts as the cognate E3 ligase that degrades AKIN10 by ubiquitination. Consequently, numerous defects associated with prl1 can be traced to AKIN10 mis-regulation, such as sugar and hormone sensitivity, and sterol synthesis (Németh et al., 1998; Flores-Pérez et al., 2010). However, AKIN10 is also found to interact with SKP1/ Arabidopsis SKP1-like1 (ASK1) subunits of a different type of ubiquitin E3 ligase, and α4/Proteasome alpha subunit D-1 (PAD1), a subunit of the proteasome (Farrás et al., 2001). Interestingly, PRL1 disrupts the binding between AKIN10 and SKP1/ASK1, suggesting these binding events are mutually exclusive (Farrás et al., 2001). Therefore, it appears that AKIN10 may mediate the degradation of other proteins by the proteasome, and possibly regulates the proteasome itself due to its physical association with it.

1.5.6 The SnRK family in plants

Aside from the SnRK1, two additional sub-families, SnRK2 and SnRK3, have evolved in plants and not found in other eukaryotes. SnRK2 and SnRK3 kinases share the kinase domain with SnRK1, but show no conservation in their regulatory domains with SnRK1 (reviewed by Coello et al., 2011). SnRK2 and SnRK3 are also functionally divergent from SnRK1, as they are unable to complement the yeast \( \Delta \)snf1 mutation (Purcell et al., 1998; Hrabak et al., 2003; Tiessen et al., 2003; McKibbin et al., 2006). In the Arabidopsis genome, three SnRK1s, ten SnRK2s and 25 SnRK3s have been identified (reviewed by Halford and Hey, 2009). SnRK1, SnRK2 and SnRK2 kinases have distinct roles in development, but recent studies have also highlighted overlapping functions.

Similar to Snf1 and AMPK, SnRK1 kinases are regulators of cellular energetic levels. SnRK1s sense cellular energy deficits from nutrient deprivation, environmental stresses and circadian rhythm (Rolland et al., 2002; Halford et al., 2003; Baena-González et al., 2007; Polge and Thomas, 2007; Baena-Gonzáles and Sheen, 2008; Wingler et al., 2009). The role of SnRK1 regulation in carbon metabolism has been characterized in different plant species. In potato tubers, SnRK1 is required for sucrose and starch synthesis (Purcell et al., 1998; McKibbin et al., 2006). Similarly, SnRK1 is also required for starch accumulation in seeds of wheat and rice (Zhang et al., 2001; Kanegae et al., 2005). As an extension to carbon metabolism regulation, SnRK kinases have also been found to regulate both biotic and abiotic responses (Hao et al., 2003; Baena-Gonzáles et al., 2007). In moss, SnRK1 is required for nutrient mobilization in
response to darkness (Thelander et al., 2004). Geminivirus transcriptional activators bind and inhibit SnRK1s, while the expression levels of SnRK1s correlate to resistance to Geminivirus infection (Hao et al., 2003).

The SnRK2 kinases regulate abiotic stress and ABA signaling. Almost all SnRK2s in rice and Arabidopsis are upregulated by osmotic stress, and three are upregulated by ABA (Boudsocq et al., 2004; Kobayashi et al., 2004). SnRK2 regulates salt stress response in Arabidopsis, wheat and soybean (Monks et al., 2001; Umezawa et al., 2004; Mao et al., 2010). The three Arabidopsis SnRK2s upregulated by ABA – SnRK2.2, SnRK2.3 and SnRK2.6 – are part of the core ABA signaling network (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). These three kinases phosphorylate and activate AREBP in the presence of ABA, and a triple knock-out of these genes leads to severe ABA insensitivity (Kobayashi et al., 2005; Furihata et al., 2006; Fujii and Zhu, 2009).

SnRK3s functions are typically calcium-dependent, since they interact with calcineurin B-like (CBL) calcium-binding proteins, and therefore are also known as CBL-interacting kinases (Guo et al., 2002). SnRK3s also contain a conserved domain required for interaction with the PP2C phosphatases, and this interaction appears to be mutually exclusive from the CBL binding (Ohta et al., 2003; Sánchez-Barrena et al., 2007). The SnRK3 kinases are mostly known for their role in salt tolerance. The best-studied SnRK3s include SnRK3.11/SALT OVERLY SENSITIVE 2 (SOS2) (Liu et al., 2000), which interacts with its cognate CBL SOS3 to activate Na/H antiporter SOS1 and alleviate salt stress (Mahajan et al., 2008).

The functional division between SnRK1, SnRK2 and SnRK3 may not be as clear as previously thought. There are several instances of functional overlap between the sub-families. For example, SnRK3s have also been implicated in ABA signaling and energy deficit response (Sano and Youssefian, 1994; Guo et al., 2002; Hu et al., 2008). In addition, SnRK2s have been shown to regulate carbon metabolism (Zheng et al., 2010). Several lines of evidence also support SnRK1 role in ABA-regulated processes. Overexpression of SnRK1 leads to ABA hypersensitivity (Jossier et al., 2009), while anti-sense SnRK1 expression in pea embryo leads to reduced storage proteins and lipids and premature germination, which resemble the lack of ABA response (Radchuk et al., 2006). As expected, genes involved in seed maturation and sugar
responses are downregulated in SnRK1 anti-sensed pea embryo, which is accompanied by a decrease in ABA (Radchuk et al., 2010a). Reduction of ABA in pea embryo by immunomodulation also leads to down-regulation of the SnRK1 regulatory β subunit (Radchuk et al., 2010b). Finally, the expression level of the tomato LeSNF4, encoding for the SnRK1 γ regulatory subunit, is upregulated by ABA or other ABA-dependent processes, such as water stress and dormancy (Bradford et al., 2003). On the other hand, conditions associated with ABA reduction, such as germination and GA treatment, downregulate LeSNF4 expression (Bradford et al., 2003). This evidence suggests the expression of the SnRK1 complex is promoted by ABA, and SnRK1 acts as a positive regulator of ABA signaling. In summary, the function of the SnRK1, SnRK2 and SnRK3 sub-families may be conserved to some degree despite the structural differences between them. Compared to yeast Snf1 and animal AMPK kinases, plant SnRK kinases are much more diverse in gene structure and function. This may reflect the different energetic demands and potential stresses faced by plants and animals.

1.6 General research objectives

Previous studies have highlighted a discrepancy between the FUS3 mRNA and protein profiles during embryogenesis; FUS3 mRNA is highly expressed during mid-to-late embryogenesis, while the protein is detected at low level up to mid-embryogenesis and is no longer present in mature embryo (Gazzarrini et al., 2004; Tsuchiya et al., 2004). This suggests post-transcriptional regulation may play a significant role in controlling FUS3 accumulation and function during embryogenesis. A decrease in the ABA/GA ratio may provide an explanation to this discrepancy, as ABA and GA stabilizes and destabilizes the FUS3 protein, respectively (Gazzarrini et al., 2004). However, the mechanism that controls FUS3 protein homeostasis remains unknown. Computational analyses conducted in the lab have shown that the FUS3 protein contains a putative PEST instability motif in the C-terminal domain, as well as more than 40 putative phosphorylation sites distributed throughout the protein, suggesting post-translational regulation may play a role in the regulation of FUS3 homeostasis (Lu, 2008). This leads us to hypothesize that FUS3 is an unstable protein, rapidly degraded at the end of embryogenesis likely through the 26S proteasome, and that the degradation is triggered by low ABA/GA ratio. In order to test this hypothesis and determine how post-translational
modifications regulate the FUS3 protein, the following research objectives have been addressed in this thesis:

1. Determine \textit{in vitro} and \textit{in vivo} whether FUS3 degradation is dependent on the proteasome, and the role of the PEST motif in FUS3 degradation (Chapter 2).

2. Identify proteins that interact with and regulate FUS3 by post-translational modification, and validate the biological relevance of the interaction (Chapter 3).

3. Characterize the role of FUS3 and the FUS3-interacting protein (AKIN10) in ABA, sugar and osmotic stress signaling (Chapter 4).

1.7 References


Eastmond, PJ., van Dijken, AJ., Spielman, M., Kerr, A., Tissier, AF., Dickinson, HG., Jones, JD., Smeekens, SC., Graham. IA. (2002). Trehalose-6-phosphate synthase 1, which


**Gomez, LD., Baud, S., Gilday, A., Li, Y., Graham, IA.** (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. *Plant J 46:* 69–84.


transcription factor mediating embryo axis formation and vascular development. *EMBO J* 17: 
1405–1411.

Harris, DL. (1946). Phosphoprotein phosphatase, a new enzyme from the frog egg. *J Biol 

Harthill, JE., Meek, SE., Morrice, N., Peggie, MW., Borch, J., Wong, BH., MacKintosh, C. 

Hawley, SA., Davison, M., Woods, A., Davies, SP., Beri, RK., Carling, D., Hardie, DG. 
(1996). Characterization of the AMP-activated protein kinase kinase from rat liver, and 
identification of threonine-172 as the major site at which it phosphorylates and activates AMP-

2420.

Henderson, JT., Li, HC., Rider, SD., Mordhorst, AP., Romero-Severson, J., Cheng, JC., 
Robey, J., Sung, ZR., de Vries, SC., Ogas, J. (2004). PICKLE acts throughout the plant to 
repress expression of embryonic traits and may play a role in gibberellin-dependent responses. 

Hey, S., Mayerhofer, H., Halford, NG., Dickinson, JR. (2007). DNA sequences from 
Arabidopsis which encode protein kinases and function as upstream regulators of Snf1 in yeast. 


Sugden, C., Crawford, RM., Halford, NG., Hardie, DG. (1999a). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5’-AMP. *Plant J* **19**: 433–439.


Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., Shinozaki, K. (2004). SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling


Chapter 2

The C-terminal domain of FUSCA3 negatively regulates mRNA and protein levels, and mediates sensitivity to the hormones abscisic acid and gibberellic acid in Arabidopsis

Previously published as:

SUMMARY

The transcription factor FUSCA3 (FUS3) controls the transition from the embryonic to the vegetative phase of development by regulating abscisic acid (ABA) and gibberellic acid (GA) levels in Arabidopsis thaliana. In a feedback loop, FUS3 accumulation is negatively and positively regulated by GA and ABA, respectively, by an uncharacterized mechanism. Here, we use a FUS3-GFP construct to show that the level of the FUS3 protein decreases dramatically during mid to late embryogenesis while its mRNA is present at high level. Deletion studies identify a C-terminal domain (CTD) that negatively regulates mRNA and protein levels and mediates sensitivity to ABA and GA. Indeed, a CTD-truncated FUS3 variant accumulates at high level and is insensitive to the destabilizing and stabilizing effects of GA and ABA, respectively. In contrast, fusion of various fragments of the CTD to GFP is sufficient to greatly reduce GFP fluorescence. The GFP-CTD fluorescence can be increased by ABA and paclobutrazol, an inhibitor of GA biosynthesis. Cell-free degradation assays show that FUS3 is a short-lived protein. FUS3 is degraded by the 26S proteasome in vitro and in vivo and the CTD affects its degradation rate. In contrast to the native form, the CTD-truncated FUS3 is unable to fully rescue the fus3-3 mutant and thus is required for FUS3 function. In conclusion, this study identifies a CTD that maintains low levels of FUS3 during embryogenesis and early germination and is required for normal FUS3 function and sensitivity to ABA and GA.
2.1 Introduction

Plants adapt to the changing environment by an intricate regulation of cellular and physiological processes. One example of such regulation is the control of the transition from embryogenesis to seed germination. During seed maturation, the embryo accumulates storage compounds and enters a period of dormancy during which it acquires desiccation tolerance (Goldberg et al., 1994; Vicente-Carbajosa and Carbonero, 2005). The breaking of dormancy is tightly controlled by endogenous and environmental signals, and allows germination to proceed under favorable conditions. The ratio of two antagonistic plant hormones, abscisic acid (ABA) and gibberellic acid (GA), plays a pivotal role in regulating seed dormancy and germination (Finkelstein et al., 2002; Finkelstein et al., 2008).

Embryos of several plant species do not undergo seed maturation, suggesting that the latter is not an obligatory step in plant development (Vicente-Carbajosa and Carbonero, 2005). Accordingly, several viviparous mutants skip the maturation process and directly enter vegetative development while still attached to the maternal tissue (Santos-Mendoza et al., 2008). In Arabidopsis, mutant screens led to the identification of several regulators of late embryonic development such as ABSCISIC ACID INSENSITIVE 3 (ABI3), LEAFY COTYLEDON 1 (LEC1), LEAFY COTYLEDON 2 (LEC2) and FUSCA3 (FUS3) (Koornneef et al., 1984; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Loss-of-function mutations in these genes result in severe defects in late embryonic functions including reduced dormancy, precocious germination and desiccation intolerance. Embryonic leaves of the lec and fus3 mutants have reduced expression of seed storage proteins (SSPs), typical embryonic markers, and express vegetative traits such as epidermal trichomes on the cotyledons (Keith et al., 1994; Meinke et al., 1994; West et al., 1994). In contrast, mutations in ABI3 do not affect epidermal trichomes, but result in seeds that are insensitive to ABA during germination (Giraudat 1992). ABI3, FUS3 and LEC2 encode transcription factors of the B3 domain family, while LEC1 encodes a HAP3 subunit of the CCAAT binding factor (Giraudat 1992; Lotan 1998; Luerssen et al., 1998; Stone 2001). These genes show an overlapping temporal pattern of expression; the LEC genes are activated during early embryogenesis, followed by FUS3 and lastly by ABI3 (Lotan et al., 1998; Stone et al., 2001; Kroj et al., 2003; Tsuchiya et al., 2004). A hierarchical and spatial network of gene regulation between these transcription factors also exists, suggesting that a complex
interaction at the transcriptional level controls late embryonic functions (Kroj et al., 2003; Kagaya et al., 2005a/b; To et al., 2006).

During germination, the expression of the *FUS3* and *LEC* genes is negatively regulated by the chromatin remodeling factor *PICKLE (PKL)* and the *VP1/ABSCISIC ACID INSENSITIVE 3-LIKE (VAL)* genes to allow seed progression through vegetative development (Ogas et al., 1999; Dean Rider et al., 2003; Suzuki et al., 2007). Loss-of-function mutations in *PKL* and triple loss-of-function mutations in the *VAL* genes result in upregulation of *FUS3/LEC2/ABI3* expression and the development of embryonic structures during vegetative growth (Ogas et al., 1999; Dean Rider et al., 2003; Suzuki et al., 2007). In agreement with this, constitutive and ectopic expression of *FUS3* and the *LEC* genes results in the expression of embryonic markers and the development of embryonic structures during vegetative growth (Lotan et al., 1998; Stone et al., 2001; Gazzarrini et al., 2004; Kagaya et al. 2005a/b). It has been shown that FUS3 controls the transition from the embryonic to the vegetative phase of development through the regulation of the ABA/GA ratio. FUS3 represses GA biosynthesis by directly binding to the promoter of GA biosynthetic genes, while its transient activation during vegetative development increases ABA levels. Accordingly, *fus3-3* loss-of-function seeds have higher GA and lower ABA levels (Nambara et al., 2000; Curaba et al., 2004; Gazzarrini et al., 2004).

Localization studies using a *FUS3:FUS3-GFP* reporter have shown that FUS3 accumulates preferentially in the epidermis, vasculature and root tips of embryos. *FUS3* expression is regulated by auxin, while the stability of the protein is governed by ABA and GA; these two hormones act antagonistically to regulate the abundance of the FUS3 protein, increasing and decreasing FUS3 level, respectively (Gazzarrini et al., 2004). These findings correlate with the fact that the FUS3-GFP protein is detected during early-mid embryogenesis, when the ABA/GA ratio in the embryo is high, but FUS3-GFP is undetected during late embryogenesis and germination when the ABA/GA ratio is low (Gazzarrini et al., 2004). Although FUS3 is an important node of interaction between different hormone signaling pathways controlling embryonic development and seed germination, the mechanism regulating FUS3 mRNA and protein abundance during embryogenesis and germination is currently unknown.
In this study we use a \textit{FUS3:FUS3-GFP} reporter to show that during embryogenesis the pattern of FUS3 protein accumulation does not follow that of its mRNA. While \textit{FUS3} mRNA is high during mid and late embryogenesis, the FUS3 protein is undetected at the latter stage. Analysis of FUS3-GFP deletion variants suggests that the N-terminal (NTD) and C-terminal (CTD) domains negatively regulate \textit{FUS3} mRNA level during embryogenesis and germination. Interestingly, the CTD is both necessary and sufficient to reduce protein level and required for efficient degradation of FUS3 through the 26S proteasome. The CTD also mediates sensitivity to the hormones ABA and GA and is required for proper FUS3 function.

\subsection*{2.2 Results}

\subsubsection*{2.2.1 Patterns of FUS3 mRNA and protein accumulation during embryogenesis and germination}

We have previously shown that a \textit{FUS3:FUS3-GFP} construct can rescue the \textit{fus3-3} embryonic and vegetative phenotypes (Gazzarrini et al., 2004; Figure 1). In these plants, the FUS3-GFP protein is present only during early and mid embryogenesis, but is not detected in mature 14 DAF embryos (Gazzarrini et al., 2004; Figure 2c). These findings contrast previous \textit{FUS3} expression studies, which show that \textit{FUS3} mRNA is present during embryogenesis up to and including 18 DAF (Luerssen et al., 1998; Kroj et al., 2003). This suggests that during embryogenesis the expression pattern of the FUS3 protein does not follow that of its mRNA. Since no expression data is available for the endogenous FUS3 protein, we investigated this discrepancy by comparing the expression patterns of FUS3-GFP mRNA and protein in \textit{FUS3:FUS3-GFP} plants.

Using qRT-PCR we first quantified \textit{FUS3} mRNA levels for both wild-type and \textit{FUS3:FUS3-GFP} plants during embryogenesis and germination (Figure 2a). Although the relative expression of wild-type plants is approximately twice that of \textit{FUS3:FUS3-GFP} plants during embryogenesis, the overall profiles were identical. More specifically, the lowest expression is found during early embryogenesis (6 DAF), peaks approximately 3 to 5 fold during mid-embryogenesis (10 DAF) and decreases slightly during late embryogenesis (14 DAF). This expression pattern corroborates expression data retrieved from available microarrays (Figure S1;
Toufighi et al., 2005). Intriguingly, it is during early embryogenesis where mRNA is least abundant, that we observed the strongest FUS-GFP signal (Figure 2c). As development continues into mid- and late embryogenesis, fluorescence from the FUS3-GFP fusion protein diminishes completely while its mRNA level is still high (Figure 2c). On immunoblots the fusion protein remains undetected (Figures 3c). During germination, the situation is less complex; the mRNA diminishes dramatically at 6 and 24 HAI and no FUS3-GFP fluorescence is detected (Figures 2a,c; Figure 3b).

To assess the activity of the FUS3 promoter used in the FUS3:FUS3-GFP construct, we made a FUS3:GFP transcriptional fusion (Figure 1b) and examined the GFP fluorescence during embryogenesis and germination (Figure 2b). The FUS3:GFP expression pattern shows that the FUS3 promoter is active throughout embryogenesis, since GFP can be readily detected at all stages (6-14 DAF; Figure 2b). This is in agreement to the mRNA expression pattern (Figure 2a), but in contrast to the fluorescence signal of FUS3:FUS3-GFP (Figure 2c). The FUS3:GFP expression pattern is similar to that of FUS3:GUS reporters described previously (Kroj et al., 2003; Tsuchiya et al., 2004). A reduced signal was observed during germination, paralleling the reduction in mRNA levels (6-24 HAI; Figure 2a and b).

We conclude that the level of FUS3 protein during embryogenesis does not follow that of its mRNA and becomes uncoupled during mid embryogenesis.

2.2.2 The C-terminal domain of FUS3 negatively regulates mRNA and protein accumulation during embryogenesis and germination

One possibility in which the level of FUS3 protein is regulated during embryogenesis is through a post-translational event, which decreases the level of the protein as the developmental program continues. A bioinformatic analysis of FUS3 predicted several putative phosphorylation sites within the NTD and CTD, as well as a C-terminal PEST degradation motif (Figure 1a). To determine whether these domains regulate FUS3 levels, we made two deletion constructs lacking either the CTD or NTD of FUS3, which were then fused to GFP. Both constructs were driven by the endogenous FUS3 promoter and were transformed into a fus3-3 background (Figure 1b). Remarkably, embryos expressing the CTD-deletion construct (FUS3:FUS3ΔC-GFP) show a dramatic increase in GFP fluorescence and protein levels when compared to
*FUS3:*FUS3-GFP embryos (Figure 2c; Figure 3a,c; Figure S2a). At the walking-stick stage (10 DAF), *FUS3:*FUS3ΔC-GFP embryos show approximately 50 to 100-fold increase in GFP fluorescence when compared to *FUS3:*FUS3-GFP embryos (Figure 3a and Figure S2a). A 50-fold increase in fluorescence intensity was also observed in *FUS3:*FUS3ΔC-GFP germinating seeds (Figure 2c and Figure 3b). In contrast, *FUS3:*FUS3ΔN-GFP embryos and germinating seeds which express the FUS3 NTD-deletion construct, show similar or slightly higher (3-fold) fluorescence intensity as *FUS3:*FUS3-GFP embryos and germinating seeds (Figures 2c and Figure 3a-b). No immunoblot signal was detected in *FUS3:*FUS3-GFP and *FUS3:*FUS3ΔN-GFP embryos, supporting the weak GFP fluorescence observed in these transgenic lines (Figure 3c).

Intriguingly, the truncated *FUS3ΔC-GFP* and *FUS3ΔN-GFP* mRNAs increase to levels higher than the full-length *FUS3-GFP* transcripts (~10-15 fold), suggesting that both terminal domains affect *FUS3* mRNA accumulation (Figure 3a,b). Only the deletion of the CTD, however, greatly enhanced GFP fluorescence and protein levels. Taken together, this suggests that the CTD negatively regulates FUS3-GFP mRNA and protein levels and warranted further investigation as to its role in FUS3 protein abundance.
**Figure 1.** FUS3 domains and deletion constructs used in this study.

(a) FUS3 amino acid sequence. The B3 domain is shown in italic. The putative PEST sequence was predicted using the PESTFIND algorithm (http://vm-bioinfo.toulouse.inra.fr/emboss/emboss.cgi/epestfind) and is underlined. Putative phosphorylation sites were identified with the NetPhos 2 algorithm (http://www.cbs.dtu.dk/services/NetPhos) and are shown in bold.

(b) Schematic representation of FUS3 domains and FUS3 constructs used in this study. 1.5 kb of the 5’ regulatory sequences of FUS3 was used to drive expression of GFP alone (FUS3:GFP). FUS3 full-length (FUS3), FUS3 C-terminal (FUS3∆C) and FUS3 N-terminal (FUS3∆N) deletion constructs are fused to GFP and the expression of the fusion constructs is driven by the FUS3 or ML1 promoters. **FUS3:FUS3-GFP** and **ML1:FUS3-GFP** constructs have been previously described (Gazzarrini et al., 2004). The same FUS3 promoter was used in all constructs. The basic B2 and B3 domains, the activation domain (A) and putative PEST sequence are shown. Numbers indicate amino acid positions.
MMVDENVETKASTLVASVDHGSGHHDHGLSASVPLLGVNVWKRRMPRQRRSSSSFNLLSFPPPPPISHVPTPLPAKIDPRKLRLFQELKNSDVSSLRRMLPKKAEEAHLPALLECEKEGIPRMEDELDDGFHVWTFKRYWPNRNSRMYVLEN TDGFVAHQLGQDFIMVQODLYSNNVYVIQRKASEEEEVINLEEDDYTNLTRIENTVNDLLLDQDFNHNNNNNNNSNSNKSYYYPVIDDVTNTESFVYD TTALENDTPLDLGGHTTTTNYYSKFGTFDGLGSVENISLDDFY.

(b)

- **FUS3:GFP**
  - FUS3 → GFP

- **FUS3:FUS3-GFP (FFG)**
  - FUS3 → B2 B3 A PEST → GFP
  - ML1 → B2 B3 A PEST → GFP

- **FUS3:FUS3ΔC-GFP (FFΔCG)**
  - FUS3 → B2 B3 A → GFP
  - ML1 → B2 B3 A → GFP

- **FUS3:FUS3ΔN-GFP (FFΔNG)**
  - FUS3 → B3 A PEST → GFP
Figure 2. Expression patterns of FUS3 transcripts and FUS3-GFP protein variants during embryogenesis and germination.

(a) qRT-PCR showing the changes in FUS3 transcript accumulation in wild-type (white bars) and fus3-3, FUS3:FUS3-GFP (black bars) at various days after flowering (DAF) and hours after imbibition (HAI). Values are averages from triplicates ± s.d.

(b) and (c) Confocal images of FUS3:GFP, FUS3:FUS3-GFP, FUS3:FUS3ΔC-GFP and FUS3:FUS3ΔN-GFP embryos at different DAF and HAI. The last three columns show paradermal optical sections. Insets show merged images (green and red channels). Red fluorescence is due to propidium iodide staining and auto fluorescence from chlorophyll. Bars in the first three columns represent 50 μm; Bars in the last 3 columns represent 20 μm. All images were taken under comparable confocal setting. (c) A higher laser intensity was used for imaging all germinating seeds (6 and 24 HAI).
(a) Relative mRNA levels for fus3-3, FUS3:FUS3-GFP compared to M.Col.

(b) Germination and Embryogenesis timelines for FUS3:GFP, FUS3:FUS3-GFP, FUS3:N-GFP, and FUS3:∆C-GFP.

(c) Images showing the expression patterns of FUS3:GFP, FUS3:FUS3-GFP, FUS3:N-GFP, and FUS3:∆C-GFP at different developmental stages.
Figure 3. Quantification of the GFP fluorescence, mRNA level and protein abundance in embryos and germinating seeds of various genotypes.

(a) and (b) Quantification of the GFP fluorescence (black bars) and relative mRNA expression levels (gray bars) in \textit{FUS3:FUS3-GFP} (\textit{FFG}), \textit{FUS3:FUS3ΔC-GFP} (\textit{FFΔCG}) and \textit{FUS3:FUS3ΔN-GFP} (\textit{FFΔNG}) lines at 10 days-after-flowering (DAF) (a) and 24 hours-after-imbibition (HAI) (b). Quantifications of GFP fluorescence and qRT-PCR on GFP-fusion transcripts were performed in walking-stick embryos and 24 HAI seeds (see Methods). Values are averages from triplicates ± s.d.

(c) Immunoblot analysis showing the levels of various GFP-fusion proteins in 10 DAF siliques of \textit{FFG}, \textit{FFΔCG} and \textit{FFΔNG} lines (numbers indicate independent transgenic lines). Immunoblots were probed with anti-GFP antibody. Ponceau staining is shown as loading control. Predicted sizes of fusion proteins are: FUS3-GFP \textasciitilde 63 kDa, FUS3ΔC-GFP \textasciitilde 54 kDa and FUS3ΔN-GFP \textasciitilde 56 kDa.
2.2.3 FUS3 C-terminal domain is sufficient to reduce protein levels

Since the deletion of the CTD greatly increases FUS3 abundance, we examined whether this domain is sufficient to reduce protein levels when fused to a heterologous protein, such as GFP. We examined GFP fluorescence in transgenic lines constitutively expressing either GFP alone (\(35S: GFP\)) or fused to the last 118 amino acids of FUS3 CTD (\(35S: GFP-CTD^{118}\); Figure 4a). Strikingly, the GFP intensity in \(35S: GFP-CTD^{118}\) embryos was on average 16 fold weaker than that observed in \(35S: GFP\) embryos (Figure 4b-c). A strong difference in protein level was also observed on immunoblot (Figure 4d) that was not attributable to a difference in mRNA levels (Figure 4e). Indeed, three \(35S: GFP-CTD^{118}\) lines accumulated GFP transcripts at similar or higher levels than \(35S: GFP\) lines. A strong difference in protein level was also observed during vegetative development (Figure S4b). These data demonstrate that FUS3 CTD\(^{118}\) is able to cause low protein accumulation.

To identify the minimal FUS3 CTD region that regulates protein accumulation we made two additional constructs, one containing the last 67 amino acids of the CTD including the PEST motif (\(35S: GFP-CTD^{67}\)) and one containing only the 37 amino acids of the PEST sequence (\(35S: GFP-CTD^{37}\)) (Figure 4a). Transgenic plants harboring the various CTD deletion constructs also show a strong decrease in GFP fluorescence, with \(35S: GFP-CTD^{67}\) and \(35S: GFP-CTD^{37}\) embryos showing on average 16 and 8-fold reduction in GFP signal, respectively (Figure 4b, c). We conclude that fusion of the PEST sequence to GFP is sufficient to strongly reduce GFP fluorescence. However, fusion of the CTD\(^{118}\) or CTD\(^{67}\) caused the strongest reduction in GFP fluorescence.
**Figure 4.** Fusion of various FUS3 C-terminal domains to GFP is sufficient to decrease GFP abundance.

(a) Schematic representation of various GFP-CTD fusion constructs driven by the 35S promoter used in this study. Numbers indicate amino acid positions.

(b) Confocal images of GFP and various GFP-CTD fusions of walking-stick embryos. Images were taken under the same confocal settings. Right panels show GFP fluorescence (green channel) of hypocotyls shown in left panels (merged images of red and green channels). Bars in left panels, 50 µm; bars in right panels, 20 µm.

(c) to (e) Quantifications of GFP fluorescence (c), protein (d) and mRNA (e) levels of various transgenic lines. (c) GFP quantifications and relative mRNA levels of *GFP* transcripts measured by qRT-PCR were performed in walking-stick embryos. Values are averages from triplicates ± s.d.

(d) Immunoblot analysis showing GFP and GFP-CTD\textsuperscript{118} accumulations in 12 days-after-flowering embryos. Immunoblots were probed with anti-GFP antibody. Ponceau staining is shown as loading control. Predicted sizes are: GFP ~ 30 kDa; GFP-CTD\textsuperscript{118} ~ 42 kDa.
(a) Schematic representation of constructs 35S:GFP, 35S:GFP-CTD<sup>118</sup>, 35S:GFP-CTD<sup>67</sup>, and 35S:GFP-CTD<sup>37</sup>.

(b) Images showing relative fluorescence intensity for 35S:GFP, 35S:GFP-CTD<sup>118</sup>, 35S:GFP-CTD<sup>67</sup>, and 35S:GFP-CTD<sup>37</sup> constructs.

(c) Graph depicting relative fluorescence intensity for different lines of constructs.

(d) Western blot analysis showing bands at 43 kDa and 34 kDa for 35S:GFP and 35S:GFP-CTD<sup>118</sup> constructs.

(e) Bar graph illustrating relative mRNA levels for different lines of constructs.
2.2.4 FUS3 degradation is mediated by the 26S proteasome pathway

The data presented so far suggest that FUS3 is a low abundant protein and its accumulation is negatively regulated by its PEST-containing CTD. Since PEST motifs are found in several unstable proteins that are degraded by the 26S proteasome pathway, we tested whether FUS3 is also degraded in a proteasome-dependent manner. Due to the very low level of FUS3 accumulation during embryogenesis and the difficulty of culturing embryos, we investigated the degradation of recombinant FUS3 in cell-free extracts prepared from 10-day-old wild-type seedlings. In the absence of the proteasome inhibitor MG132, GST-tagged FUS3 was degraded rapidly and decreased by 75% and 98% within 30 min and 2 h, respectively, while MG132 greatly delayed the degradation of GST-FUS3 (Figure 5a). In contrast, GST-FUS3ΔC was degraded at a slower rate and decreased by 44% and 87% within 30 min and 2h, respectively. Quantification of the degradation kinetics in the absence of MG132 revealed half-lives (t_{1/2}) of approximately 15 and 45 minutes for GST-FUS3 and GST- FUS3ΔC, respectively (Figure 5b).

To test whether FUS3 degradation follows the proteasome pathway and is mediated by the CTD in vivo, we generated transgenic plants that overexpress FUS3 and FUS3ΔC during vegetative development under the control of the ML1 promoter (ML1:FUS3-GFP and ML1:FUS3ΔC-GFP; Figure 1b and Gazzarrini et al., 2004). We exposed ML1:FUS3-GFP and ML1:FUS3ΔC-GFP seedlings to MG132 for 2 and 6 hours (Figure 5c). Two experiments were conducted with similar results and one is shown in Figure 5c. After 6 hours of treatment, FUS3-GFP levels were 3 fold higher in the presence of MG132 (lane 4), compared to the control sample at the same time (lane 3), while FUS3ΔC-GFP levels increased only 1.1-1.7 fold in the presence of MG132 (lane 8), compared to the control sample at the same time (lane 7; Figure 5c). These results indicate that FUS3 is a short-lived protein that is processed in a proteasome-dependent manner. They also suggest that the CTD affect the rate of degradation through the 26S proteasome.

We compared GFP fluorescence, protein and mRNA levels in 4 DAG seedlings and found that FUS3ΔC-GFP fluorescence and protein levels are approximately 6 fold higher than those of FUS3-GFP protein (Figure 5c,d and S3b). In contrast, FUS3ΔC-GFP mRNA accumulates to a slightly higher (2.5 fold) level than FUS3-GFP mRNA (Figure 5d). Similar results were obtained in three independent transgenics (Figure S3a). This indicates that in 4 DAG
*ML1:FUS3ΔC-GFP* seedlings the deletion of the CTD plays a major role in increasing FUS3 protein level and a minor function in mRNA level.

During embryogenesis, *ML1:FUS3ΔC-GFP* embryos show a much higher GFP fluorescence (approximately 20 to 100 fold) and protein level when compared to *ML1:FUS3-GFP* embryos (Figure 6a,b,c and Figure S2b). Interestingly, immunoblots show that FUS3ΔC-GFP accumulates to a much higher level when its expression is driven by the FUS3 promoter (*FUS3:FUS3ΔC-GFP*) compared to the ML1 promoter (*ML1:FUS3ΔC-GFP*; Figure 6c). This is possibly due to a lower activity of the *ML1* promoter during embryogenesis in comparison to the *FUS3* promoter and/or to a positive feedback regulation of FUS3 on its own expression (see Discussion). It is noteworthy to mention that three bands accumulated in *FUS3:FUS3ΔC-GFP* embryos; a lower molecular weight band migrating at approximately the expected size of FUS3ΔC-GFP (54 kDa), and two higher molecular weight bands of weaker intensity (Figure 3c and 6c and Figure S4). In *ML1:FUS3ΔC-GFP* embryos and seedlings, however, only the lower molecular weight band is detected. The lack of expression of the higher molecular weight bands in this line could be due to a lower expression level of the FUS3ΔC-GFP construct or a different translational or posttranslational regulation of FUS3ΔC-GFP in the two transgenic lines. The origin of the multiple bands is, at the moment, unknown.
Figure 5. FUS3 degradation is mediated by the proteasome pathway.  

(a) Immunoblots showing GST-FUS3 and FUS3∆C degradation in the presence or absence of the proteasome inhibitor MG132. Recombinant GST-fusion proteins were expressed and purified from *E. coli* and then added to the wild-type protein extracts for the indicated times. Immunoblots were probed with anti-GST antibody. Ponceau stain is shown as loading control. Experiments were repeated twice with similar results, one is shown here.  

(b) Half-life plot for the degradation of GST-FUS3 and FUS3∆C shown in (a).  

(c) Immunoblots showing FUS3-GFP and FUS3∆C-GFP protein levels in 4-day-old transgenic plants treated with CHX in the presence or absence of MG132 for the indicated times. Fold changes in protein levels are shown and were calculated with respect to the CHX 2h ML1:FUS3-GFP control (lane 1) and normalized to a cross-reacting band (*). Immunoblots were probed with anti-GFP antibody.  

(d) Quantification of GFP fluorescence (black bars) and relative mRNA expression levels (gray bars) in *ML1:FUS3-GFP* and *ML1:FUS3∆C-GFP* seedlings. GFP fluorescence was measured on leaf primordia of 4 DAG seedlings and qRT-PCR on *GFP*-fusion transcripts was performed in 4 DAG seedlings. Values are averages from triplicates ± s.d.
(a) Mock + MG132

 GST-FUS3

 GST-FUS3ΔC

(b) Mock + MG132

 GST-FUS3 remaining

 GST-FUS3ΔC remaining

(c) ML1:FUS3-GFP  ML1:FUS3ΔC-GFP

 WT

 FUS3-GFP

 FUS3ΔC-GFP

 Relative intensity

(d) Relative fluorescence intensity

 ML1: FUS3-GFP  ML1: FUS3ΔC-GFP
Figure 6. Deletion of the C-terminal domain increases FUS3 abundance in *ML1:FUS3ΔC-GFP* embryos.

(a) Confocal images of *ML1:FUS3-GFP (MFG)* and *ML1:FUS3ΔC-GFP (MFΔCG)* torpedo (first column) and walking-stick (second and third columns) embryos. The third column shows paradermal optical sections. Insets show merged images (red and green channel). Bars in first and second columns, 50 µm; Bars in third column, 20 µm.

(b) Quantification of GFP fluorescence in *MFG* and *MFΔCG* walking-stick embryos. Bars indicate s.d.

(c) Immunoblot analysis showing the levels of GFP-fusion proteins in 10 DAF siliques of *FUS3:FUS3-GFP (FFG)*, *FUS3:FUS3ΔC-GFP (FFΔCG)*, *ML1:FUS3-GFP (MFG)* and *ML1:FUS3ΔC-GFP (MFΔCG)* lines. Blots were probed with anti-GFP antibody. Ponceau staining is shown as loading control.
2.2.5 FUS3 sensitivity to ABA and GA is mediated by its C-terminal domain

Using the \( ML1:FUS3-GFP \) construct, we have previously shown that the FUS3 protein is stabilized by ABA and destabilized by GA (Gazzarrini et al., 2004). To test whether FUS3 sensitivity to these two hormones is mediated by its CTD, we first exposed 3-day-old \( ML1:FUS3-GFP \) and \( ML1:FUS3\Delta C-GFP \) seedlings to GA and ABA for 4 days. As expected, \( ML1:FUS3-GFP \) leaf primordia exposed to GA show a significant decrease in GFP signal, whereas those exposed to ABA show a stronger signal (Figure 7a). In contrast, no change in GFP intensity was observed in \( ML1:FUS3\Delta C-GFP \) leaf primordia exposed to the same treatments (Figure 7a; Figure S5). No significant change in GFP mRNA levels was observed in the transgenic lines exposed to the hormones (Figure 7b).

We then exposed \( 35S:GFP \) and \( 35S:GFP-CTD^{118} \) seedlings to ABA and paclobutrazol (PAC), an inhibitor of GA biosynthesis and found an increase in GFP fluorescence when \( 35S:GFP-CTD^{118} \) seeds were germinated for 3 days on paclobutrazol or 7 days on ABA (Figure 7c). Quantification of GFP protein levels in these transgenic plants shows that paclobutrazol, but not ABA, can increase GFP-CTD\(^{118}\) protein level in whole seedlings (Figure 7d). Interestingly, \( GFP \) and \( GFP-CTD^{118} \) mRNA levels were reduced approximately 2-fold in seedlings treated with ABA (Figure 7e), possibly explaining the lack of effect of ABA on GFP-CTD\(^{118}\) protein level on immunoblots (Figure 7d, lane 7). It is also possible that the effect of ABA is spatially restricted (to the epidermis of the hypocotyls) and only detectable by confocal microscopy. Altogether these data suggest that the CTD mediates FUS3 sensitivity to ABA and GA.
Figure 7. Regulation of FUS3 levels by ABA and GA is mediated by its CTD.

(a) Confocal images of leaf primordia of ML1:FUS3-GFP and ML1:FUS3ΔC-GFP seedlings germinated for 3-4 days on MS and transferred for 4 days onto MS media alone, or supplemented with 10 µM GA or ABA. Merged images of green and red channel are shown. Bars represent 10 µm. All images were taken under comparable confocal settings.

(b) qRT-PCR showing FUS3 transcript levels in ML1:FUS3-GFP (gray bars) and ML1:FUS3ΔC-GFP (black bars) 3-day-old seedlings exposed to 10 µM ABA or GA for 4 days. Values are averages from triplicates ± s.d.

(c) Confocal images of the epidermis of the hypocotyls of 35S:GFP and 35S:GFP-CTD seedlings germinated on MS, 1 µM ABA or 1 µM paclobutrazol for 3 or 7 days. Bars represent 20 µm. All images were taken under comparable confocal settings.

(d) Immunoblots showing GFP and GFP-CTD levels in transgenic plants exposed to ABA and paclobutrazol as described in (c). Immunoblots were probed with anti-GFP antibody. Ponceaus stain and the cross reacting band (*) are shown as loading controls.

(e) Relative mRNA levels of GFP transcripts measured by qRT-PCR in transgenic plants exposed to ABA and paclobutrazol as described in (c). Average ratios of fold changes relative to MS controls of two biological replicas are shown.
**Figure 1.**

(a) Immunofluorescence images showing localization of ML1:FUS3-GFP and ML1:FUS3ΔC-GFP in cells treated with MS, GA, and ABA. Scale bars represent 10 μm.

(b) Relative mRNA levels of ML1:FUS3-GFP and ML1:FUS3ΔC-GFP in MS, GA, and ABA treatments. Error bars indicate standard deviation.

(c) Representative images showing expression of 35S::GFP and 35S::GFP-CTD118 in MS, GA, and ABA treatments. Scale bars represent 20 μm.

(d) Western blot analysis of 35S::GFP and 35S::GFP-CTD118 in WT, MS, PAC, ABA, MS, PAC, and ABA treatments. Ponceau staining for loading control.

(e) Relative mRNA levels of 35S::GFP and 35S::GFP-CTD118 in MS, PAC, and ABA treatments. Error bars indicate standard deviation.
2.2.6 The FUS3 C terminus contains an important determinant of protein function

Targeted and constitutive expression of FUS3 to the epidermal layer using the ML1:FUS3-GFP construct rescues fus3-3 embryonic defects, delays growth and flowering time, and converts leaves into cotyledon-like organs (Gazzarini et al., 2004). The truncated FUS3ΔC-GFP is highly abundant during embryonic and vegetative development; thus we predicted that both FUS3:FUS3ΔC-GFP and ML1:FUS3ΔC-GFP would rescue fus3-3 and display strong vegetative phenotypes. Surprisingly, ML1:FUS3ΔC-GFP and FUS3:FUS3ΔC-GFP could only partly rescue fus3-3 embryonic defects, such as premature germination of green seeds, desiccation intolerance of dry seeds and overall seedling growth (Figure 8a-c). In agreement with this, the accumulation of SSPs, which are important for desiccation tolerance and early seedling growth, was only partly rescued in these lines (Figure S6). This finding corroborates previous results showing that a similar truncated form of FUS3 transiently induced in seedlings is unable to fully activate downstream SSP genes (Kagaya et al., 2005a).

During early vegetative development, ML1:FUS3ΔC-GFP and FUS3:FUS3ΔC-GFP were able to rescue the ectopic development of trichomes on most of fus3-3 cotyledons, but could not suppress trichome development on rosette leaves (Figure 8d-h; Figure S7). At later stages, ML1:FUS3ΔC-GFP and FUS3:FUS3ΔC-GFP plants developed rounder and smaller rosette and cauline leaves, resembling ML1:FUS3-GFP leaves (Figure 8i-p). However, these phenotypes were not as severe as those observed in ML1:FUS3-GFP lines and the adult plants were similar to wild type. Finally, ML1:FUS3ΔC-GFP plants grown under a long day cycle grew slower and were late bolting, similar to ML1:FUS3-GFP plants, but were not dwarf in stature (Figure 8q).

In summary, the truncated and more abundant FUS3ΔC-GFP variant could only partially rescue fus3-3 embryonic defects and was unable to cause phenotypes as strong as the full-length FUS3 when ectopically expressed during vegetative development. This indicates that FUS3 CTD is required for normal FUS3 function.
**Figure 8.** FUS3ΔC partly rescues *fus3*-3 embryonic and vegetative phenotypes.  
(a) to (c) % seed germination (gray bars) and seedling growth (black bars) of wild-type, *fus3*-3, *FUS3:FUS3-GFP* (*FFG*), *FUS3:FUS3ΔC-GFP* (*FFΔCG*), *ML1:FUS3-GFP* (*MFG*) and *ML1:FUS3ΔC-GFP* (*MFΔCG*) lines. (a) *FFΔCG* partly rescues precocious germination of *fus3*-3 immature (green) seeds. (b) and (c) *FFΔCG* and *MFΔCG* partly rescue desiccation intolerance of *fus3*-3 dry seeds. Averages of % germinating seeds or % seedling growth ± s.d. from three plates are shown.  
(d) to (q) Vegetative phenotypes of various lines. (d) to (h) *FFΔCG* and *MFΔCG* rescue trichome defects on *fus3*-3 cotyledons but do not suppress trichome development of rosette leaves. Arrows in (e) point to ectopic development of trichomes on *fus3*-3 cotyledons. *MFG* seedlings (f) develop glabrous rosette leaves, while *FFΔCG* (g) or *MFΔCG* (h) seedlings bear trichomes on rosette leaves.  
(i) to (m) *FFΔCG* and *MFΔCG* plants develop smaller and rounder rosette leaves, similar to *MFG*.  
(n) to (p) *MFG* and *MFΔCG* plants develop smaller and rounder cauline leaves in comparison to the elongated wild-type cauline leaves (see arrows and insets).  
(q) Late flowering phenotype of *MFG* and *MFΔCG* plants.
2.3 Discussion

The regulation of growth and development requires coordinated control of protein synthesis, localization and degradation in all organisms. Transcription factors are an important example of proteins whose level and localization in the cell is tightly controlled. Although a complex regulatory network controlling the *LEAFY COTYLEDON* expression patterns during embryogenesis has been proposed (Kroj et al., 2003; Kagaya et al., 2005a/b; To et al., 2006), very little is known about the regulation of these master embryonic regulators at the protein level. To obtain a better understanding of the regulation of the FUS3 protein during embryogenesis and germination, in this study we have A) used the *FUS3:FUS3-GFP* construct to compare FUS3 mRNA and protein accumulations and B) studied the role of FUS3-GFP variants in the regulation of FUS3 protein expression.

2.3.1 The expression pattern of the FUS3 protein does not correlate with that of its mRNA

In this study, we show that in *FUS3:FUS3-GFP* plants the *FUS3-GFP* mRNA peaks at mid and late embryogenesis (10-14 DAF), while the FUS3-GFP protein is detected only up to the walking-stick stage (10 DAF) and not at the mature stage (14 DAF). At these later stages the mRNA is still high, indicating that FUS3 protein levels do not correlate with changes in the mRNA levels and become uncoupled during mid-late embryogenesis. Furthermore, the FUS3-GFP fluorescence was weak and the fusion protein was not detected on immunoblots at 6, 10 or 14 DAF, suggesting that the level of the FUS3-GFP protein is maintained at low levels throughout embryonic development.

*FUS3* expression driven by its native promoter, which comprises 1.5 kb upstream of the FUS3 start site, recapitulates the expression pattern of the endogenous *FUS3* mRNA, since similar trends were found when measuring endogenous *FUS3* transcript in wild type (Figure 2a and S1). However, at 10 and 14 DAF *FUS3-GFP* mRNA was lower than the endogenous *FUS3* mRNA, suggesting the existence of enhancer regions further upstream the 1.5 kb promoter used here and/or increased stability of the endogenous *FUS3* versus the *FUS3-GFP* mRNA. The *FUS3p:FUS3-GFP* construct lacks the *FUS3* introns and 3’UTR regions, which may affect
FUS3 stability/level. It is unlikely that the promoter used in this study does not drive sufficient expression of FUS3 during late embryogenesis that can be detected at least by confocal microscopy. Firstly, similar mRNA levels are found at 10 and 14 DAF, both of which are much higher than those at 6 DAF, yet fluorescence can be detected at 6 and 10 DAF, but not at 14DAF. Secondly, using a FUS3:GFP transcriptional reporter, which shows an expression pattern similar to that of two FUS3:GUS reporters previously described (Kroj et al., 2003; Tsuchiya et al., 2004), a high GFP signal can be readily detected throughout embryogenesis even in mature embryos. Since the FUS3:FUS3-GFP construct is able to rescue the fus3-3 mutant phenotypes, including those related to late embryogenesis functions, it is most likely that this construct expresses FUS3 at a level comparable to that found endogenously.

2.3.2 The CTD is necessary and sufficient to reduce protein levels

Protein abundance is determined by transcriptional and translation rates coupled with the stability of individual proteins (Van Der Kelen et al., 2009). FUS3 contains several predicted phosphorylation sites at the NTD and CTD and a putative C-terminal PEST degradation motif (degron), which is a region rich in proline (P), glutamate (E), serine (S), threonine (T) present in several unstable proteins (Rechsteiner and Rogers, 1996). In this study we have provided evidences that the PEST-containing CTD is necessary and sufficient to negatively regulate protein levels and is required for efficient degradation of FUS3 through the 26S proteasome pathway. Indeed, a FUS3ΔC variant lacking the CTD accumulates in the cell up to 100 fold higher than its native form, depending on the promoter used and the stage of development. A higher level of FUS3ΔC versus FUS3 was found during embryogenesis, compared to germinating seeds or seedlings, suggesting that the CTD may control FUS3 abundance in a developmentally regulated manner. It has been shown that degradation of the closely related B3 transcription factor ABI3 is mediated by the 26S proteasome (Zhang et al., 2005). Our study demonstrates that FUS3 is a short-lived protein, with a half-life of approximately 15 minutes measured in cell-free degradation assays. Deletion of the CTD decreases FUS3 degradation rates, with a half-life of approximately 45 minutes for FUS3ΔC. We also show that FUS3 degradation is regulated by the 26S proteasome both in vitro and in vivo. It is possible that FUS3ΔC cannot interact, or interacts with lower efficiency, with factors responsible for FUS3
degradation through the 26S proteasome. Interestingly, MG132 treatments did not restore FUS3-GFP level to the levels of FUS3ΔC-GFP in vivo. This suggests that other mechanisms beside protein degradation control the increase in protein levels. Since translational regulation plays a major role together with protein degradation in regulating protein levels, it will be interesting to test whether the CTD also affects translational rates/efficiency.

The importance of the CTD in regulating FUS3 abundance is further exemplified with the GFP-CTD chimeras, which showed a decrease in GFP protein accumulation and fluorescence intensity. Thus, the FUS3 CTD is sufficient to induce low protein accumulation when fused to a stable protein such as GFP. Although the 37 amino acids of the PEST sequence alone are sufficient to reduce GFP fluorescence (8 fold), longer CTD sequences (CTD118 and CTD67) are more effective (16 fold). This suggests that sequences outside the PEST region could be important to negatively regulate protein levels, for example by allowing better interactions with proteins recruiting FUS3 for degradation and/or responsible for posttranslational modifications leading to degradation. It is unlikely that the CTD causes misfolding of GFP, which would lead to aggregation and/or degradation of the fusion proteins (Link et al., 2005). Firstly, GFP fluorescence was found in the nuclei and cytoplasm of 35S:GFP and all 35S:GFP-CTD transgenic plants, albeit with different fluorescence intensity and no aggregation of GFP or GFP sequestration in specific compartments was found. Secondly, two bands were detected on immunoblots for GFP and GFP-CTD during embryogenesis, the smaller band possibly corresponding to a cleavage product, however only one band was found during vegetative development for both constructs (Figure S4).

2.3.3 The NTD and CTD negatively regulates FUS3 mRNA accumulation

Our data point to a possible role of the CTD and NTD in the regulation of FUS3 expression. In FUS3:FUS3ΔC-GFP and FUS3:FUS3ΔN-GFP plants, deletion of either terminal domain causes an increase in the accumulation of FUS3 transcripts (10-20 fold) during embryogenesis and germination, suggesting that both domains negatively regulate FUS3 mRNA levels. However, in ML1:FUS3ΔC-GFP seedlings (4 DAI) deletion of the CTD can induce only a minor increase in FUS3ΔC mRNA (2.5 fold). This suggests that the CTD-mediated regulation of FUS3 mRNA
levels may also be developmentally controlled. It is also possible that *FUS3* 5’UTR sequences, which are absent in *ML1:FUS3-GFP* and *ML1:FUS3ΔC-GFP* constructs, play a major role in regulating *FUS3* mRNA accumulation and/or stability. Interestingly, fusion of the CTD to GFP did not cause a decrease in *GFP-CTD* mRNA, the latter accumulates to a similar or higher level than that of GFP. Thus, the CTD alone is not sufficient to reduce the mRNA level when fused to GFP.

The CTD and NTD regions of the *FUS3* mRNA may negatively regulate *FUS3* mRNA levels by posttranscriptional regulation, which affects mRNA stability, transport and/or localization. Since the *FUS3* CTD alone had little effect in mRNA levels in the ML1 and 35S-driven constructs, it is possible that other *FUS3* mRNA sequences are required for the NTD and CTD-mediated downregulation of transcript level. Alternatively, both terminal domains may influence transcriptional regulation of *FUS3*. Based on promoter reporter studies in different mutant backgrounds, To et al. (2006) proposed that *FUS3* expression during embryogenesis is positively regulated by *FUS3*/*LEC2*/*ABI3* in a combinatorial fashion, as *FUS3* expression is reduced in *fus3 lec2* double mutant, completely lost in the *fus3 lec2 abi3* triple mutant, but not altered in *fus3*. One possible explanation for the increase in *FUS3*:FUS3ΔC-GFP and *FUS3*:FUS3ΔN-GFP mRNA is that the truncated FUS3ΔC and FUS3ΔN variants interfere with the yet uncharacterized mechanism by which FUS3/LEC2/ABI3 increase *FUS3* expression, perhaps due to aberrant interactions with negative regulators of *FUS3* transcription. This would also explain why the lack or presence of the CTD had little effect on the mRNA levels of ML1 and 35S-driven constructs, respectively.

2.3.4 The CTD mediates ABA and GA responses and is required for *FUS3* function

*FUS3* has been shown to induce ABA and repress GA syntheses (Curaba et al., 2004; Gazzarrini et al., 2004). In turn, ABA stabilizes while GA destabilizes the *FUS3* protein, respectively (Gazzarrini et al., 2004). Therefore, a decrease in the ABA/GA ratio during late embryogenesis and germination has been proposed to destabilize *FUS3*, which is undetected at these stages despite the presence of *FUS3* mRNA (Gazzarrini et al., 2004; Figure 2). Our data indicates that the *FUS3* CTD mediates sensitivity to ABA and GA, as deletion of this domain renders
FUS3ΔC-GFP resistant to GA-induced destabilization and ABA-mediated stabilization. Supporting this mechanism, the fluorescence of the GFP-CTD chimera can be increased by ABA and paclobutrazol treatments, indicating that the FUS3 CTD responds to changes in ABA/GA levels. The stabilizing effect of ABA on GFP-CTD seems to be more spatially-restricted (confined to the epidermis of the hypocotyls) and weaker than that of paclobutrazol. This is possibly due to the fact that ABA treatments cause approximately a two-fold reduction in mRNA levels and that paclobutrazol can inhibit not only GA biosynthesis but also ABA degradation, thus having a synergistic effect on FUS3 stability (Saito et al., 2006). It is possible for ABA and GA to regulate a common factor to control FUS3 degradation. Alternatively, ABA and GA could operate through different pathways including regulating the syntheses of each other and thus altering the ABA/GA ratio. Finally, ABA and GA could affect translation efficiency of FUS3. Notably, FUS3 mRNA and ABA levels progressively increase during embryogenesis and are highest during mid-embryogenesis (Gazzarrini et al., 2004; Figure 2). The temporal and spatial development of hormonal gradients during embryogenesis and germination is expected to play a role in controlling FUS3 abundance.

Phenotypic analysis of the various transgenic lines expressing the deletion constructs shows that the CTD is required for proper FUS3 function, since the stable FUS3ΔC is unable to fully rescue the fus3-3 mutant and to induce the strong dominant phenotypes observed with full-length FUS3 during vegetative development. This can be explained by the fact that FUS3ΔC lacks the entire CTD, which includes the activation domain. These results support previous findings showing that a FUS3 CTD-deletion variant lacking the entire CTD is unable to activate FUS3 downstream target genes, such as the seed storage protein At2S3, when ectopically and transiently expressed during vegetative development (Kagaya et al., 2005). Finally, it is also possible that a high accumulation of FUS3ΔC interferes with the function of other regulators of mid-late embryogenesis, such as LEC2 and ABI3. This could disrupt a complex network of regulatory processes, since all three embryonic regulators possess a conserved B3 DNA-binding domain and can bind to the same regulatory elements, although with different affinities (Suzuki et al., 1997; Monke et al., 2004; Braybrook et al., 2006). Interestingly, a fus3 T-DNA insertion mutant has been shown to be a true null mutant and to display weaker embryonic phenotypes compared to the fus3-3 mutant (Tiedemann et al., 2008). This difference of penetrance may be
due to the activity of a C-terminal truncated neomorphic FUS3 protein potentially made in fus3-3. This could also explain the inability of FUS3ΔC to fully rescue fus3-3.

Taken together, our data suggests that FUS3 is a short-lived and low abundant protein and that its accumulation in the cell is under tight control and regulated at multiple levels. Although our data is compatible with a model in which the FUS3 protein is maintained at a low expression level during embryogenesis and germination by posttranscriptional and posttranslational regulation involving the CTD, additional pathways could also contribute to inhibition of FUS3 protein expression at these stages. Translational regulation plays an important role in controlling protein abundance. The translation of individual transcripts can be affected by global repression of protein synthesis, developmental programs, environmental stimuli and hormones (Kawaguchi and Bailey-Serres, 2002; Kawaguchi and Bailey-Serres, 2005). In the future, it will be interesting to investigate whether mRNA translation, transport and localization affect FUS3 accumulation and localization and the role of the CTD in these processes.

2.4 Experimental procedures

2.4.1 Plant material, growth conditions and seed germination

Arabidopsis seeds (Columbia ecotype) of various genotypes were germinated on half-strength Murashige and Skoog (MS) media containing 5 mM MES (pH 5.7) (Sigma). For germination assays, 300-600 seeds were surface sterilized, chilled for 5 days and germinated for 7 days at room temperature under constant light. Experiments were repeated at least twice. Seedlings transferred to soil were grown in controlled environmental chambers at 21 °C under constant light. Seedlings used for the flowering time experiment were grown under a regime of 16 hours light at 22 °C and 8 hours dark at 18 °C. For in vivo MG132 experiments, 4 DAG seedlings were transferred to liquid MS media containing 5 mM MES, 100 µM CHX (Sigma), and 50 µM MG132 (Peptides International) or DMSO as mock treatment for the indicated times.

2.4.2 Cell-free degradation assays

Cell-free degradation assays were conducted essentially as described in Wang et al. (2009). Ten-day-old seedlings were ground in liquid nitrogen, resuspended in degradation buffer (25 mM
Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 5 mM DTT, 4 mM PMSF, 10 mM ATP, 100 µM cyclohexamide) and clarified by two 10-min centrifugation steps at 20,000 g at 4 ºC. Protein concentration was determined by the Bradford protein assay (BioRad). Equal amounts of extracts were transferred to individual tubes and incubated at room temperature with or without 40 µM MG132, for the indicated times. For each assay, 100 ng of recombinant E. coli–purified GST-FUS3 and GST-FUS3ΔC was incubated in 100 µL of cell extract (containing 200 µg total proteins) obtained from 10-day-old wild-type seedlings. Reactions were stopped by adding SDS sample buffer. See Supplementary Experimental Procedure for cloning and expression of the GST-fusion proteins.

2.4.3 Immunoblot analysis
Siliques and seedlings were crushed in liquid nitrogen and homogenized in extraction buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, 1 mM PMSF (Bioshop), 1X plant protease inhibitor cocktail (Sigma). Dry or germinating seeds were homogenized in extraction buffer containing 100 mM Tris-HCl (pH 8), 0.5% SDS, 10% glycerol, 2% β-mercaptoethanol, 1 mM PMSF and 1X plant protease inhibitor cocktail. The tissue extracts were boiled for 3 minutes, centrifuged and the supernatant was collected. The concentrations of the protein extracts were measured by Bradford assay using Quick Start Bradford dye reagent (BioRad). GFP and GFP-fusion proteins were detected using a rabbit anti-GFP polyclonal antibody (Abcam). GST-fusion proteins were detected using a HRP conjugated to goat anti-GST polyclonal antibody (GE Healthcare). SuperSignal West Pico chemiluminescent substrate (Pierce) was used for band detection.

2.4.4 Expression analysis
Total RNA was extracted using RNeasy miniprep kit (Qiagen), according to manufacturer’s instructions. RNA was precipitated with 20% isopropanol, 0.24 M sodium citrate and 0.16 M NaCl, followed by a second precipitation with 2 M LiCl. 1 µg of RNA was treated with RNase-free DNaseI (Fermentas) to eliminate genomic DNA contamination. First strand cDNA was synthesized according to manufacturer’s protocols (Invitrogen). Quantitative reverse-transcription PCR (qRT-PCR) was performed using SYBR Green (BioRad). Efficiency of each pair of primers was determined based on its standard curve. The mean value of three replicates was normalized using ACTIN 7 as the internal control. Results are plotted as the ratio to the
lowest detected level. Two independent experiments were conducted with similar results and one is shown. Primers used for qRT-PCR are listed in Table S1.

2.4.5 Confocal microscopy

Confocal microscopy was performed using Zeiss LSM 510 META confocal microscope. GFP was excited at 488 nm, and detected at 505-530 nm (green channel). Fluorescence from propidium iodide and autofluorescence from chlorophyll were detected at ≥ 585 nm (red channel). All samples were mounted either in water or in 25 µg/mL propidium iodide with 0.1% Sil-wet.

2.4.6 Quantification of GFP fluorescence

GFP quantification was performed using Carl Zeiss LSM510 version 3.2 SP2 software. Confocal images of walking-stick embryos or epidermal cells of leaf primordia, taken under comparable laser settings, were used for measurement of GFP intensities. For each image, three equal-sized areas were selected and the average GFP intensity was calculated. The average background intensity measured in wild type or fus3-3 samples was subtracted from the average intensity calculated from the samples.

2.4.7 Quantification of immunoblot images

Band intensities on Western blots were quantified using ImageJ (http://rsb.info.nih.gov/ij/index.html). Pixel values were measured on equal sized areas and normalized against the non-specific bands in each lane. The intensity values shown in the paper are the ratios relative to the reference treatment(s).

2.4.8 Supplementary experimental procedure

2.4.8.1 Cloning and generation of transgenic plants

The FUS3ΔC and FUS3ΔN constructs were created by PCR using FUS3 7Z/pGEM as template (Gazzarrini et al., 2004). T7-for and EcoRI-FUS3ΔC-rev primers were used to amplify FUS3ΔC, HindIII-FUS3ΔN-fwd and Fus3-EcoRI-R (Gazzarrini et al., 2004) primers were used to amplify FUS3ΔN. The PCR fragments were subcloned into pBluescript (pBS) by replacing the FUS3 fragment of the FUS3-GFP/pBS plasmid previously described (Gazzarrini et al.,
The resulting \textit{FUS3\textDelta C-GFP} and \textit{FUS3\textDelta N-GFP} fragments were excised and cloned into \textit{pBI101} by replacing the \textit{FUS3-GFP} fragment of \textit{FUS3:FUS3-GFP/pBI} and \textit{ML1:FUS3-GFP/pBI} plasmids, previously described (Gazzarrini \textit{et al.}, 2004). To clone the \textit{FUS3-GFP} transcriptional fusion construct, GFP was amplified by PCR using \textit{FUS3-GFP/pBS} as template, and KpnI-XhoI-GFP-fwd and T7-for primers. The PCR fragment was cloned into \textit{pBI101} by replacing the \textit{FUS3\textDelta C-GFP} fragment from \textit{FUS3:FUS3\textDelta C-GFP/pBI} plasmid. To construct \textit{35S:GFP-CTD\textsuperscript{118}}, \textit{35S:GFP-CTD\textsuperscript{67}} and \textit{35S:GFP-CTD\textsuperscript{37}}, the \textit{CTD\textsuperscript{118}}, \textit{CTD\textsuperscript{67}}, \textit{CTD\textsuperscript{37}} fragments were amplified by PCR using primer sets shown in Figure S8. The resulting PCR products were cloned into \textit{pEGAD} vector and sequenced (Cutler \textit{et al.}, 2000). All constructs were transformed into Arabidopsis by floral dip (Clough and Bent, 1998). Transgenic \textit{FF\textDelta CG}, \textit{FF\textDelta NG} and \textit{MF\textDelta CG} plants were generated in a \textit{fus3-3} background and \textit{FUS3:GFP} plants were generated in a wild type (Col) background. These transgenic lines were selected on media containing 25\textmu g/mL kanamycin (Sigma). \textit{35S:GFP}, \textit{35S:GFP-CTD\textsuperscript{118}}, \textit{35S:GFP-CTD\textsuperscript{67}}, \textit{35S:GFP-CTD\textsuperscript{37}} lines were generated in a wild-type (Col) background and selected on media containing 50 \textmu g/mL glufosinate ammonium salt (Crescent Chemical). Eight to fifteen transgenic lines were generated per construct and screened for GFP fluorescence. Three representative lines per construct were chosen for further characterization.

\subsection*{2.4.8.2 Cloning and purification of GST-fusion constructs}

For purification of recombinant GST-FUS3 and GST-FUS3\textDelta C proteins, FUS3 and FUS3\textDelta C were amplified by PCR using \textit{FUS3 7Z/pGEM} as template (Gazzarrini \textit{et al.}, 2004) and the following primers: FUS940-SmaI-F and FUS940-EcoRI-R3, for amplification of full-length FUS3, EcoRI-FUS3-pGEX5’ and FUS-XhoI-pEG202-R1, for amplification of FUS3\textDelta C (see Table I for primers sequences). FUS3 and FUS3\textDelta C PCR fragments were cloned into pGEX-2TK and pGEX-6P-3 vectors (GE Healthcare), respectively. The GST-fusion constructs were expressed in \textit{E. coli} strain BL21 and purified using Glutathione resin (Sigma-Aldrich) according to the manufacture’s protocol.

\subsection*{2.5 Acknowledgement}

We thank Dan Riggs, Kevin Breitkreuz and Dario Bonetta for critical reading of the manuscript. This work was supported by NSERC and CFI grants to S.G.
2.6 Supplementary Table and Figures

**Figure S1.** FUS3 mRNA levels during embryogenesis and germination.

a) Raw data of FUS3 (At3g26790) relative mRNA levels during embryogenesis and germination were obtained from The Bio-array resource database (http://bar.utoronto.ca; Toufighi et al., 2005). Average of replicate treatments ± s.d. are shown.

b) 'electronic fluorescent pictographic' (eFP) representation of FUS3 expression pattern during embryogenesis using the eFP browser at The Bio-array resource database (http://bar.utoronto.ca; Toufighi et al., 2005). Drawing of silique and seed stages corresponding to those shown in (a).

c) Heat map showing colors corresponding to averages of relative mRNA levels used in (b).
(a) Relative mRNA levels during embryogenesis.

(b) Relative mRNA levels during germination.

(c) Images illustrating different stages of embryogenesis and germination.
Figure S2. Quantification of GFP fluorescence in embryos of various transgenic lines.

(a) Relative fluorescence intensities measured in fus3-3 walking-stick embryos harboring FUS3: FUS3-GFP (FFG) or FUS3: FUS3ΔC-GFP (FFΔCG) constructs.

(b) Relative fluorescence intensities measured in walking-stick embryos of ML1: FUS3-GFP (MFG) and six ML1: FUS3ΔC-GFP (MFΔCG) transgenic lines.

Three equal sized areas on the cotyledons of walking-stick embryos were selected for quantification and the average intensities are shown. Bars indicate averages ± s.d.
**Figure S3.** Quantification of FUS3-GFP mRNA levels and GFP fluorescence in *ML1:FUS3-GFP* and *ML1:FUS3ΔC-GFP* seedlings

a) Relative mRNA expression levels (gray bars) was measured by qRT-PCR in 4-days-old *ML1:FUS3ΔC-GFP* and *ML1:FUS3-GFP* seedlings. Relative fluorescence intensity (black bars) was quantified in leaf primordia of 4-days-old *ML1: FUS3ΔC-GFP* and *ML1: FUS3-GFP* seedlings. Numbers indicate different independently transformed lines. Values are averages from triplicates ± s.d.

b) Confocal images of *ML1:FUS3-GFP* and *ML1:FUS3ΔC-GFP* 4 DAG seedlings. Right column shows paradermal optical sections of the leaf primordia shown in the left column.
a) Relative fluorescence intensity and relative mRNA levels for different samples.

ML1: FUS3-GFP

ML1: FUS3ΔC-GFP

#1, #9, #13, #42, #d6, #13, #22

b) Fluorescence images of ML1:FUS3ΔC-GFP and ML1:FUS3-GFP.
Figure S4. Accumulations of GFP and GFP-FUS3 variants in embryos and germinating seedlings.

a) Immunoblot analysis showing FUS3ΔC-GFP accumulation in 10 DAF ML1:FUS3ΔC-GFP and FUS3:FUS3ΔC-GFP embryos. Immunoblots were probed with anti-GFP antibody. Top panel shows a short film exposure showing three bands, a predominant lower molecular weight band and two higher molecular weight bands (see arrows) of weaker intensity. Middle panel shows a longer film exposure of the same blot. Ponceau staining is shown as loading control.

b) Immunoblot analysis showing GFP and GFP-CTD\textsuperscript{118} levels in 15 DAG seedlings of 35S:GFP and 35S:GFP-CTD\textsuperscript{118}, respectively. A single band is detected in 35S:GFP and 35S:GFP-CTD\textsuperscript{118} lines. Numbers indicate independent transgenic lines. Star indicates a cross-reacting band. Immunoblots were probed with anti-GFP antibody. Ponceau staining is shown as loading control.
(a) ML1:FUS3ΔC-GFP

FUS3ΔC-GFP short exposure

FUS3ΔC-GFP long exposure

Ponceau staining

(b) 35S:GFP-CTD118

43 -

34 -

GFP-CTD118

GFP

Ponceau staining
Figure S5. Quantification of GFP fluorescence in \textit{ML1: FUS3-GFP} and \textit{ML1: FUS3ΔC-GFP}

Relative fluorescence intensity quantified in three \textit{ML1: FUS3ΔC-GFP} and \textit{ML1: FUS3-GFP}
leaf primordia treated with or without hormones for 4 days. Three areas of equal size were
selected on the leaf primordia for quantification and the average intensities are shown. Bars
indicate s.d.
ML1: FUS3-GFP

ML1: FUS3ΔC-GFP
**Figure S6.** Accumulation of seed storage proteins in mature seeds of various genotypes.
SDS-PAGE gels of proteins isolated from mature seeds of wild type (WT), *fus3-3*, *FUS3:FUS3ΔC-GFP* (*FFΔCG*), *FUS3:FUS3-GFP* (*FFG*), *ML1: FUS3-GFP* (*MFG*) and *ML1: FUS3ΔC-GFP* (*MFΔCG*) lines. Seeds were allowed to dry for 6 weeks. Proteins were extracted from approximately 100 seeds of each genotype, quantified by Bradford assay and equal amounts of total protein extracts were loaded on a 15% SDS-PAGE gel. Proteins were visualized by Coomassie staining. 12S and 2S seed storage proteins are indicated on the left. These experiments were repeated twice. Numbers below genotypes denote individual transgenic lines.
Figure S7. Phenotype penetrance quantification of trichomes present on the cotyledons of various genotypes.

Graph showing the % of seedlings of wt, fus3-3 and four independently transformed FUS3:FUS3ΔC-GFP (FFΔCG) lines that develop ectopic trichomes on the cotyledons seven days after germination. Bars indicate s.d.
% of seedlings
with trichomes on the cotyledons

- fus3-3
- wt
- FFΔCG d2
- FFΔCG d4
- FFΔCG d3
- FFΔCG d6
Table S1. Primers used in this study
FUS3\textsubscript{190}-for and FUS3\textsubscript{190}-rev primers were used to amplify 190 bp of *FUS3* and ACT7\textsubscript{190}-for and ACT7\textsubscript{190}-rev were used to amplify 190 bp of *ACTIN7* as reference. GFP\textsubscript{546}-for and GFP\textsubscript{546}-rev primers were used to amplify 546 bp of *GFP* and ACT7\textsubscript{555}-for and ACT7\textsubscript{555}-rev were used to amplify 555 bp of *ACTIN7* as reference.
**Table S1.** Primers used in this study

<table>
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<th>Primer Name</th>
<th>Sequence 5’ – 3’</th>
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<td>KpnI-XhoI-GFP-fwd</td>
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<tr>
<td>T7-for</td>
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2.6 References


Chapter 3

AKIN10 and FUSCA3 interact to control lateral organ development and phase transitions in Arabidopsis

Previously published as:

SUMMARY

The Snf1/AMPK/SnRK1 kinases act as sensors of energy status in eukaryotes. Despite their important role in regulating cellular responses to metabolic stress, only few SnRK1 substrates have been identified. Using yeast-two hybrid screens, we have isolated AKIN10 as an interactor of the B3-domain transcription factor FUSCA3 (FUS3), an essential regulator of seed maturation in Arabidopsis. Pull-down and BiFC assays confirm the interaction in vitro and in planta, respectively. In-gel kinase assays show that AKIN10 phosphorylates FUS3 and that the N-terminal domain of FUS3 is required for AKIN10 phosphorylation. Mutations of three serines (fus3S55A/S56A/S57A) within a partial SnRK1 consensus sequence in the N-terminal region of FUS3 greatly reduces FUS3 phosphorylation by AKIN10, indicating that these serines are the predominant AKIN10 target sites. In a cell-free system AKIN10 positively regulates FUS3 stability, as overexpression of AKIN10 delays the degradation of the recombinant FUS3. Plants overexpressing AKIN10 show delayed seed germination, vegetative growth and flowering time, indicating that AKIN10 antagonizes the embryonic-to-vegetative and vegetative-to-reproductive phase transitions. Furthermore, overexpression of AKIN10 alters cotyledon, silique and floral organs development, suggesting that AKIN10 regulates lateral organ development. Genetic interaction studies show that the fus3-3 mutation partially rescues the phase transition and organ development defects caused by AKIN10 overexpression. Taken together, these findings indicate that FUS3 and AKIN10 physically interact and share overlapping pathways to regulate developmental phase transitions and organogenesis in Arabidopsis.
3.1 Introduction

During the early stages of embryogenesis, the zygote undergoes a series of cell divisions leading to pattern formation. The hormone auxin plays an essential role in the establishment of apical-basal polarity and coordinates organ development (Bowman and Floyd, 2008). During mid-late embryogenesis the embryo enters a maturation phase, during which it synthesizes storage compounds in preparation to enter desiccation and dormancy. Seed storage reserves, such as proteins and lipids, are used in the early days post-germination to sustain growth of the young seedling. The hormones abscisic acid (ABA) and gibberellic acid (GA) play predominant and opposite roles in the control of seed dormancy and germination. The breaking of seed dormancy and the onset of germination are important checkpoints during the life cycle of angiosperms, as the seeds integrate environmental and endogenous signals to coordinate growth under favorable conditions.

In Arabidopsis, the control of seed dormancy and germination is governed by hormonal changes and an intricate network of transcriptional regulation, including two clades of the B3 domain superfamily of transcription factors (Suzuki and McCarty, 2008). The CCAAT-binding LEAFY COTYLEDON1 (LEC1) gene, together with the B3 domain ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3) and LEAFY COTYLEDON2 (LEC2) genes, are expressed during embryogenesis and induce late embryonic development (Lotan et al., 1998; Stone et al., 2001; Kroj et al., 2003; Tsuchiya et al., 2004). A complex and interdependent regulatory network between these genes, not yet fully understood, controls their expression patterns and functions (Kroj et al., 2003; Kagaya et al., 2005a/b; To et al., 2006).

Loss- and gain-of-function mutations in FUS3 result in heterochronic developmental shifts (Keith et al., 1994; Nambara et al., 2000; Gazzarrini et al., 2004). Indeed, the fus3-3 mutant develops embryos that bypass the maturation phase and enter vegetative development precociously. As a result, fus3-3 embryos accumulate less seed storage proteins, are less dormant and desiccation intolerant (Keith et al., 1994). In contrast, ectopic expression of FUS3 during vegetative development (ML1:FUS3) results in the prolongation of the embryonic program. ML1:FUS3 plants produce hyperdormant seeds, develop cotyledon-like organs throughout vegetative growth and delay flowering (Gazzarrini et al., 2004; Lu et al., 2010).
FUS3 controls developmental phase transitions by negatively regulating GA, while positively regulating ABA syntheses; these hormones negatively and positively regulate the stability of the FUS3 protein, respectively (Nambara et al., 2000; Curaba et al., 2004; Gazzarrini et al., 2004). Recently, the C-terminal domain (CTD) of FUS3 has been shown to be responsible for the instability of the FUS3 protein and to be involved in hormone sensitivity (Lu et al., 2010). FUS3 expression is induced by auxin, while auxin biosynthetic genes are positively regulated by FUS3, thus establishing a positive feedback regulation during embryonic development (Gazzarrini et al., 2004; Yamamoto et al., 2010). Ultimately, these studies demonstrate that FUS3 is a point of convergence of several hormone-signaling pathways during seed development.

Given the importance of the B3/LEC1 genes during embryogenesis, very little is known about the protein complexes that they form. So far, only few ABI3 interacting proteins (AIPs) have been identified in Arabidopsis, and only the Opaque2-like bZIP factor BLZ2 has been identified as an HvFUS3 interactor in Barley (Hordeum vulgare L.; Kurup et al., 2000; Zhang et al., 2005; Moreno-Risueno et al. 2008).

Members of the Sucrose non-fermenting1 (Snf1)-related protein kinase1 (SnRK1) are activated in response to starvation or metabolic stress, similarly to their animal (AMPK) and yeast (Snf1) counterparts, and regulate gene expression at a global level (Baena-Gonzalez and Sheen, 2008; Sheen 2010). In addition, several studies have demonstrated that SnRK1s are required for the synthesis of proteins and starch in storage organs, processes that are not induced by starvation or energy limitation (Halford and Hey, 2009). Thus, it appears that SnRK1s sense the energy status as opposed to respond solely to energy deprivation. Interestingly, recent studies have linked the catalytic subunit of the SnRK1 complex to hormone signaling and seed development in plants (Radchuk et al., 2006; Jossier et al., 2009; Radchuk et al., 2010). SnRK1-antisense pea (Pisum sativum) seeds have maturation defects including reduced storage products, precocious germination and reduced desiccation tolerance, phenotypes correlating with a downregulation of PsFUS3 and PsABI3 expression levels and a reduction in ABA accumulation in this mutant (Radchuk et al., 2006; Radchuk et al., 2010). The catalytic and regulatory subunits of the SnRK1s have been shown to be involved in hormone metabolism and signaling (Bradford 2003;
Baena-Gonzalez et al., 2007; Jossier et al., 2009; Radchuk et al., 2010). Thus, SnRK1s are also emerging as important nodes in hormone signaling and metabolism.

Considering the magnitude of reprogramming in gene expression induced by changes in SnRK1 levels, only few substrates were shown to be phosphorylated by SnRK1 (Baena-Gonzalez et al., 2007; Halford and Hey, 2009). In this study we have identified the SnRK1 kinase AKIN10 as a FUS3 interactor in yeast two-hybrid (Y2H) screens. The interaction between FUS3 and AKIN10 is confirmed in vitro and in planta. We also show that AKIN10 phosphorylates FUS3 and acts as a positive regulator of FUS3 level. Our biochemical and genetic interaction studies indicate that FUS3 and AKIN10 interact to regulate lateral organ development and phase transitions in Arabidopsis.

3.2 Results

3.2.1 Identification of AKIN10 as a FUS3-interacting protein

To elucidate the role of FUS3 during embryonic development and phase transition, we identified FUS3 interacting proteins using the Y2H system. Due to the presence of the activation and PEST-instability domains at the CTD of FUS3, the full-length FUS3 bait was not suitable for Y2H screens (Lu et al., 2010). These domains were removed and two deletion constructs were used as the baits (Figure 1a): FUS3(ΔC), lacking the activation domain and PEST-instability region, and FUS3(N90), comprising only the first 90 amino acids of FUS3. Both constructs were stably expressed in yeast and did not autoactivate the reporters. Yeast containing bait and reporter plasmids were transformed with a cDNA library made from Arabidopsis flowers, siliques and germinating seeds. From 10^6 transformants per screen, AKIN10 was recovered in 65 clones using FUS3(N90) and in 3 clones using FUS3(ΔC). Since the AKIN10 clone was truncated at the 5’-end, lacking a portion of the kinase domain (Figure 1a; AKIN10(ΔN)), we generated a full-length AKIN10 prey to validate the interaction (Figure 1a). Co-expression of both FUS3 baits with the full-length AKIN10 prey results in yeast growth on selective media (Figure 1b). These results indicate that FUS3 and AKIN10 physically interact and that the N-terminal region of FUS3 and the C-terminal region of AKIN10 are sufficient for the interaction.
3.2.2 FUS3 interacts with AKIN10 \textit{in vitro} and \textit{in planta}

To validate the Y2H results, pull-down assays were performed. Both AKIN10 and AKIN10(ΔN) from soluble yeast extracts were pulled down by GST-FUS3, but not by GST alone (Figure 1c). To test whether FUS3 could bind AKIN10 from plant extracts, we generated 17 transgenic plants overexpressing \textit{AKIN10 (35S:AKIN10-HA)} and selected 4 lines showing transcript levels higher than WT (Figure S2a). Indeed, AKIN10-HA from soluble extract of three independent \textit{35S:AKIN10-HA} plants was also pulled down by GST-FUS3, but not by GST alone, indicating that \textit{in vitro} FUS3 interacts with AKIN10 expressed in either yeast or plants (Figure 1c).

We next tested the interaction between AKIN10 and FUS3 \textit{in planta}. First, we monitored the subcellular localization of each protein in \textit{Nicotiana benthamiana}, and found that both AKIN10-YFP and FUS3-GFP strongly localize to the nuclei and weakly to the cytoplasm of epidermal cells (Figure 1d). AKIN10-YFP fluorescence was also detected in punctate aggregates of unknown origin, which did not colocalize with autofluorescence from the chloroplasts (Figure 1d; Figure S1). To determine whether FUS3 interacts with AKIN10 \textit{in planta}, we employed bimolecular fluorescence complementation (BiFC; Figure 1e). Coexpression of the N-terminal half of YFP fused to FUS3 (nYFP-FUS3) and the C-terminal half of YFP fused to AKIN10 (cYFP-AKIN10) in \textit{N. benthamiana} leaves resulted in fluorescence predominantly in the nuclei of epidermal cells. No interactions were detected between AKIN10 and MYB49, an unrelated transcription factor, and between FUS3 and SnRK3.15, a different SnRK kinase, confirming the specificity of the interaction. These data indicate that FUS3 interacts with AKIN10 \textit{in planta} and that the interaction occurs predominantly in the nuclei.
**Figure 1.** FUS3 interacts with AKIN10 in Y2H, *in vitro* and *in planta.*

(a) Schematic diagrams of FUS3 and AKIN10 domains and deletion constructs used in the Y2H. The basic B2 and B3 domains, the activation domain (A) and the putative PEST sequences of FUS3 are shown. The protein kinase domain (PK), ubiquitin-associated domain (UBA), γ-subunit associated domain (γ) and β-subunit associated domain (β) of AKIN10 are shown. Numbers indicate amino acid positions.

(b) FUS3 interacts with AKIN10 in Y2H. Rows, FUS3 bait constructs fused to the LexA DNA-binding domain (BD); columns, prey constructs fused to the activation domain (AD). Vector containing AD alone acts as negative control.

(c) *In-vitro* interaction of FUS3 with AKIN10. Pull-down assays using GST-FUS3 and GST as the baits with cell extracts from yeast expressing full-length AKIN10-HA or AKIN10(ΔN)-HA (top two rows), or from three independent 35S:AKIN10-HA plants (bottom three rows). Input: AKIN10 from cell extracts only. AKIN10 was detected using anti-HA antibody.

(d) Localization of FUS3 and AKIN10 in *N. benthamiana* pavement cells transiently expressing GFP-FUS3 or YFP-AKIN10.

(e) BiFC interaction assays in *N. benthamiana* pavement cells co-expressing nYFP-FUS3 and cYFP-AKIN10 or nYFP-MYB49 and cYFP-AKIN10, showing interaction between FUS3 and AKIN10, but not between MYB49/AKIN10 and FUS3/SnRK3.15. Labels above each panel indicate the filter channel imaged. DIC, differential interference contrast image.
(a) Protein domains and mutations:

- **FUS3**
  - B2
  - B3
  - PEST domain

- **FUS3(ΔC)**
  - B2
  - B3

- **FUS3(N90)**
  - B2
  - 271
  - 535

- **AKIN10**
  - PK
  - UB
  - γ
  - β
  - PEST domain

(b) Proteins and interactions:

- **BD-FUS3(N90)**
- **BD-FUS3(ΔC)**

(c) Mass spectrometry and kD:

- **AKIN10-HA (yeast)**
- **AKIN10(ΔN)-HA (yeast)**
- **AKIN10-HA #7 (plant)**
- **AKIN10-HA #10 (plant)**
- **AKIN10-HA #11 (plant)**

(d) Fluorescence images:

- **DIC**
- **GFP/YFP Merge**
- **GFP-FUS3**
- **YFP-AKIN10**

(e) Fluorescence images with additional constructs:

- **nYFP-FUS3 + cYFP-AKIN10**
- **nYFP-MYB49 + cYFP-AKIN10**
- **nYFP-FUS3 + cYFP-SnRK3.15**
3.2.3 FUS3 is phosphorylated by AKIN10

To test whether FUS3 is a substrate of AKIN10, we performed in-gel kinase assays using recombinant FUS3 as the substrate and cell extracts from WT and 35S:AKIN10-HA seedlings. We attempted to isolate akin10 loss-of-function mutants, however none of the T-DNA insertion lines we tested were null mutants, including the akin10-1 mutant previously characterized (Fragoso et al., 2009; Figure S2).

In-gel kinase assays using GST-FUS3 as the substrate show one strong band of ~60 kD in WT and 35S:AKIN10-HA cell extracts and one band of ~66 kD only in 35S:AKIN10-HA cell extracts (Figure 2b-i, c-i). The MW of these bands corresponds to the sizes of the endogenous AKIN10 and the AKIN10-HA fusion proteins detected on immunoblots using anti-AKIN10 and anti-HA antibodies (Figure 2e). Thus, the 60 kD band likely represents the kinase activities of the endogenous AKIN10 and possibly the closely related AKIN11, both of which have a MW of ~60 kD and are the most active SnRK1 kinases in seedling extracts (Zhang et al., 2009). Furthermore, AKIN10-HA immunoprecipitated from 35S:AKIN10-HA extract shows only the ~66 kD band (Figure 2c-i), confirming the ~66 kD phosphorylation activity is indeed contributed by AKIN10-HA. Very weak 40-45 kD kinase activities are detected in WT and 35S:AKIN10-HA, suggesting that other kinases phosphorylate FUS3 (Figure 2b-i, 2c-i). No kinase activity is present in the negative control (GST), indicating that the signals are not due to kinase autophosphorylation (Figure 2b-iv).

To identify the region of FUS3 required for AKIN10-mediated phosphorylation, we made two deletion constructs; one lacking the C-terminal region (GST-FUS3∆C) and one lacking the N-terminal region (GST-FUS3∆N) flanking the B3 domain (Figure 2a,d). Of the two constructs, only GST-FUS3∆C is phosphorylated by AKIN10 (Figure 2b-ii, 2b-iii). Interestingly, the 45 kD band is much more evident using the GST-FUS3∆C substrate, suggesting that the CTD may negatively regulate the activity of these smaller kinase(s). In contrast, when the GST-FUS3∆N fusion protein is used as the substrate the 60 and 66 kD kinase activities are barely detectable. Thus, FUS3 phosphorylation by AKIN10 requires FUS3 N-terminal region.

Among the 47 serines (S) and threonines (T) sites present in the FUS3 sequence, the NetPhos2 algorithm predicted 14 S and 5 T to be phosphorylated (Blom et al., 1999; Lu et al., 2010;
Figure S3a,b). Among these, only the sequences around S55, S56, S57 and S194 partially match the SnRK1 consensus sequence established thus far, with slight differences in the positions of the predicted hydrophobic residues (Figure 2f). Furthermore, these predicted SnRK1 target motifs are highly conserved in orthologous FUS3 proteins from different plant species (Figure S3c,d). Simultaneous mutations of S55, S56 and S57 into alanines (fus3S55A/S56A/S57A) dramatically reduce the 60 and 66 kD kinase activities, suggesting that FUS3 is phosphorylated at one or more of these residues (Figure 2c-ii). In contrast, no change in phosphorylation activity is detected in the fus3S194A mutant (Figure 2c-iii). In agreement with this, simultaneous mutation of all four serines (fus3S55A/S56A/S57A/S194A) does not further reduce the phosphorylation activity detected in fus3S55A/S56A/S57A (Figure 2c-iv). Lastly, mutation of S24/S26 (fus3S24A/S26A) does not change FUS3 phosphorylation (Figure 2c-v). These sites are located in the FUS3 N-terminal region and are highly predicted to be phosphorylated, but do not match the SnRK1 consensus sequence (Figure S3b).

Taken together, these data indicate that FUS3 is phosphorylated at one or more of the S55/S56/S57 residues, and that phosphorylation of FUS3 by AKIN10 requires FUS3 N-terminal region. It also suggests that FUS3 is phosphorylated by ~40-45 kD kinases.
Figure 2. Phosphorylation of recombinant FUS3 variant proteins by in-gel kinase assays.
(a) Schematic diagrams of the GST-fusion constructs used in the in-gel kinase assays.
(b), (c) In-gel kinase assays conducted with proteins extracted from 7-day-old WT and 35S:AKIN10-HA seedlings or immunoprecipitated AKIN10-HA (c-i). The arrowheads in (b) and (c) indicate the expected AKIN10 and AKIN10-HA kinase activities.
(d) Immunoblots showing GST-FUS3 variants used as the substrates in the kinase assays.
(e) Immunoblot showing AKIN10 and AKIN10-HA in WT and 35S:AKIN10-HA plants. Endogenous AKIN10 (MW ~60 kD) was detected in both genotypes with anti-AKIN10 antibody (top); AKIN10-HA (MW ~65 kD) was detected with anti-HA antibody (bottom) and anti-AKIN10 antibody (top).
(f) Predicted SnRK1 consensus sequence (top) and putative SnRK1 target sites within the FUS3 sequence (bottom). Phosphorylated serines (S) and threonines (T) are in bold and underlined. Basic amino acids are in black, hydrophobic amino acids are in grey.
3.2.4 AKIN10 overexpression delays FUS3 degradation

Using a cell-free degradation system, we have previously shown that FUS3 is rapidly degraded via the 26S proteasome, suggesting that protein turnover plays a major role in the regulation of FUS3 level (Lu et al., 2010). In light of this, we investigated whether AKIN10 affects the degradation rate of FUS3 using the same cell-free system. GST-FUS3 degradation is delayed in 35S:AKIN10-HA compared to WT (Figure 3a,b). GST-FUS3 is still present after 60-90 minutes of incubation in 35S:AKIN10-HA extract, whereas it is nearly completely degraded in WT extract at these times. These results indicate that overexpression of AKIN10 increases FUS3 stability.
Figure 3. Overexpression of *AKIN10* delays FUS3 degradation.

(a) Immunoblot showing the degradation of GST-FUS3 in a cell-free system. GST-FUS3 was incubated with cell extracts from WT and 35S:*AKIN10-HA* 7-day-old seedlings for the indicated times. Three experiments were conducted and one representative is shown.

(b) Degradation kinetics of GST-FUS3. Averages of three experiments ± s.d. are shown. (*) Denotes significant difference from WT (p<0.05).
(a) 

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Time (min.)

- GST-FUS3
- Ponceau

(b) 

% GST-FUS3 remaining

WT

35S:AKIN10-HA

Time (min.)
3.2.5 Genetic interaction between AKIN10 and FUS3 in the regulation of phase transitions

Our data so far indicates that AKIN10 phosphorylates FUS3 and delays FUS3 degradation. To study the biological role of this interaction, we first characterized the 35S:AKIN10-HA mutants phenotypically and then tested the genetic interaction with fus3-3. The 35S:AKIN10-HA plants generated in this study show delayed flowering and senescence, confirming previous findings (Figure 4a; Baena-Gonzalez et al., 2007). In addition to delayed flowering, 35S:AKIN10-HA plants also produce more leaves than WT at bolting, suggesting that both vegetative and reproductive development were delayed (Figure 5b). Interestingly, we also found that 35S:AKIN10-HA plants produce seeds that germinate slower than WT (Figure 4b). Collectively, these phenotypes indicate that AKIN10 negatively regulates the embryonic-to-vegetative and vegetative-to-reproductive phase transitions. Similarly, plants overexpressing FUS3 during vegetative and reproductive development show delayed germination, growth and flowering time (Gazzarrini et al., 2004). Thus, AKIN10 and FUS3 may regulate developmental phase transitions through overlapping pathways.

To test this, we crossed fus3-3 into 35S:AKIN10-HA plants. Indeed, fus3-3 35S:AKIN10-HA plants flower at a similar time as WT and produce a similar number of leaves at bolting, suggesting that fus3-3 rescues the 35S:AKIN10-HA defects in vegetative and reproductive phase transitions (Figure 5a,b). Furthermore, fus3-3 35S:AKIN10-HA seeds germinate precociously when still green and immature, become desiccation intolerant when dry and develop cotyledons with trichomes, similarly to fus3-3 (Figures 5c, 6k-l). Notably, the fus3-3 seed phenotypes can be rescued by the introduction of a FUS3:FUS3-GFP construct previously described, suggesting that these phenotypes are due to the lack of a functional FUS3 (Figures 4b and 5c; Gazzarrini et al., 2004; Lu et al., 2010). Immunoblots confirm the presence of the AKIN10-HA protein in the fus3-3 35S:AKIN10-HA double mutant, ruling out the possibility of transgene silencing (Figure 5d).

Altogether, the genetic interaction data corroborates the physical interaction results and indicates that FUS3 and AKIN10 share overlapping pathways to negatively regulate the embryonic-to-vegetative and vegetative-to-reproductive phase transitions.
**Figure 4.** Delayed seed germination and flowering time of 35S:AKIN10-HA plants.

(a) Late flowering phenotype of two 35S:AKIN10-HA transgenic plants.

(b) Germination rates of dry seeds from various genotypes. Averages from three experiments ± s.d. are shown. (*) Denotes significant difference from WT (p<0.001).
(a) WT 35S:AKIN10-HA

(b) Germination rate over days after imbibition (D.A.I.) for WT, fus3-3, 35S:AKIN10 (#7), 35S:AKIN10 (#10), FUS3:FUS3-GFP
Figure 5. Genetic interaction between AKIN10 and FUS3 during phase transition.

(a) Late flowering phenotype of 35S:AKIN10-HA (#7) is rescued in fus3-3 35S:AKIN10-HA plants. Image showing plants of various genotypes and of the same age grown on soil.

(b) Bolting time (top) and total number of rosette leaves emerged at bolting (bottom) measured in soil-grown plants of various genotypes. D.A.I., days after imbibition. Values are averages from N=6–10 ± s.d. (*) Denotes significant difference from WT (p<0.0001).

(c) Germination and growth rates of immature green seeds of various genotypes. Averages from three experiments ± s.d. are shown.

(d) Immunoblot showing AKIN10-HA protein in plants of various genotypes. The same level of AKIN10-HA protein is found in 35S:AKIN10-HA and rescued fus3-3, 35S:AKIN10-HA plants. Ponceau-S stain is shown as the loading control. AKIN10-HA was detected with anti-HA antibody.
(a) Plant phenotypes of WT, fus3-3, 35S:AKIN10, fus3-3 35S:AKIN10

(b) Bar graphs showing age at bolting (D.A.I.) for different genotypes.

(c) Bar graphs showing % green seed germination and % seedling growth for different genotypes.

(d) Western blot analysis showing AKIN10-HA expression levels.
3.2.6 Genetic interaction between FUS3 and AKIN10 in lateral organ development

A number of morphological defects are also found in lateral organs of all three 35S:AKIN10-HA lines generated, including cotyledons, flowers and siliques. Approximately 44-57% of 35S:AKIN10-HA seedlings develop defective cotyledons, which included altered number of cotyledons, abnormal cotyledon margins, arrested cotyledon development and major defects leading to developmental arrest or failure to germinate (Figure 6b-j). The latter include mp-like seedlings or seedlings lacking the basal and/or the apical domains (Figure 6h-j). We characterized two lines in more detail and found that 12-13% have an altered number of cotyledons, which included monocotyledon, polycotyledon and cotyledon fusion (Figure 6m). Seedlings with altered cotyledon number often display altered leaf phyllotaxy. Upon a closer inspection, cotyledons defects are also observed at a much lower frequency in fus3-3 and they include tricotyledon (Figure 6m) and mp-like seedlings. The fus3-3 cotyledon defects are completely rescued by the introduction of the FUS3:FUS3-GFP construct, suggesting that they are linked to the loss of FUS3 (Figure 6m).

We then tested whether the 35S:AKIN10-HA cotyledon defects could be rescued by fus3-3. Interestingly, these defects are scored at a much lower frequency in fus3-3 35S:AKIN10-HA, suggesting that they are partially dependent on FUS3 (Figure 6m).

During reproductive development, abnormal number, development and phyllotaxy of floral organs are present in early flowers of 35S:AKIN10-HA plants (Figure 6n). Finally, 35S:AKIN10-HA plants develop aborted or shorter siliques, which also display an altered phyllotaxy. Interestingly, fus3-3 can partially rescue these phenotypes, as fus3-3 35S:AKIN10-HA plants develop siliques that are similar to WT or show only a moderate phenotype (Figure 6o).

Collectively, these data indicate that FUS3 and AKIN10 interact to regulate lateral organ development.
Figure 6. Genetic interaction between \textit{AKIN10} and \textit{FUS3} in lateral organ development.

(a) WT seedling.

(b)-(j) Defects in cotyledon morphology and number of \textit{35S:AKIN10-HA} seedlings include polycotyledon (b, c), monocotyledon (d), fusion or lobed cotyledons (e), cotyledon margin defects (f), arrested cotyledons (g), lack of cotyledons and root (h), \textit{mp}-like (i, j). \textit{fus3-3} (k) and \textit{fus3-3 35S:AKIN10-HA} tricotyledon bearing trichomes (l). Black and white images are cleared seedlings showing the venation pattern. White arrowheads indicate trichomes.

(m) Frequency of cotyledon defects in seven-day-old seedlings of various genotypes. (*) Denotes significant difference from \textit{fus3-3 35S:AKIN10-HA} (p<0.05). (**) Denotes significant difference from \textit{FUS3:FUS3-GFP} and WT (p<0.05).

(n) WT and \textit{35S:AKIN10-HA} floral organs showing \textit{35S:AKIN10-HA} petal, stamen and carpel fusions. Black and white images are cleared petals to show the venation patterns. White arrowheads point to primary veins.

(o) Inflorescences of plants of various genotypes showing partial rescue of \textit{35S:AKIN10-HA} siliques defects in \textit{fus3-3 35S:AKIN10-HA} plants. \textit{35S:AKIN10-HA} siliques abort at different levels in mildly (left), or strongly (right) affected inflorescences. \textit{fus3-3 35S:AKIN10-HA} plants show partial rescue of siliques development.
(a) (c) (d) (e) (f) (g) (h) (i) (j) (k) (l) (m) (n) (o)

WT 35S:AKIN10-HA

35S:AKIN10-HA fus3-3

35S:AKIN10-HA

% Seedlings with altered cotyledon number

WT fus3-3 (FUS3:FUS3-GFP) 35S:AKIN10-HA 35S:AKIN10-HA

WT fus3-3 35S:AKIN10-HA 35S:AKIN10-HA
3.2.7 FUS3 and AKIN10 expression patterns

The physical and genetic interactions between AKIN10 and FUS3 indicate that these proteins act in overlapping pathways to regulate embryonic, vegetative and reproductive development. A survey of the expression profiles of these two genes in publicly available microarrays shows that AKIN10 is constitutively expressed throughout development including the seed, where FUS3 mRNA is found at its highest level (Figure S4). Thus, the interaction between these two genes in the regulation of embryonic development and dormancy/germination is biologically relevant. FUS3 is also expressed in floral organs, highest in stamens and pollen, which may explain the ability of fus3-3 to rescue 35S:AKIN10-HA silique development and fertility (Figure S4).

Although microarrays data show that FUS3 is expressed at low level during vegetative growth, using qRT-PCR and a transcriptional reporter (FUS3p:GFP) we have shown that FUS3 is expressed in the cotyledons and hypocotyls of germinating seedlings (Lu et al., 2010). Using qRT-PCR and the same FUS3p:GFP reporter, FUS3 expression can be detected at least up to 7 days not only in the cotyledons and hypocotyls, but also in the leaf primordia and shoot apical meristem (Figure 7a,d-i). A second promoter-reporter (FUS3p:GUS) previously described shows a similar pattern of FUS3 expression (Figure 7b,c; Tsuchiya et al., 2004). This indicates that FUS3 is also expressed during early stages of vegetative growth and thus may play a role in vegetative development and phase transition.

Since AKIN10 acts as a positive regulator of FUS3, and ectopic expression of FUS3 shows phenotypes similar to those seen in 35S:AKIN10-HA transgenic plants (Gazzarrini et al., 2004; Baena-Gonzalez et al., 2007, Jossier et al. 2009), 35S:AKIN10-HA phenotypes may be attributed to altered FUS3 expressions. In order to test this hypothesis, FUS3 expression in 7 day-old seedlings was measured by qRT-PCR (Figure S5). No statistically significant differences were found in FUS3 transcript levels between all three 35S:AKIN10-HA transgenic lines and WT, suggesting FUS3 expression was not dramatically altered in 35S:AKIN10-HA seedlings.
**Figure 7.** FUS3 expression pattern during vegetative development.

(a) qRT-PCR showing *FUS3* mRNA levels in WT seedlings 1-7 days after imbibition (DAI).

(b), (c) GUS activity in the hypocotyls, cotyledons and emerging leaf primordia of 5 DAI *FUS3p:GUS* seedlings. The right panel shows a higher magnification of the leaf primordia shown in the left panel (see arrow).

(d)-(h) Confocal images showing GFP fluorescence in the hypocotyl (d), leaf primordia of 5 DAI (e) and 7 DAI (g) seedlings, epidermis of the cotyledon (f), SAM (i) and trichomes (h) of 5 and 7 days-old *FUS3p:GFP* seedlings. GFP fluorescence is present in the vasculature (d) and all cell types of the epidermis.

LP, leaf primordia; SAM, shoot apical meristem; HYP, hypocotyls.
ve mRNA levels

(a)

D.A.I.

Relative mRNA levels

0 10 20 30 40 50

1 2 3 5 7

(b) (c)

FUS3p:GUS

LP LP LP

sam

LP

FUS3p:GFP

COT

LP

LP

LP

LP

SAM

LP

SAM
3.3 Discussion

3.3.1 AKIN10 interacts with FUS3 in Y2H, in vitro and in planta

The B3 domain transcription factors, FUS3, ABI3 and LEC2, are master regulators of seed maturation and germination. They are required for the synthesis of storage compounds, the establishment of dormancy and desiccation tolerance, and are important nodes in hormone biosynthethic and signaling pathways. Despite their pivotal role during seed maturation, very little is known about the regulation of these proteins and their interacting partners. In this study, we have identified the SnRK1 kinase AKIN10 as an interactor of FUS3 in Y2H screens and shown that AKIN10 phosphorylates FUS3 and delays its degradation. FUS3 and AKIN10 interact genetically to regulate seed development and organogenesis, while inhibiting developmental phase transitions.

In Y2H screens using two different FUS3 baits, 68 clones encoding AKIN10 were found to interact with FUS3. In yeast, the N-terminal domain of FUS3 and the C-terminal domain of AKIN10 are sufficient for interaction. FUS3 also interacts with AKIN10 in vitro and in planta. BiFC assays show that these proteins interact predominantly in the nuclei of N. benthamiana epidermal cells. This is in agreement with the subcellular localization of AKIN10 and FUS3 in N. benthamiana and Arabidopsis (Gazzarrini et al., 2004; Bitrian et al., 2010). Although AKIN10 was previously found in the chloroplasts when expressed under the 35S promoter (Fragoso et al., 2009), AKIN10-YFP does not co-localize with the chloroplasts in N. benthamiana epidermis. It is possible that AKIN10 is localized to different compartments and that the subcellular localization of AKIN10 is differentially regulated in various tissues and under various conditions. Nuclear and cytoplasmic localizations of the SnRK/Snf1/AMPK have been shown in different plant species and other organisms. AKIN10 has been detected in the nuclei of Arabidopsis plants stably expressing AKIN10-GFP under the control of its endogenous promoter (Bitrian et al., 2010). Moreover, ZmAKIN10 and ZmAKIN11 have been detected in the nuclei and cytoplasm of onion epidermal cells (Lopez-Paz et al., 2009). Similarly, two different AMPK isoforms, a1 and a2, have been shown to localize preferentially to the nucleus and cytosol, respectively, and to shuttle between the nucleus and the cytoplasm in response to stress (Salt et al., 1998). Finally, the Snf1 protein kinase is localized in the cytoplasm during
growth of *S. cerevisiae* in high glucose and accumulates in the nucleus in response to glucose limitation (Vincent et al., 2001). Thus, our finding that the AKIN10-FUS3 interaction occurs predominantly in the nuclei is physiologically relevant.

### 3.3.2 AKIN10 phosphorylates FUS3

Using in-gel kinase assays, recombinant FUS3 is predominantly phosphorylated by a ~60 kD kinase which corresponds to the size of the endogenous AKIN10. Although we could not isolate an *akin10* null mutant, we immunoprecipitated a HA-tagged variant of AKIN10 (AKIN10-HA; MW ~66 kD) to demonstrate that FUS3 is indeed phosphorylated by AKIN10 (Figure 2c-i). Since AKIN10 and AKIN11 share very similar MWs, expression profiles and together contribute to the majority (95%) of the SnRK1 kinase activity detected in Arabidopsis, it is likely that AKIN11 also phosphorylates FUS3 (Zhang et al., 2009).

We mutated serines that closely match the SnRK1 consensus motif (h-X-R/K/H-X2-S/T-X3-h; Halford et al., 2003; Figure 2) and found that AKIN10 phosphorylates FUS3 at one or more of the S55/S56/S57 residues located at the N-terminal domain of FUS3, but not S194. Since weak 66 kD kinase activity could still be detected in the fus3\(^{S55A/S56A/S57A}\) and fus3\(^{S55A/S56A/S57A/S194A}\) mutants, we can deduce that other unidentified sites of FUS3 are also phosphorylated by AKIN10; these additional sites are either non-conventional sites that do not match the SnRK1 consensus, or do not score high with the Netphos2 algorithm. We can also surmise that AKIN10 can phosphorylate S/T that do not perfectly match the consensus target motif established thus far. A recent survey of the phosphoproteome has identified several phosphorylated proteins that partially match the SnRK1 consensus target motif, suggesting the existence of a larger number of AKIN10 substrates than previously anticipated (de la Fuente van Bentem et al., 2008). Intriguingly, FUS3 is weakly phosphorylated by ~40-45 kD kinases. This was evident when using the truncated FUS3(ΔC), which was phosphorylated more efficiently than the full-length FUS3 by these smaller kinases. Possibly, the CTD of FUS3 may serve as a regulatory domain inhibiting the phosphorylation of FUS3 by these kinases.

Phosphorylation plays an important role in regulating protein stability and function. We have previously shown that FUS3 is a short-lived protein rapidly degraded by the 26S proteasome (Lu et al., 2010). Interestingly, overexpression of AKIN10 delays FUS3 degradation by a yet
uncharacterized mechanism. At the moment we do not know whether the delayed degradation of FUS3 in 35S:AKIN10-HA is a direct or indirect effect. FUS3 has 47 S/T distributed throughout the protein, 19 of which are highly predicted to be phosphorylated. Mutation of additional S/T sites may be required to fully understand the role of phosphorylation in FUS3 regulation. Phosphorylation of FUS3 by AKIN10 may have other roles than regulating FUS3 stability. Interestingly, the AKIN10 target motif in FUS3 is located in the B2 domain, which in ABI3 has been shown to be important for DNA-binding specificity and protein interaction (Nag et al., 2005; Zhang et al., 2005). Thus, phosphorylation of FUS3 by AKIN10 may modulate similar functions/processes.

3.3.3 AKIN10 and FUS3 function interactively to promote seed maturation, dormancy and inhibit developmental phase transitions

Overexpression of FUS3 or AKIN10 result in more dormant seeds, late flowering and late senescing plants, suggesting that both genes negatively regulate developmental phase transitions (Figure 4; Figure 5; Gazzarrini et al., 2004; Baena-Gonzalez et al., 2007; Lu et al., 2010). Since the AKIN10 overexpression phenotypes are rescued in a fus3-3 mutant background, we conclude that AKIN10 requires a functional FUS3 to alter phase change. The genetic interaction reinforces the physical interaction of these proteins and indicates that AKIN10 and FUS3 function in overlapping signaling pathways, promoting seed maturation, dormancy and inhibiting developmental phase transitions. Our findings are in agreement with previous studies conducted in pea, where SnRK1-antisense seeds have maturation defects including a decrease in the synthesis of storage proteins, precocious germination and reduced desiccation tolerance. In these plants, PsFUS3 and PsABI3 are downregulated, explaining the viviparous and desiccation intolerance phenotypes (Radchuk et al., 2006). Since FUS3 activates its own promoter (To et al., 2006), a lack of positive regulators such as AKIN10 would decrease FUS3 level, thus causing the defects in dormancy and desiccation tolerance. However, FUS3 transcript level remains relatively unchanged in 35S:AKIN10-HA seedlings (Figure S5). Perhaps post-translational stabilization of FUS3 alone is not sufficient to promote its own expression by positive feedback. It is also possible that other yet-unidentified AKIN10 targets regulate FUS3 and ABI3 expression, such that the interaction is more complex than a simple positive regulation. Our
findings and those of Radchuk et al. (2006) indicate that AKIN10 may regulate seed maturation by modulating FUS3 level and thus may act as a sensor of stored energy in the seed. This parallels the role proposed for AMPK in mammals (McBride and Hardie, 2009).

Genetic and molecular studies have shown that FUS3 and AKIN10 positively regulate ABA metabolism (Nambara et al., 2000; Gazzarrini et al., 2004; Radchuk et al., 2006; Jossier et al., 2009; Radchuk et al., 2010; Yamamoto et al., 2010). Overexpression of AKIN10 and FUS3 result in ABA-hypersensitive phenotypes that are dependent on the ABA biosynthetic gene ABA2 (Gazzarrini et al., 2004; Jossier et al., 2009). Thus, AKIN10 and FUS3 could interact to control the synthesis of ABA during embryogenesis and regulate dormancy and germination (Figure 8). Furthermore, overexpression of FUS3 delays germination and flowering time, which is partly dependent on the downregulation of GA biosynthesis and on the increase in ABA level (Gazzarrini et al., 2004). Since delayed flowering in 35S:AKIN10-HA plants requires FUS3, both genes could interact to control vegetative-to-reproductive phase transitions by regulating the GA and ABA syntheses and/or signaling pathways. Interestingly, in tomato seeds ABA and GA positively and negatively regulate the expression of the regulatory subunit LeSNF4 of the SnRK1 complex, respectively. In contrast, expression of the AKIN10 ortholog LeSNF1 is unresponsive to hormonal treatment (Bradford et al., 2003). This suggests a complex hormonal regulation of the SnRK1 complex at the transcriptional and posttranslational level and warrants further investigation.

The function of FUS3 has been characterized predominantly in the seeds, due to the phenotypes of the fus3 mutants and to the high expression of this gene in the seed. The SnRK1 genes are also expressed in the seeds, thus the interaction of FUS3 and AKIN10 in the regulation of embryonic development (cotyledons) and seed dormancy is biologically relevant. However, the rescue of the 35S:AKIN10-HA late flowering phenotype by fus3-3 was unexpected, given the fact that FUS3 is not highly expressed during vegetative development and that fus3-3 flowers at a similar rate as WT under normal growth conditions. Two possible scenarios can explain this result. Firstly, fus3-3 rescues 35S:AKIN10-HA vegetative phenotypes indirectly and the rescue is a consequence of the fact that fus3-3 seeds bypass the maturation and dormancy programs and germinate right after completing early-mid embryogenesis. This would imply that a developmental program regulating flowering time is activated during maturation/dormancy and
is dependent on \( FUS3 \). Secondly, \( FUS3 \) is expressed post-embryonically at a low level and plays a subtle/redundant role during vegetative and reproductive growth. In agreement with this, using qRT-PCR and two transcriptional reporters (\( FUS3p:GFP \) and \( FUS3p:GUS \)), we show that \( FUS3 \) is indeed expressed at least up to 7 days post-germination in the epidermis and vasculature of the cotyledons, hypocotyls, leaf primordia and also in the SAM (Figure 7; Lu et al., 2010). This suggests that \( FUS3 \) may have a function during early stages of vegetative growth and may affect vegetative and reproductive phase transitions in response to endogenous, i.e. hormones, or environmental signals. The fact that overexpression of \( FUS3 \) delays vegetative growth and flowering time suggests that this gene can regulate vegetative and reproductive phase transitions (Gazzarrini et al., 2004).

3.3.4 Interaction between AKIN10 and FUS3 regulates lateral organ development

In this study we have uncovered a role for \( AKIN10 \) and \( FUS3 \) in organogenesis. Plants overexpressing \( AKIN10 \) show several defects in the development of lateral organs, including cotyledons, flowers, leaves and siliques. \( fus3-3 \) partially rescues the \( 35S:AKIN10-HA \) cotyledon and silique phenotypes, suggesting that \( AKIN10 \) and \( FUS3 \) interact to regulate lateral organ development.

The defects in cotyledon development caused by \( AKIN10 \) overexpression resemble mutants affected in auxin transport, synthesis, localization and signaling, and include radialized cotyledons, pleiocotyly, partially or completely fused cotyledons, or failure to develop cotyledons (Bowman and Floyd, 2008; Chandler 2008). Similar phenotypes can be mimicked by the inhibition of polar auxin transport, suggesting that auxin is required for the establishment of bilateral symmetry during embryogenesis (Liu et al., 1993; Hadfi et al., 1998). Since auxin activates \( FUS3 \) and \( AKIN10 \) promoters and \( FUS3 \) positively regulates auxin biosynthesis, aberrant cotyledon development in loss- and gain-of-function \( FUS3 \) and \( AKIN10 \) mutants could be due to changes in auxin synthesis, localization or response (Figure 8; Gazzarrini et al., 2004; Jossier et al., 2009; Yamamoto et al., 2010).

Recent studies in Drosophila and mammalian cells have demonstrated that the role of AMPK in energy sensing is coupled with fundamental cell biological functions, such as cell polarity and
cell division (Williams and Brenman, 2008). Indeed, mutants affected in AMPKα and the AMPK activating kinase LKB1 both display mitotic and polarity defects, the former in epithelial cell polarity and the latter in neuronal cell polarization (Williams and Brenman, 2008). Similarly, our findings uncovered implications for FUS3-mediated SnRK1 role in organogenesis, including the establishment of bilateral symmetry in seedlings and radial symmetry in flowers. Thus, plant and animal SnRK1/AMPK may use common regulatory mechanisms to sense the energy status and ultimately coordinate essential and basic cellular functions, such as cell division, growth and differentiation.
Figure 8. Model of FUS3 and AKIN10 interaction during embryogenesis.
Proposed model explaining the interaction between FUS3, AKIN10 and the hormones ABA and auxin during embryonic development.

During early embryogenesis, FUS3 and AKIN10 interact to regulate cotyledon development. Auxin induces the expression of *FUS3* and *AKIN10* (Gazzarrini et al., 2004; Radchuk et al., 2010) and both genes then regulate auxin metabolism and signaling genes (Gazzarrini et al., 2004; Baena-Gonzalez et al., 2007; Radchuk et al., 2010; Yamamoto et al., 2010), establishing a feedback regulation that controls auxin level and distribution and regulates cotyledon development.

During mid embryogenesis, FUS3 and AKIN10 act interactively to control late embryonic functions. FUS3 positively regulate ABA synthesis and both genes regulate ABA biosynthetic genes (Nambara et al., 2000; Gazzarrini et al., 2004; Jossier et al., 2009; Yamamoto et al., 2010); an increased level of ABA activates AKIN10 promoter and stabilizes FUS3 (Gazzarrini et al., 2004; Radchuk et al., 2010), promoting the synthesis of storage compounds, the establishment of dormancy and desiccation tolerance. FUS3 and AKIN10 regulate cotyledon development and seed maturation also through independent pathways.
AKIN10

FUS3

auxin

ABA

cotyledon development

pattern formation

dormancy

maturation

dormancy

pattern formation

cotyledon development

FUS3

AKIN10
3.4 Experimental procedures

3.4.1 Plant material, growth conditions, seed germination and statistics

Germination assays and growth of Arabidopsis plants (Columbia) of various genotypes were previously described (Lu et al., 2010). For germination assays of green seeds, 100-300 green-immature seeds were harvested from yellow siliques, sterilized and germinated for 7 days at room temperature under constant light. Seed germination refers to seedlings emerged from the seed coat and with open cotyledons, while growth refers to seedlings with developed true leaves. Seedlings transferred to soil were grown in controlled environmental chambers at 21°C under constant light. Seedlings used for the flowering time experiment were grown under a regime of 16 hours light at 21°C and 8 hours dark at 18°C. Statistical analysis for bolting assays was conducted using non-paired $t$ test, while paired $t$ test was used for statistic evaluation of all other experiments.

3.4.2 Generation of transgenic plants

The AKIN10-HA construct was created by PCR amplification of At3G01090.2 and cloned into pEGAD (Cutler et al., 2000), where GFP was replaced by 3xHA. The $35S:AKIN10-HA$ construct was transformed into Arabidopsis WT by floral dip. The transgenic lines were selected on 50 $\mu$g/ml glufosinate ammonium salt (Crescent Chemical). Seventeen transgenic lines were screened for $AKIN10$ mRNA and protein levels. Four representative lines were chosen for further characterization.

3.4.3 Yeast two-hybrid

Saccharomyces cerevisiae strain EGY48 ($MATa$, $his3$, $trp1$, $ura3$, 6 $lexAop-LEU2$), and the $pEG202$, $pJG4-5$ and $pSH18-34$ vectors were used for the yeast two-hybrid assay (Clontech). To generate the LexA-FUS3 bait constructs, $FUS3(N90)$ and $FUS3(\Delta C)$ were amplified by PCR and cloned into $pEG202$. EGY48 cells harboring the $pSH18-34$ reporter vector and the $LexA-FUS3(N90)$ or $LexA-FUS3(\Delta C)$ bait plasmids were transformed with 50 $\mu$g of a cDNA library made from Arabidopsis siliques, flower buds and germinating seeds (Norclone Biotech
Laboratories). Transformants were plated on media lacking uracil, histidine, tryptophan, and leucine and supplemented with 80 µg/ml X-gal, 2% (w/v) galactose and 1% (w/v) raffinose. The cDNA inserts were PCR-amplified from the yeast colonies using primers listed in Figure S6 and the PCR products were digested with RsaI or HaeIII, sorted based on fragment sizes and sequenced. Putative clones were retransformed into yeast to confirm the interaction. See Appendix 6.1 for detailed experimental procedures.

3.4.4 Pull-down assays

Extraction of yeast and plant proteins was performed as described (Sambrook and Russel, 2001; Lu et al., 2010). 300 µl yeast or plant extract containing the prey proteins and 50 µg of purified bait proteins were each separately incubated with 50 µl of glutathione-agarose (Sigma) in 1 ml of binding buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.2% glycerol, 0.6% Triton X-100, 0.5 mM β-mercaptoethanol) in 4°C for 2 hours. Glutathione agarose were collected by centrifugation at 12,000 g in 4°C for 2 minutes. Pre-absorbed prey protein extract were then incubated with bait-bound glutathione agarose in 4°C for 2 hours. Glutathione agarose were then washed 6 times with the washing buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.6% Triton X-100). Pulled-down proteins were detected by immunoblotting using a rabbit anti-HA polyclonal antibody (Cedarlane) and a donkey anti-rabbit HRP secondary antibody (Jackson Immuno Research).

3.4.5 Bi-molecular fluorescent complementation, confocal microscopy and GUS-staining

The nYFP-FUS3, cYFP-AKIN10 and YFP-AKIN10 constructs were made by cloning the FUS3 CDS into the nYFP-vector and the AKIN10 CDS into cYFP-vector and YFP-vector via the Gateway system (Invitrogen). Donor vector of AKIN10 (pENTR223) was obtained from ABRC. Donor vector of MYB49 was obtained from D. Desveaux (Park et al., 2009). Donor vector of FUS3 was made by amplifying the FUS3 CDS by PCR and cloning the PCR product into pDON201 vector. The GFP-FUS3 construct was made by PCR amplification of FUS3 CDS and subsequent cloning of the PCR product into pEGAD. The BiFC constructs were transformed into Agrobacterium tumefaciens strain GV2260. N. benthamiana plants were agro-infiltrated as previously described (Lewis et al., 2008). Plants were imaged after 48 hours by
confocal microscopy as described (Lu et al., 2010). GUS staining was performed as in Donnelly et al., (1999). See Appendix 6.2 for detailed experimental procedures.

3.4.6 Cell-free degradation assays, qRT-PCR and immunoblots

Cell-free degradation assays, qRT-PCR, immunoblots and band quantifications were conducted as previously described (Lu et al, 2010). AKIN10 was detected with anti-AKIN10 antibody (Cedarlane) and AKIN10-HA was detected with anti-HA antibody (Cedarlane).

3.4.7 Cloning and purification of GST- and HA-fusion constructs

GST-FUS3 and GST-FUS3ΔC constructs were previously described (Lu et al., 2010). To generate GST-FUS3ΔN, a FUS3 fragment lacking the first 270 nucleotides of the CDS was amplified by PCR and cloned into the pGEX-6P-3 vector (GE Healthcare). To generate the various GST-fus3 mutants, site-directed mutagenesis (Agilent) was performed on the GST-FUS3 construct (Figure S6). The GST constructs were expressed in E. coli and purified using glutathione resin (Sigma). AKIN10-HA was immunoprecipitated using anti-HA antibody (Cedarlane) and protein-A/G agarose (Santa Cruz) according to manufacturer’s instruction and Weigel and Glazebrook (2002).

3.4.8 In-gel kinase assay

In-gel kinase assays were essentially performed as described in Liu et al. (2008). Proteins (15 µg) extracted from 7-day-old seedlings were resolved by SDS-PAGE in 8% gels casted with 0.5 mg/ml GST or GST-fusion proteins. See Appendix 6.3 for detailed procedures.

3.5 Acknowledgements

We thank K. Breitkreuz for critical reading of the manuscript, D. Desveaux for BiFC and 35S:YFP vectors, C. Hasenkampf for pDON201 vector and S. Patel, N. Rajakulendran, L. Yip, M. Ly and A. Chan for assistance in the lab. This work was supported by NSERC and CFI grants to S.G.
3.6 Supplementary Figures

Figure S1. Localization of AKIN10-YFP in *N. benthamiana* cells.
Top two rows: epidermis of *N. benthamiana* leaves transiently expressing AKIN10-YFP showing fluorescence in the nuclei, cytoplasm and punctate aggregates of unknown origin.
Bottom row: epidermis of *N. benthamiana* leaves transiently coexpressing nYFP-FUS3 and cYFP-AKIN10 in BiFC assays, showing YFP fluorescence in the nuclei and cytoplasm of pavement cells.
The AKIN10-YFP fluorescence (first column) does not co-localize with the chloroplasts autofluorescence shown in the red channel (second column).
Labels above each panel indicate the filter channel imaged. DIC, differential interference contrast image.
35S:AKIN10-YFP

35S:AKIN10-YFP

AKIN10-cYFP + FUS3-nYFP
**Figure S2.** Molecular characterization of SnRK1 mutants.

(a) RT-PCR showing expression of the full-length AKIN10 transcript in WT and four independent 35S:AKIN10-HA plants. ACTIN7 is used as the control.

(b) Three members of the SnRK1 family exist in Arabidopsis, AKIN10 (At3g01090), AKIN11 (At3g29160) and AKIN12 (At5g39440). The akin10-1 (SALK_127939), akin10-2 (SAIL_834_G03) and akin11 (WiscDsLox320B03) T-DNA insertion mutants were obtained from the SALK collection (Alonso et al., 2003) and homozygous lines were identified by PCR using gene-specific and T-DNA specific primers (Figure S6). Arrowheads indicate primer locations used in RT-PCR shown in (c).

(c) RT-PCR amplification of ~200bp of AKIN10, AKIN11 and AKIN12 in WT, akin10-1, akin11 and 35S:AKIN10-HA (#7). Primer sequences are listed in Figure S6. cDNA from seedlings was used as the template in lanes 1-5 and genomic DNA in lane 6. RNA extraction and cDNA synthesis was previously described (Lu et al., 2010). ACTIN7 is shown as the control. No AKIN11 transcript could be amplified in akin11, suggesting that it is a null mutant. Although akin10-1 was previously shown to be a true null mutant (Fragoso et al. 2009), AKIN10 CDS was amplified in akin10-1, while the 3-UTR was not. No AKIN12 mRNA could be amplified in all genotypes, probably due to the very low level of expression measured in microarray data (Figure S4).

(d) Immunoblot showing AKIN10 (b) and AKIN10-HA (c) protein levels in seedlings of WT, akin10-1, akin10-2, akin11 and 35S:AKIN10-HA (#7). Ponceau-S stain is shown as control. AKIN10 was detected with anti-AKIN10 antibody and AKIN10-HA was detected with anti-HA antibody. Predicted MW of AKIN10 ~60 kD, of AKIN10-HA ~65 kD. The AKIN10 protein was detected in WT, akin10-1 and akin10-2 mutants, confirming that they are not null mutants. The AKIN10 protein was also detected in the akin10-1 akin11-1 double mutant, ruling out the possibility that the AKIN10 antibody detects the AKIN11 protein due to cross reactivity and confirming that the akin10-1 mutant is not a null mutant.
Figure S3. Prediction of S/T phosphorylation sites in FUS3.
(a) FUSCA3 amino acid sequence.
(b) Prediction of phosphorylated serine/threonine in FUS3 using the NetPhos2 algorithm. Residues with a score above the threshold (>0.5) are predicted to be phosphorylated and are indicated by 'S' (serine) or 'T' (threonine). Among the 14 serines and 5 threonines predicted to be phosphorylated, only S55, S56, S57 and S194 match SnRK1 consensus sequence and are indicated by arrows. Column 2: position of the residue being analyzed; column 3: sequence context (shown as a 9-residue sequence centered on the residue being analyzed); column 4: output score (value in the range [0.000-1.000]); column 5: assignment (scores above the threshold of 0.5 are assigned as *S* or *T*).
(c)-(d) Conservation of S55/S56/S57 (c), S194 (d) and flanking basic/hydrophobic amino acids in FUS3 orthologs from different plant species. Basic amino acids are in black, while hydrophobic amino acids are in gray.
Arabidopsis thaliana (NP_566799.1)
Populus trichocarpa (XP_002298665.1)
Ricinus communis (XP_002512863.1)
Vitis vinifera (XP_002275489.1)
Medicago truncatula (ABD32571.1)

Arabidopsis thaliana (NP_566799.1)
Populus trichocarpa (XP_002298665.1)
Ricinus communis (XP_002512863.1)
Vitis vinifera (XP_002275489.1)
Medicago truncatula (ABD32571.1)
**Figure S4.** Expression analysis of *FUS3* and *SnRK1* genes throughout development. (a)-(d) Relative mRNA levels of *FUS3*, *AKIN10*, *AKIN11* and *AKIN12* during embryonic development (a), reproductive development (b), vegetative development (c) and epidermis (d) were obtained from microarray data available at The Bio-array resource database (http://bar.utoronto.ca; Toufighi et al., 2005). FUS3 is expressed at low level throughout vegetative development. Relatively higher expression of *FUS3* detected in floral organs, senescing and cauline leaves, older leaves (leaves 4-6) is boxed. Increased expression is also detected in the epidermis of stem.
Figure S5. *FUS3* transcript accumulation in WT and *35S:AKIN10-HA* transgenic seedlings. qRT-PCR showing *FUS3* mRNA levels in WT and three independent *35S:AKIN10-HA* transgenic seedlings.
Relative mRNA level

WT  #7  (10)  (11)

35S:AKIN10-HA
Figure S6. Primer sequences used in this study.
<table>
<thead>
<tr>
<th>Primers to genotype <em>akin10</em></th>
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<td>AKIN10_LP: ACCACACGTTGGAAACTTTTG</td>
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3.7 References


Chapter 4

Overlapping and distinct roles of AKIN10 and FUSCA3 in ABA and sugar signalling during seed germination

Previously published as:

ABSTRACT

The Arabidopsis B3-domain transcription factor FUSCA3 (FUS3) is a master regulator of seed maturation and also a central modulator of hormonal responses during late embryogenesis and germination. Recently, we have identified AKIN10, the Arabidopsis ortholog of Snf1 (Sucrose Non-Fermenting-1)–Related Kinase1 (SnRK1), as a FUS3-interacting protein. We demonstrated that AKIN10 physically interacts with and phosphorylates FUS3 at its N-terminal region, and genetically interacts with FUS3 to regulate developmental phase transition and lateral organ growth. Snf1/AMPK/SnRK1 kinases are important sensors of the cellular energy level, and they are activated in response to starvation and cellular stress. Here we present findings that indicate FUS3 and AKIN10 functionally overlap in ABA signalling, but play different roles in sugar responses during germination. Seeds overexpressing FUS3 and AKIN10 both display ABA-hypersensitivity and delayed germination. The latter is partly dependent on de novo ABA synthesis in both genotypes, as delayed germination can be partially rescued by the ABA biosynthesis inhibitor, fluridone. However, seeds and seedlings overexpressing FUS3 and AKIN10 show different sugar responses. AKIN10-overexpressing seeds and seedlings are hypersensitive to glucose, while those overexpressing FUS3 display overall defects in osmotic stress, primarily during seedling growth, as they show increased sensitivity towards sorbitol and glucose. Hypersensitivity to sugar and/or osmotic stress during germination are partly dependent on de novo ABA synthesis for both genotypes, although are likely to act through distinct pathways. This data suggests that AKIN10 and FUS3 both act as positive regulators of seed responses to ABA, and that AKIN10 regulates sugar signalling while FUS3 mediates osmotic stress responses.
4.1 Introduction

Seed formation is a critical adaptation in the plant life cycle, as it allows plants to temporarily cease growth in adverse conditions until the environment becomes favourable. During the maturation phase of embryogenesis, the embryo accumulates nutrient reserves, acquires desiccation tolerance and enters a stage of dormancy. The transition from embryonic to vegetative development (germination) is tightly regulated by the hormones abscisic acid (ABA), which promotes dormancy and inhibits germination, and gibberellic acid (GA), which has the opposite effect of breaking dormancy and stimulating germination (Koorneef et al., 2002; Finkelstein et al., 2008). In Arabidopsis, B3-domain transcription factors of the AFL (ABSCISIC ACID INSENSITIVE3, FUSCA3, LEAFY COTYLEDON2) family act as master regulators of late embryogenesis, as loss- and gain-of-function mutations in these genes greatly affect seed maturation (Suzuki and McCarty, 2008).

Genetic and molecular analyses indicate FUSCA3 (FUS3) inhibits the transition from the embryonic to the vegetative phase of development by promoting ABA accumulation while inhibiting GA biosynthesis (Gazzarrini et al., 2004). The loss-of-function mutant, fus3-3, bypasses dormancy and enters postembryonic development prematurely due to a lower ABA/GA ratio (Keith et al. 1994; Curaba et al., 2000; Nambara et al. 2000; Gazzarrini et al., 2004). Conversely, ectopic expression of FUS3 post-embryonically (ML1:FUS3) delays seed germination and plant development by increasing ABA level while repressing GA biosynthesis (Gazzarrini et al., 2004; Chiu et al., 2012). The stability of the FUS3 protein also appears to be tightly regulated by ABA, GA and the 26S proteasome (Gazzarrini et al., 2004; Lu et al., 2010).

In our recent study, AKIN10, was identified as an interactor of FUS3 from yeast two-hybrid screens (Tsai and Gazzarrini, 2012). AKIN10 belongs to the Sucrose-Non-Fermenting 1 (Snf1)-related kinase1 (SnRK1) family and acts as a central regulator of cellular energetics in plants (Halford and Hey, 2009). Our results indicate AKIN10 physically interacts with and phosphorylates FUS3 at its N-terminal region, and delays its degradation in a cell-free system. Overexpression of AKIN10 (35S:AKIN10) causes delays in developmental phase transitions (germination and flowering) and defects in lateral organ formation. These phenotypes can be partially rescued by the fus3-3 mutation, suggesting FUS3 and AKIN10 act in overlapping
pathways to regulate developmental phase transitions and lateral organ development (Tsai and Gazzarrini, 2004). Interestingly, SnRK kinases also regulate stress responses, hormonal and sugar signalling pathways by affecting transcription at the global level (Bradford et al., 2003; Radchuk et al., 2006; Baena-González et al., 2007; Jossier et al., 2009; Radchuk et al., 2010). This prompted our investigation of the role of AKIN10 and FUS3 in ABA and sugar responses during germination.

4.2 Results

We first tested germination rates (radicle protrusion) of two independent lines overexpressing AKIN10 (35S:AKIN10) and FUS3 (ML1:FUS3) on ABA (see Material and Methods). Both 35S:AKIN10 and ML1:FUS3 transgenic plants were previously shown to delay seed germination on minimal MS medium (Baena-González et al., 2007; Chiu et al., 2012) with ML1:FUS3 showing a greater germination delay than 35S:AKIN10 (Figure 1). The delayed germination has been attributed to the heightened sensitivity to and level of ABA in ML1:FUS3 seeds (Gazzarrini et al., 2007; Chiu et al., 2012), but the cause of delay remains unknown for 35S:AKIN10 seeds. To test whether altered ABA sensitivity contributes to the 35S:AKIN10 germination delay, wild type (WT), ML1:FUS3 and 35S:AKIN10 seeds were germinated on a low concentration of ABA (0.2 µM). ABA delayed WT germination, though germination rates recovered to approximately 80% in 5 days (Figure 1). ML1:FUS3 germination was more sensitive to ABA compared to WT, and reached only 20-50% after 5 days of treatment (Figure 1a). 35S:AKIN10 germination was also hypersensitive to ABA, but recovered to approximately 80% within 5 days of treatment (Figure 1b). Germination rates tested on 0.4 µM ABA show that both genotypes are slightly hypersensitive to this concentration of ABA compare to WT (Figure 1c). This suggests that both AKIN10 and FUS3 positively regulate ABA sensitivity during germination.

In order to dissect the role of ABA in the delayed germination of FUS3- and AKIN10-overexpressing seeds, WT, ML1:FUS3 and 35S:AKIN10 germination rates were assayed in media supplemented with 10 µM fluridone, an inhibitor of phytoene desaturase which reduces ABA synthesis (Chamovitz et al., 1993; Grappin et al., 2000). If delayed germination of the transgenic lines is due to increased ABA synthesis, then fluridone should rescue this delay.
Germination rates of WT, *ML1:FUS3* and *35S:AKIN10* seeds were higher in the presence of fluridone compared to untreated seeds (Figure 1a, b), suggesting *de novo* ABA synthesis negatively regulates germination in all genotypes. However, *ML1:FUS3* and *35S:AKIN10* seeds still germinated later than WT on fluridone (Figure 1a,b), and their rates did not increase even at a higher concentration of fluridone (50 µM). This suggests *de novo* ABA synthesis alone cannot explain the delayed germination phenotype of *ML1:FUS3* and *35S:AKIN10* seeds.
**Figure 1.** Overexpression of *FUS3* or *AKIN10* leads to ABA hypersensitivity and delayed germination, which is partly dependent on *de novo* ABA synthesis.

Germination (radicle protrusion) kinetics of seeds from WT and two independent lines of *ML1:FUS3* (a) or *35S:AKIN10* (b) on MS media or MS supplemented with 0.2 µM ABA or 10 µM fluridone (FLU). WT germination is significantly higher (P<0.01) than *35S:AKIN10* on 10 µM FLU at 2 days after imbibition (DAI).

(c) ABA dose-response curves for WT, *ML1:FUS3* and *35S:AKIN10* seed germination (radicle protrusion) 2 days after imbibition. WT germination is higher (P<0.001) than *ML1:FUS3* and *35S:AKIN10* at 0.4 µM ABA. Averages from 3 experiments ± SD are shown. 100-150 seeds were used in each experiment.
ABA and sugar signalling pathways are intricately related and share common downstream signalling components (Gazzarrini and McCourt, 2001; Cheng et al., 2002; Gibson, 2005). Since AKIN10 is known to partake in sugar signalling (Hardie, 2007; Halford and Hey, 2009) and since FUS3 expression is regulated by sugar (Tsukagoshi et al., 2007), we investigated the role of FUS3 and AKIN10 in sugar signalling during germination. WT, ML1:FUS3 and 35S:AKIN10 germination rates were assayed two days after imbibition on 3% glucose or 3% sorbitol as an osmotic control. At the concentration tested glucose, but not sorbitol, significantly reduced WT seed germination, as previously described (Figure 2a) (Dekkers et al., 2004; Gibson, 2005). 35S:AKIN10 germination was similar to WT on sorbitol, but showed hypersensitivity on glucose (Figure 2a). Surprisingly, ML1:FUS3 germination was reduced by sorbitol, while the effect of glucose varied between the two transgenic lines (Figure 2a). To better understand the effect of exogenous glucose application, seedlings growth rates (cotyledon expansion) were assayed 4 days after imbibition in the presence of the sugars. In this case, cotyledon expansion of both ML1:FUS3 transgenic lines was hypersensitive to both sorbitol and glucose, whereas 35S:AKIN10 cotyledon expansion was inhibited specifically by glucose (Figure 2b). These results indicate that overexpression of FUS3 during germination causes hypersensitivity towards osmotic stress, whereas overexpression of AKIN10 leads to hypersensitivity specifically towards glucose. We next tested whether the osmotic hypersensitivity of ML1:FUS3 and glucose hypersensitivity of 35S:AKIN10 seeds during germination are dependent on increased ABA synthesis. Germination rates were assayed 2 days after imbibition on 10 µM fluridone in the presence and absence of 3% glucose or sorbitol. In both cases, fluridone was able to partially restore the germination delay imposed by sorbitol and glucose on ML1:FUS3 and glucose on 35S:AKIN10 (Figure 3). These results indicate the hypersensitivity of ML1:FUS3 seeds to osmotic stress and glucose hypersensitivity of 35S:AKIN10 seeds are both partially dependent on de novo ABA synthesis.
**Figure 2.** Overexpression of *FUS3* or *AKIN10* leads to different responses to sugar during seed germination and seedling growth.

(a) Germination (radicle protrusion) of WT, *ML1:FUS3* and *35S:AKIN10* seeds 2 days after imbibition on MS ± 3% sorbitol (sor) or 3% glucose (glc).

(b) Seedling growth (cotyledon expansion) of WT, *ML1:FUS3* and *35S:AKIN10* seeds 4 days after imbibition on MS ± 3% sor or 3% glc. Averages from 3 experiments ± SD are shown. 100-150 seeds were used in each experiment.
(a) % Germination (radicle protrusion)

(b) % Seedling growth (cotyledon expansion)

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<td>3% sor</td>
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Figure 3. Osmotic stress hypersensitivity of \textit{ML1:FUS3} seeds and glucose hypersensitivity of \textit{35S:AKIN10} seeds are both partially dependent on \textit{de novo} ABA synthesis.

Germination (radicle protrusion) of \textit{35S:AKIN10} and \textit{ML1:FUS3} seeds 2 days after imbibition on MS, 10 \(\mu\)M fluridone (FLU), 3\% sorbitol (sor) \(\pm\) 10 \(\mu\)M FLU, and 3\% glucose (glc) \(\pm\) 10 \(\mu\)M FLU. Averages from 3 experiments \(\pm\) SD are shown. 100-150 seeds were used in each experiment.
% Germination (radicle protrusion)

35S:AKIN10 (11)

ML1:FUS3 (12)

MS  FLU  FLU + sor  sor  FLU + glc  glc
4.3 Discussion

Collectively, the data shown here demonstrate a positive role of *AKIN10* and *FUS3* in ABA responses during germination, as well as distinct roles in sugar and osmotic stress responses during seed germination and seedling growth. Indeed, FUS3 plays an inhibitory role under osmotic stress, while AKIN10 inhibitory role is specific for glucose. These data complement previous findings showing that plants overexpressing *AKIN10* display defects in post-embryonic development and root elongation on exogenous ABA and glucose (Baena-González et al., 2007; Jossier et al., 2009). Notably, the germination of seeds overexpressing *AKIN10* was previously shown to be unaffected by 3 µM ABA (Jossier et al., 2009). This is likely due to the high concentration of ABA used by Jossier et al. (2009), and that we recorded germination rates over multiple time points after imbibition.

Although fluridone was able to elevate the germination kinetics in seeds of both genotypes on MS media, it was not sufficient to fully recover the delayed germination of *ML1:FUS3* and *35S:AKIN10* seeds to the WT level. This suggests de novo ABA synthesis is only partially responsible for *ML1:FUS3* and *35S:AKIN10* delayed germination. It is possible that overexpression of *FUS3* or *AKIN10* already increases ABA level during embryogenesis, thus increasing seed dormancy. This is likely the case for FUS3, considering that a short activation of FUS3 indeed increases ABA level in *ML1:FUS3-GR* seedlings, while loss-of-function *fus3-3* embryos contain less ABA (Nambara et al., 2000; Gazzarrini et al., 2004). The germination delay of *35S:AKIN10* seeds was more closely, but not completely, inhibited by fluridone. Although no differences in ABA level were previously found between WT and AKIN10-overexpressing seedlings (Jossier et al., 2009), this does not exclude the possibility of higher ABA accumulation in *35S:AKIN10* embryos compared to WT. In addition, ABA accumulation prior to germination may also explain why fluridone was unable to fully restore *ML1:FUS3* and *35S:AKIN10* reduced germination in the presence of glucose. Alternatively, ABA-independent pathways may be activated by FUS3 and AKIN10 during germination.

In conclusion, the data presented here indicate both FUS3 and AKIN10 act as positive regulators of ABA signalling during germination, although showing different sensitivity to the hormone, but they play different roles in sugar and osmotic stress signalling. *AKIN10* is
involved in glucose-specific pathway(s), while FUS3 modulates osmotic stress responses. Both sugar and osmotic responses regulated by AKIN10 and FUS3 are partly dependent on de novo ABA synthesis, likely through distinct pathways. It remains to determine if phosphorylation of FUS3 by AKIN10 is required to modulate seed sensitivity to ABA.

4.4 Material and methods

Arabidopsis seeds of WT (Col-0), FUS3-overespressing (ML1:FUS3-GFP; Gazzarrini et al., 2004; Lu et al., 2010) and AKIN10-overexpressing (35S:AKIN10-HA; Tsai and Gazzarrini, 2012) lines were vernalized and germinated as previously described (Lu et al., 2010). ABA, fluridone, sorbitol and glucose supplemented in the media at the concentrations specified in each experiment. Germination was considered positive when the radicle had emerged from the seed, while seedling growth was scored positive when the cotyledons were expanded. The ML1:FUS3 construct was previously shown to rescue the fus3-3 embryonic phenotypes, including desiccation intolerance of the seeds, and to ectopically express FUS3 post-embryonically (Gazzarrini et al., 2004; Lu et al. 2010). In contrast, 35S:FUS3 does not rescue the fus3-3 mutant and causes co-suppression when transformed in a WT background. Therefore, the 35S promoter could not be used to overexpress FUS3. Conversely, 35S:AKIN10 has been shown to overexpress AKIN10 post-embryonically (Tsai and Gazzarrini, 2012). Since ML1 and AKIN10 expression levels are very similar throughout development, as measured in several microarrays (eNorthern; http://bar.utoronto.ca), we did not attempt to overexpress AKIN10 using the ML1 promoter.

4.5 References


Chapter 5

Discussion
*FUS3* has been characterized as a master regulator of late embryogenesis and maturation by controlling the synthesis of ABA and GA. These hormones, in turn, regulate the level of the FUS3 protein. We have proposed post-translational regulatory mechanisms that may explain how FUS3 protein level is regulated by hormones. Two pathways appear to regulate FUS3 protein levels: one promotes rapid FUS3 degradation through the proteasome, is mediated by the PEST motif and stimulated by GA; the other delays FUS3 degradation, is mediated by AKIN10 and stimulated by ABA. In addition, new roles for *FUS3* and AKIN10 have been identified. *FUS3* and AKIN10 were shown to genetically interact to inhibit two major developmental phase transitions: embryonic to vegetative and vegetative to reproductive phases of development. Furthermore, FUS3 and AKIN10 are positive regulators of ABA signaling and both differ in their response to osmotic stress during germination, suggesting these processes are regulated by independent pathways. Finally, *FUS3* and AKIN10 also genetically interact to regulate lateral organ development.

5.1 The roles of *FUS3*, *AKIN10* and auxin in cotyledon development

*FUS3* function has been mainly characterized during mid-late embryogenesis. However, *FUS3* is already expressed at the globular stage embryo during cotyledon initiation, and its expression is promoted by auxin, though the role of *FUS3* at this stage isn’t entirely clear (Gazzarrini et al., 2004). Our results suggest *FUS3* genetically interacts with AKIN10 to regulate cotyledon development, as defects in cotyledon numbers caused by overexpression of AKIN10 are partially dependent on a functional *FUS3* allele (Chapter 3). Auxin plays a pivotal role in the initiation and development of cotyledons and their vasculature (reviewed by Chandler et al., 2008). Both *FUS3* and AKIN10 regulate the expression of auxin synthesis genes, and mutants affected in auxin synthesis, signaling and transport cause similar cotyledon defects (Baena-González et al., 2007; Chandler, 2008; Yamamoto et al., 2010). This, in addition to our results, suggests *FUS3* likely acts downstream of AKIN10 to regulate cotyledon number and morphology by controlling auxin synthesis, perception and/or transport. Auxin in turn activates FUS3 expression to establish a feed-forward regulation (Figure 1). Auxin accumulation and PIN transporter localization patterns in *akin10* and *fus3* mutants can be determined to validate this hypothesis.
Surprisingly, a small but significant portion of fus3-3 seedlings also show aberrant cotyledon number, despite this mutation being able to partially rescue the cotyledon number defect of 35S:AKIN10 (Chapter 3). Similarly, silencing instead of overexpression of PsSnRK1 in pea embryo also causes defects in cotyledon morphology and symmetry (Radchuk et al., 2006; Radchuk et al., 2010b). Thus, loss-of-function mutations in FUS3 and SnRK1 silencing can both lead to cotyledon defects similar to overexpression of AKIN10. This implies cotyledon development control by FUS3 and AKIN10 is complex, and does not simply correlate to FUS3 or AKIN10 expression level.

5.2 Post-translational regulation and homeostasis of FUS3

FUS3 mRNA is present throughout embryogenesis, and the FUS3 promoter is active even in early seedlings. However, the FUS3 protein is undetectable by the end of embryogenesis, suggesting FUS3 protein level is actively reduced by an unknown post-transcriptional regulatory mechanism (Gazzarrini et al., 2004; Tsuchiya et al., 2004). Although ABA increases while GA decreases FUS3-GFP fluorescence in seedlings ectopically expressing FUS3, the molecular mechanisms of these regulations remain elusive (Gazzarrini et al., 2004). We have demonstrated that FUS3 is a short-lived protein, whose degradation is dependent on the proteasome and regulated by a C-terminal PEST degradation motif; this PEST motif is also required for the GA-induced and ABA-inhibited instability of the FUS3 protein (Chapter 2). Based on these findings, it is plausible that GA stimulates FUS3 degradation by the proteasome pathway using a PEST-dependent mechanism (Figure 1). Consistent with our findings, PEST-mediated protein degradation is indeed often achieved by the proteasome (Rechsteiner and Rogers, 1996). Interestingly, the closely related ABI3 is ubiquitinated by the E3 ligase AIP2 for degradation (Zhang et al., 2005). Therefore, FUS3 is likely degraded by ubiquitin-dependent pathway as well. However, the molecular link between PEST-mediated FUS3 degradation and GA is still unclear. Often the PEST motif needs to be phosphorylated to trigger proteolysis, and several potential phosphorylation sites are indeed present on the FUS3 PEST (Rechsteiner and Rogers, 1996; Chapter 3). Some of these sites may be phosphorylated or dephosphorylated in response to GA, thereby rendering FUS3 susceptible to ubiquitination and thus degradation. None of
these phosphorylation sites match the SnRK consensus target motif, suggesting SnRKs are unlikely to be the kinases that directly regulate GA-dependent FUS3 degradation.

The regulatory relationship between SnRK1 and GA is not well documented in Arabidopsis, but in pea SnRK1 has been shown to repress GA synthesis, and the tomato SnRK1 γ subunit LeSNF4 expression is repressed by GA (Radchuk et al., 2006; Bradford et al., 2009). Therefore, in Arabidopsis AKIN10 may also regulate GA synthesis and/or signaling, thereby potentially controlling FUS3 degradation (Figure 1). However, the PEST motif mediating FUS3 degradation is at the CTD, while the AKIN10 phosphorylation sites are at the FUS3 NTD. It is possible that AKIN10-mediated FUS3 phosphorylation may cause conformational changes that affect the C-terminus, possibly exposing or concealing the PEST motif.

ABA increases FUS3 protein level by an unknown mechanism (Gazzarrini et al., 2004; Chapter 2). Our results have helped elucidate this mechanism by identifying AKIN10 as a positive regulator of FUS3 stability, as AKIN10 overexpression delays FUS3 degradation (Chapter 3). This, in addition to the positive role AKIN10 plays in ABA signaling, makes AKIN10 the prime candidate as the molecular link that connects FUS3 increased accumulation in the presence of ABA (Radchuk et al., 2006; Jossier et al., 2009; Radchuk et al., 2010b; Chapter 4). Together, a hypothesis can be formed with AKIN10 being the upstream element that relays ABA signaling to FUS3, thereby delaying FUS3 degradation (Figure 1).

The mechanism of how AKIN10 delays FUS3 degradation remains in question (Chapter 3). AKIN10 was shown to phosphorylate the FUS3 N-terminal region, making phosphorylation the simplest hypothesis to explain how AKIN10 delays FUS3 degradation; perhaps phosphorylated FUS3 adapts a conformation that is less recognized by the ubiquitin ligase or the proteasome. However, this hypothesis needs to be empirically determined. It is possible that FUS3 phosphorylation by AKIN10 alters properties of FUS3 other than its turnover, such as FUS3 DNA-binding activity or sub-cellular localization. On the other hand, AKIN10 may phosphorylate other proteins or promote the expression of other genes, which in turn delays FUS3 degradation by indirect mechanisms (Figure 1).
Since AKIN10 belongs to multigene SnRK kinase family, AKIN10 may not be the only kinase that interacts with and phosphorylates FUS3. Evidently, not all potential SnRK1 phosphorylation sites on FUS3 were shown to be phosphorylated by AKIN10 (Chapter 3). One such other candidate that may phosphorylate FUS3 is the SnRK1 kinase AKIN11, the closest homologue of AKIN10 (Halford and Hey, 2009). Indeed, AKIN10 is expressed in the ovule while AKIN11 is expressed in the embryo proper, and therefore AKIN11 is more likely to interact with FUS3 in developing embryos (Bitrián et al., 2011). SnRK2 kinases are also strong candidates for mediating FUS3 phosphorylation, as their role in ABA signaling is much better defined than that of SnRK1s (reviewed by Halford and Hey, 2009; Cutler et al., 2010). Furthermore, SnRK2 kinases share part of the target site motif with SnRK1, thus sites phosphorylated by AKIN10 can potentially be phosphorylated by SnRK2 kinases as well (Halford and Hey, 2009). Conversely, SnRK kinases may interact and phosphorylate structurally-related AFL genes with similar downstream effects, as they share similar functions during late embryogenesis. Although ABI3 does not appear to be phosphorylated by SnRK2 kinases (Nakashima et al., 2009), other permutations of SnRK-AFL interactions remain uncharacterized.
**Figure 1.** Model of *FUS3* and *AKIN10* regulation of embryogenesis and germination by hormonal control

Proposed model depicting the regulatory relationships between *FUS3*, *AKIN10*, hormones auxin, ABA and GA, and their roles in embryogenesis and germination. Pointed arrows denote positive regulation; flat-ended arrows denote negative regulation. Solid lines denote signaling or transcriptional regulation, dashed lines denote protein-protein interaction and post-translational regulation, dotted lines denote indirect protein level regulation.

Auxin promotes the expression of *FUS3* and *AKIN10* (Gazzarrini et al., 2004; Radchuk et al., 2010a). *FUS3* and *AKIN10* promote YUC gene expression and repression of *SUR1*, respectively (Baena-González et al., 2007; Yamamoto et al., 2010), suggesting a potential mechanism for *FUS3* and *AKIN10* regulation of cotyledon development. ABA stabilizes the FUS3 protein while promotes expression of the tomato SnRK1 γ subunit (Brocard-Gifford et al., 2003; Gazzarrini et al., 2004; Chapter 2). *FUS3* promotes ABA synthesis, and both *FUS3* and *AKIN10* positively regulate ABA synthesis and/or perception (Nambara et al., 2000; Gazzarrini et al., 2004; Jossier et al., 2009; Yamamoto et al., 2010; Chapter 4). ABA in turn promotes seed maturation while inhibits seed germination. GA represses the expression of the tomato SnRK1 γ subunit and reduces FUS3 protein levels by its PEST motif during germination, while *FUS3* represses GA synthesis during embryogenesis (Brocard-Gifford et al., 2003; Gazzarrini et al., 2004; Yamamoto et al., 2010). ABA and GA also antagonize each other by FUS3-independent mechanism (reviewed by Razem et al., 2006; Weiss and Ori, 2007). AKIN10 interacts and phosphorylates FUS3, and contributes to decreasing FUS3 degradation (Chapter 3). Finally, SnRK2 kinases may also phosphorylate FUS3 in an ABA-dependent manner.
5.3 *AKIN10* and *FUS3* regulate developmental phase transitions by hormonal control

*FUS3* regulates the timing of developmental programs through hormonal control. *FUS3* inhibits seed germination by repressing GA biosynthesis while increasing ABA level; *FUS3* also delays vegetative growth and flowering by inhibiting GA biosynthesis (Gazzarrini et al., 2004; Chapter 2). Here, we have shown that *AKIN10* also negatively regulates seed germination and flowering time, and that delayed flowering phenotype caused by overexpression of *AKIN10* is *FUS3*-dependent (Chapter 3). Since *FUS3* and the SnRK1-activating kinases (SnAKs) are both expressed in the SAMs and negatively regulate cell division (Gazzarrini et al., 2004; Shen and Hanley-Bowdoin, 2006; Lumba et al., 2012; Chapter 3), it is possible that *AKIN10* and *FUS3* attenuate cell division to delay vegetative growth and flowering (Figure 2).

Recently, *FUS3* was shown to negatively regulate vegetative phase transitions by repressing ethylene signaling (Figure 2). Loss-of-function *fus3*-3 plants prematurely develop rosette leaves with abaxial trichomes and greater blade-to-petiole ratio, which are hallmark features of older leaves; these phenotypes can be corrected by chemically or genetically inhibiting ethylene signaling (Lumba et al., 2012). On the contrary, overexpression of *FUS3* represses the expression of ethylene synthesis and signaling genes, thereby delaying vegetative development (Lumba et al., 2012). This indicates that ethylene promotes vegetative phase transition, and that repression of ethylene pathway by *FUS3* delays vegetative phase transition. Ethylene also positively regulates senescence (reviewed by Lim et al., 2007). Ethylene-insensitive mutants show delayed senescence while ethylene synthesis is increased in senescing leaves (Oh et al., 1997; van der Graaff et al., 2006) (Figure 2). The role of FUS3 in senescence has yet to be investigated, but loss- and gain-of-function studies suggest *AKIN10* negatively regulates senescence (Baena-Gonzáles et al., 2007; Cho et al., 2012; Chapter 3) (Figure 2). It would be interesting to determine whether the physical and/or genetic interaction between FUS3 and AKIN10 controls or is regulated by ethylene signaling as a means of regulating these developmental processes.
**Figure 2.** Model of *FUS3* and *AKIN10* regulation of vegetative development by hormonal control.

Proposed model depicting the regulatory relationships between *FUS3*, *AKIN10*, hormones GA and ethylene, and their roles in vegetative development. Pointed arrows denote positive regulation; flat-ended arrows denote negative regulation. Solid lines denote signaling or transcriptional regulation; dashed lines denote protein-protein interaction and post-translational regulation.

*AKIN10* negatively regulates flowering and senescence (Baena-Gonzáles et al., 2007; Chapter 3). AKIN10 phosphorylates FUS3, and contributes to decreasing FUS3 degradation (Chapter 3). FUS3 in turn represses the synthesis of GA and ethylene action (Gazzarrini et al., 2004; Yamamoto et al. 2010; Lumba et al., 2012). GA and ethylene positively regulates vegetative development, flowering and senescence.
5.4 The role of AKIN10 and FUS3 in abiotic stress signaling

The seed maturation process is comparable to abiotic stress response in several aspects. Seeds lose the majority of their water content during maturation, therefore maturing seeds likely experience drought stress and osmotic stress associated with water loss (Egli and TeKrony, 1997). Mature seeds of annual plants must overwinter, thus seeds inevitably experience cold stress as well. In addition, plants exposed to abiotic stresses and maturing seeds are associated with a rise in ABA level, and consequently both maturing seeds and vegetative tissues undergoing abiotic stress or treated with ABA share comparable transcriptional profiles (reviewed by Qin et al., 2011). Our results showed both FUS3 and AKIN10 act in overlapping pathways to relay different abiotic stress signals to generate an ABA response and delay germination. Overexpression of AKIN10 enhances glucose sensitivity, while overexpression of FUS3 leads to hypersensitivity towards osmotic stress (Chapter 4) (Figure 3). Despite these different responses, both sensitivity changes are dependent on ABA, as inhibition of ABA synthesis partially rescues the germination hypersensitivity in both cases (Chapter 4). Consequently, overexpression of either AKIN10 or FUS3 confers ABA hypersensitivity during germination (Chapter 4) (Figure 3). The ABA elevation in response to osmotic stress is well-documented (Yamaguchi-Shinozaki and Shinozaki, 2006). On the other hand, sugar and ABA signaling pathways are known to share common signaling components, while excessive sugar also triggers ABA accumulation (Arenas-Huertero et al., 2000; Gibson, 2005; Rook et al., 2006). FUS3 and AKIN10, therefore, converge different types of stress stimuli into a common ABA response, leading to growth arrest manifested as delayed germination (Figure 3).

Seed germination at high temperature can be lethal for the developing seedling and results in seedling growth arrest (Tamura et al., 2006; Toh et al. 2008). Recently, FUS3 mRNA and protein levels were shown to increase during germination at high temperature (Chiu et al., 2012) (Figure 3). In addition, seeds overexpressing FUS3 are hypersensitive to high temperature and do not germinate, but remain viable and recover when transferred to ambient temperature (Chiu et al., 2012). FUS3 actively protects the embryo from premature germination at high temperatures by increasing ABA synthesis and delaying germination, indicating that FUS3 plays
a role in heat stress responses. Similar to *FUS3*, *AKIN10* has been proposed to regulate abiotic stress responses such as darkness, hypoxia and herbicide (Baena-González et al., 2007; Baena-González and Sheen, 2008; Jossier et al., 2009). Considering *AKIN10* positively regulates *FUS3* level, it will be interesting to test whether *AKIN10* and/or other SnRK kinases increase *FUS3* protein accumulation in response to heat stress. ABI3 participates in heat stress response as well, as it has been shown to drive the expression of the heat stress transcription factor A9 (HsfA9) in an ABA-dependent manner; HsfA9 in turn promotes the expression of various heat-shock proteins (Kotak et al., 2007). It is possible that other AFL genes also show conserved function in heat stress response.
Figure 3. Model of FUS3- and AKIN10-mediated stress responses during germination.

Proposed model depicting FUS3 and AKIN10 perceiving stress stimuli to inhibit germination, mediated by ABA. Pointed arrows denote positive regulation; flat-ended arrows denote negative regulation. Solid lines denote signaling or transcriptional regulation, dashed lines denote protein-protein interaction and post-translational regulation, dotted lines denote indirect protein level regulation.

Abiotic stress such as excessive glucose, osmotic or heat stress elevate ABA level (Yamaguchi-Shinozaki and Shinozaki, 2006; Arenas-Huertero et al., 2000); excessive glucose and heat can also indirectly cause osmotic stress. Excessive glucose also triggers ABA accumulation. ABA in turn derepresses SnRK2 kinases to inhibit seed germination. Heat stress also increases the mRNA and protein levels of FUS3 (Chiu et al., 2012). FUS3 promotes the synthesis of ABA, while ABA in turn increases FUS3 protein level, both FUS3 and AKIN10 positively regulate ABA responses, while ABA promotes the expression of SnRK1 γ subunit (Nambara et al., 2000; Bradford et al., 2003; Gazzarrini et al., 2004; Jossier et al, 2009; Chapter 2; Chapter 4). In addition, FUS3 and AKIN10 may inhibit germination by ABA-independent mechanisms as well (Chapter 4). AKIN10 phosphorylates FUS3, which may contribute to increasing FUS3 protein accumulation (Chapter 3). SnRK2 kinases may also phosphorylate FUS3 to relay ABA signal, since SnRK2 share phosphorylation target motif with AKIN10 (Halford and Hey, 2009).
The involvement of AFL genes in abiotic stress responses also sheds new light on the evolution of this subfamily and seed dormancy. Primitive plants such as algae, moss and fern do not form seeds, instead progress immediately to vegetative growth upon the completion of embryogenesis (Harada, 1997; Vicente-Carbayosa and Carbonero, 2005). The lack of seed formation and dormancy limits the growth of these plants in humid environments and less adverse conditions. Interestingly, ABI3/VP1-like B3 transcription factors have been identified in the genomes of moss *Physcomitrella patens*, green algae *Chlamydomonas reinhardtii* and *Volvox carteri* (Romanel et al., 2009). This brings the question of what the functions of these ABI3/VP1 factors are in moss and algae if they don’t form seeds. The characterization of AFL as osmotic, drought, salt and/or heat stress response regulators, together with the interaction of FUS3 with AKIN10, which also regulates stress response, suggests the ancestral B3 transcription factor may be primarily involved in abiotic stress response (Romanel et al. 2009).

In closing, the convergence between seed maturation and abiotic stress response pathways, as demonstrated by the FUS3-AKIN10 physical and genetic interactions, provides new insights about the evolution of seed formation. Based on these findings, it can be hypothesized that seed formation and dormancy program may be derived from the abiotic stress response pathway. Understanding how the seed maturation programs is re-activated post-embryonically in response to stress may shed light on how stress response pathways have evolved to regulate seed development and dormancy.

5.5 References


Appendix

Supplementary experimental procedures
6.1 Yeast two-hybrid screen

6.1.1 Yeast strain and growth media

_Saccharomyces cerevisiae_ strain EGY48 (MATα, his3, trp1, ura3, 6 lexAop-LEU2) (Clontech) was used for all yeast two-hybrid experiments. All yeast growth media were made as described by Rose et al., 1990.

6.1.2 Testing for auto-activation of the bait constructs

The _pSH18-34_ plasmid (Clontech) harbouring the LacZ reporter was used to transform _Saccharomyces cerevisiae_ strain EGY48. Yeast transformation was performed essentially as described by Gietz and Woods, 2002. EGY48 cells were grown in 5 ml of YPD media and harvested from overnight culture by centrifugation. The pellet was then resuspended in the following components in the listed order: 240 µl 50% (w/v) polyethylene glycol 3500, 36 µl 1 M lithium acetate, 10 µl 10 mg/ml boiled salmon sperm carrier DNA (Sigma), 1 µg _pSH18-34_ plasmid DNA in 74 µl sterile double-distilled water (ddH2O). The transformation mixture was mixed by gentle pipetting until homogeneous, and incubated in a 42 °C water bath for 1~3 hours, mixing occasionally by inversion. Yeast cells were harvested from the transformation mixture by quick centrifugation. The supernatant was removed; the pellet was resuspended in 100 µl sterile ddH2O, and plated on yeast nitrogenous base (YNB) –Ura media with 2% (w/v) glucose. The plates were incubated at 30 °C for 48 hours.

Subsequently, the _FUS3ΔC-BD/pEG202_ and _FUS3(N90)-BD/pEG202_ constructs were used to individually transform EGY48[_pSH18-34_], to establish EGY48[_pSH18-34, FUS3ΔC-BD/pEG202_], and EGY48[_pSH18-34, FUS3(N90)-BD/pEG202_], respectively, using the transformation protocol described above.

To test for FUS3 bait auto-activation properties, EGY48[_pSH18-34, FUS3ΔC-BD/pEG202_] and EGY48[_pSH18-34, FUS3(N90)-BD/pEG202_] colonies were resuspended in sterile ddH2O, and 5 µl were used to inoculate YNB –Ura/–His/–Leu media with 2% (w/v) galactose and 1% (w/v) raffinose, and YNB ura- his- media with 2% galactose, 1% raffinose, 1x buffered (BU) salt and 80 µg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (Xgal). Autonomously activating
bait constructs trigger LEU2 expression, thus allowing growth in leu- media, and beta-galactosidase activity, thus generating blue precipitate in the presence of Xgal.

6.1.3 cDNA library construction and amplification

The Arabidopsis cDNA library was made from mRNA extracted from Arabidopsis flower buds, siliques and imbibed seeds. The mRNA was converted to cDNA and cloned in the pJG4-5 plasmid (Clontech) by Norclone Biotech Laboratories.

100 µl of the *Escherichia coli* cDNA library glycerol stock were diluted in 20 ml of Luria broth (LB) media, and the titre of the dilution was determined by serial plating, while the diluted stock was kept in 4 °C. Once the titre had been determined, 5 x 10^5 colony forming units (CFU) of the diluted cDNA library glycerol stock were plated on LB media with 100 µg/ml ampicillin, using 20 petri dishes (150 mm) with 2.5x10^4 CFU on each, and incubated for 16 hours at 37 °C. Two ml of sterile ddH2O was added on the plate and colonies were harvested using a scraper. The cells were pooled and washed with sterile ddH2O, and the cDNA library was extracted from the pellet using the Plasmid Maxi Kit (Qiagen) according to manufacturer’s instructions.

6.1.4 cDNA library transformation and selection for interaction

50 µg of the cDNA library was used to transform the EGY48[pSH18-34, FUS3AC-BD/pEG202] and EGY48[pSH18-34, FUS3(N90)-BD/pEG202] strains. Yeast transformation was performed essentially as described by Gietz et al., 1997. 5 ml of EGY48[pSH18-34, FUS3AC-BD/pEG202] or EGY48[pSH18-34, FUS3(N90)-BD/pEG202] overnight culture in YNB –Ura/--His media with 2% glucose was used to inoculate 500 ml of pre-warmed yeast peptone dextrose (YPD) media, and incubated in 30 °C with agitation until OD_{600} = 0.5~0.6. Yeast cells were harvested by centrifugation (3000 rpm, 4 °C for 5 minutes) and washed once with sterile ddH2O. The pellet was resuspended in 30 ml of 0.1 M lithium acetate, split into 50 aliquots of 600 µl each, then incubated in 30 °C for 15 minutes. Cells were harvested by quick centrifugation, the supernatants were discarded, and to the pellet the following components were added in the order listed: 480 µl 50% (w/v) polyethylene glycol 3500, 72 µl 1 M lithium acetate, 5 µl 10 mg/ml boiled salmon sperm carrier DNA (Sigma), 1 µg cDNA library in 120 µl sterile ddH2O. The transformation mixtures were mixed by gentle pipetting until homogeneous, and incubated in a water bath first at 30 °C for 30 minutes, then at 42 °C for 45~60 minutes, mixing occasionally
by inversion. Yeast cells were harvested from the transformation mixtures by quick centrifugation. Supernatants were removed, and pellets were resuspended in 300 µl sterile ddH₂O to be plated on YNB ura- his- trp- media with 2% glucose. The plates were incubated at 30 °C for 48 hours.

The colonies harbouring the cDNA library were harvested by adding 2 ml of sterile ddH₂O to each plate, and the colonies were collected using a scraper. Cells harbouring the library were pooled and washed with sterile ddH₂O. The pellet was resuspended in a glycerol solution [65% (v/v) glycerol, 0.1 M MgSO₄, 25 mM Tris-HCl pH 8] and stored at -80 °C.

To select for FUS3-interacting proteins, 2x10⁶ CFU of cells harbouring the cDNA library were plated on YNB –Ura/–His/–Trp/–Leu media with 2% galactose, 1% raffinose, 1x BU and 80 µg/ml Xgal. The plates were incubated at 30 °C and colonies were picked after 3~7 days of incubation.

6.1.5 Sequence analysis of the FUS3-interacting protein

The sequences coding for the putative FUS3-interacting proteins were amplified by colony polymerase chain reaction (PCR) using the BCO1 and BCO2 primers. The PCR products were digested by HaeIII or Rsal (New England Biolabs), separated by electrophoresis in 1.5 % agarose gel and sorted by their restriction patterns. Clones with identical HaeIII and Rsal restriction patterns were considered to be identical; duplicated clones were discarded and only one clone from each restriction class was kept for further analyses.

The AD-prey/pJG4-5 library clones were isolated from yeast. EGY48[pSH18-34, FUS3ΔC- BD/pEG202, AD-prey/pJG4-5] or EGY48[pSH18-34, FUS3(N90)-BD/pEG202, AD-prey/pJG4-5] cells were cultured in YNB ura- his- trp- media with 2% glucose overnight and then harvested by quick centrifugation. The pellets were washed in ddH₂O, then resuspended in 200 µl digestion mix [1% (v/v) β-mercaptoethanol, 0.1 M Tris-HCl pH 7.5, 0.01 M ethylenediaminetetraacetic acid (EDTA) pH 8, 1000 U lyticase (Sigma)], and incubated at 37 °C for 1~2 hours. 200 µl of lysis solution [0.2 M NaOH, 1% (w/v) SDS] were added to the mixtures and incubated in 65 °C for 20 minutes then cooled on ice. 200 µl of 3M potassium acetate were added to the mixtures and incubated on ice for 15 minutes. The mixtures were
centrifuged for 3 minutes; the supernatants were collected, to which 0.6 volumes of iso-propanol was added. The mixtures were incubated in room temperature for 15~30 minutes to precipitate the DNA. The mixtures were centrifuged for 10 minutes; the supernatants were discarded and the pellets were washed once with 1 ml 70% ethanol. The pellets were air-dried, and subsequently resuspended in 50 µl TE buffer.

To isolate the pJG4-5 plasmids from total yeast DNA, the yeast DNA collected were used to transform E. coli strain KC8 (Clontech) by electroporation using the MicroPulser™ electroporator (BioRad). Transformed KC8 cells were streaked on trp- M9 media (Lech and Brent, 1987) with 100 µg/ml ampicillin, and incubated in 37 °C for 24 hours. pJG4-5 plasmids were then extracted from E. coli colonies using the EZ-10 Spin Column Plasmid DNA Kit (Bio Basic) according to the manufacturer’s instructions.

Isolated pJG4-5 plasmids harbouring the coding sequences of putative FUS3-interacting proteins were sequenced using the BCO1 and BCO2 primers. The sequences were translated and clones that were out-of-frame with the upstream activation domain were deemed to be false positives and not kept for further analyses. Sequences in-frame with the upstream activation domain were used for BLAST searches from National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the proteins they encode. pJG4-5 plasmids encoding putative FUS3-interacting proteins were used to transform EGY48[pSH18-34, FUS3AC-BD/ pEG202] and EGY48[pSH18-34, FUS3(N90)-BD/pEG202] cells to repeat the interaction analyses as described above.

6.2 Agro-infiltration for *Nicotiana benthamiana* transient expression

Agro-infiltration was performed essentially as described in Lewis et al., 2008. *Agrobacterium tumefaciens* strains GV2260 harbouring the protein construct of interest and Tobacco Etch Virus enhancer (TEV) were separately streaked on LB media supplemented with 50 µg/ml rifampicin and either 100 µg/ml spectinomycin for all BiFC constructs, or 50 µg/ml kanamycin for 35S:FUS3-GFP and 35S:YFP-AKIN10 constructs. Agrobacterium was incubated at 30 °C for 48
hours. 5 ml of LB media with the appropriate antibiotics were inoculated with single Agrobacterium colonies, incubated at 30 °C with agitation for ~20 hours. 500 µl of these overnight cultures were used to inoculate 25 ml of LB media supplemented with 10 mM MES pH 5.7 and 20 µM acetosyringone (in DMSO) with the appropriate antibiotics, incubated at 30 °C with agitation for ~20 hours. Cells were harvested by centrifugation (4 k rpm, room temperature for 25 minutes), the pellet was washed with 12.5 ml infiltration medium (10 mM MgCl₂, 10 mM MES pH 5.7, 150 µM acetosyringone in DMSO). Pellets were resuspended with infiltration medium to OD₆₀₀ no more than 0.4, and these cultures were incubated at room temperature with agitation overnight.

The abaxial side of leaves from 3-week-old *Nicotiana benthamiana* plants were sprayed with water 1 hour before infiltration to help stomata opening. Equal parts of Agrobacterium cultures of all constructs to be co-infiltrated and Agrobacterium culture harbouring TEV were mixed, and these mixtures were used to infiltrate the abaxial side of *N. benthamiana* leaves using a 1 ml syringe without needle. Infiltrated leaves were observed by confocal microscopy 48 hours after infiltration. Images were taken on a Zeiss LSM 510 META confocal microscope equipped with a Zeiss oil immersion 40x objective lens and an argon laser. GFP and YFP were excited at 488 nm and emitted fluorescence was collected through a 505-530 nm band pass filter. Chloroplast autofluorescence was collected through a 585 nm long pass filter.

6.3 In-gel kinase assay

In-gel kinase assay was performed essentially as described by Liu et al., 2008. GST, GST-FUS3 or GST-fus3 were used as the substrates and incorporated in the gel. Substrate-incorporated SDS-PAGE gel was casted by making the resolving gel [1.5 ml 40% acrylamide/bis-acrylamide mix, 1.25 ml 1.5 M Tris-HCl pH 8.8, 0.5 mg purified phosphorylation substrate (eluted in 50 mM Tris-HCl pH 9.5) plus ddH₂O to 2.182 ml, 25 µl 20% SDS, 40 µl freshly made ammonium persulfate, 3 µl Tetramethylethylenediamine (TEMED)] followed by the stacking gel (0.25 ml 40% acrylamide/bis-acrylamide mix, 0.625 ml 1.5 M Tris-HCl pH 6.8, 1.5875 ml ddH₂O, 12.5 µl 20% SDS, 20 µl freshly made ammonium persulfate, 5 µl TEMED).
7-day-old Arabidopsis seedlings were harvested in liquid nitrogen, homogenized to fine powder, and resuspended in extraction buffer [100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM sodium orthovanadate, 10 mM NaF, 50 mM β-glycerophosphate, 10 mM DTT, 1 mM PMSF, 1x Protease Inhibitor for plant tissue (Sigma), 5% (v/v) glycerol] at 1 mg of tissue per 1 µl buffer. The plant homogenates were centrifuged at 20 k rpm at 4°C for 10 minutes, the supernatants were collected and centrifuged again to remove the finer cell debris. The protein concentration of the extract was determined using Bradford reagent (BioRad).

15 µg of protein extract were separated by SDS-PAGE using the gel incorporated with the substrate at no more than 100 V. After electrophoresis, the gel was washed 3 times, each 30 minutes in 100 ml of washing buffer (25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM sodium orthovanadate, 5 mM NaF, 0.5 mg/ml bovine serum albumin, 0.1 % Triton X-100) at room temperature. Subsequently, the gel was washed 3 times in 100 ml renaturation buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM sodium orthovanadate, 5 mM NaF) at 4 °C, with each wash at least 30 minutes.

The gel was washed in 100 ml of reaction buffer (25 mM HEPES pH 7.5, 2 mM EGTA, 12 mM MgCl₂, 1 mM DTT, 0.1 mM sodium orthovanadate) at room temperature for 30 minutes. To initiate the phosphorylation reaction, the gel was then incubated in 30 ml reaction buffer plus 200 nM ATP and 50 µCi of γ-32P-ATP (3000µCi/mmol, Perkin Elmer) for 1.5 hours at room temperature with agitation. To terminate the reaction, the gel was washed for 30 minutes in termination solution [5% trichloroacetic acid (w/v) and 1% sodium pyrophosphate (w/v)] 5 times, or until the termination solution is free of radioactivity. The gel was then dried on Whatman paper, and then exposed to light-sensitive film at -80 °C for ~24 hours prior to autoradiography.

6.4 References


